NEW PERSPECTIVES ON PEDIATRIC ACUTE LEUKEMIA

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NEW PERSPECTIVES ON PEDIATRIC ACUTE LEUKEMIA

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EDITORIAL
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Editorial: New Perspectives on Pediatric Acute Leukemia

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Editorial on the Research Topic

New Perspectives on Pediatric Acute Leukemia

The scenario of pediatric acute leukemia has changed extremely rapidly over these last decade and the pediatric hematologists are now facing new challenges related to the biology of the disease, the prognostic classifications of patients, and the consequent risk-based targeted approach. Acute lymphoblastic leukemia (ALL) in children has always represented a paradigm of success, and the recent advent of large-scale genomic studies and novel immunotherapy-based approaches have further revolutionized the perspective on this disease. Pediatric acute myeloid leukemia (AML) still suffers from a lower cure rate if compared to ALL, due to a still high incidence of recurrence and of severe and dose-limiting short- and long-term toxicities. Nevertheless, much has been learned about the biology of the disease through studies of specific recurrent genetic lesions and the outcomes for these children are progressively improving thanks to the great collaborative efforts of the main pediatric AML groups worldwide.

The Research Topic "New perspectives a in Pediatric Acute Leukemia" includes innovative and original contributions on multiple aspects of pediatric leukemia and gives to the readers the possibility to have an organic and comprehensive overview of novel insights related to the biology of the disease. Original reports aimed at exploring and clarifying the prognostic value of specific recurrent molecular markers are included. A focus on how to refine the patient's risk stratification is covered, with reports on the outcome of specific subgroups of patients such as children with Down Syndrome (DS) or with acute promyelocytic leukemia (APL). Lastly, this collection gives also the opportunity to go deeply into the discovery of novel strategies for targeted therapeutic intervention, from the modeling of pediatric acute leukemia in mice toward a three-dimensional (3D) cell-based drug discovery approach.

The review articles by Kuhlen et al. and Lonetti et al. point out the role of several new drugs targeting key molecular pathways involved in leukemia growth and proliferation that have been developed and approved. These include kinase and proteasome inhibitors, epigenetic and enzyme targeting, as well as apoptosis regulators. Experimental and clinical evidences are comprehensively described and deeply discussed in these contributions, giving a wide and complete overview on the state of the art of novel compounds and targeted therapies currently under evaluation. The contribution by Mercher and Schwaller, provides important insights on new targeted approaches, mainly for AML, but additionally, this review also shed the light on emerging biological concepts of extreme interest. Of particular interest are the data provided on specific subgroups of aggressive AML where the disease phenotype is dependent on the appropriate expression and activity of the driver fusion oncogenes in a particular window of opportunity during fetal development.

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A novel perspective on the scenario of leukemia drug discovery is also given by the contribution of Cartledge Wolf and Langhans, where interesting dynamic interactions between leukemic cells and the bone marrow environment are explored. The review shows how the interaction between leukemic cells, stromal cells, and the extracellular matrix plays critical roles in the development, progression, and relapse of AML as well as in drug response and the development of resistance.

Moving to the risk based stratification, important aspects related to specific subgroups of patients are also covered. Ksiazek et al. reported an unexpectedly high frequency of the fusion gene transcript resulting from translocation t(10;11)(p12;q23) involving MLL gene, considered an unfavorable prognostic factor, in the Poland pediatric patients affected by AML. As this data was also reported by other reports, it seems that this fusion gene transcript could be relatively frequent in specific populations, opening new considerations on the peculiar biology of this MLL-rearranged leukemia. These characteristics should be taken into account in the analysis of the frequency of recurrent genetic features of pediatric AML.

The paper by Czogala et al. reports the treatment results and genetic characteristics of children with DS affected by AML treated from 2005 to 2019 with two different specific protocols. The study confirms that reduced-intensity protocols are very effective in DS patients with AML without affecting the treatment efficacy. In addition, a significant decrease in treatment-related mortality was noticed, without an increase in relapse. Similar considerations can be made for the other report by the same group (Czogała et al.) reporting the treatment results of a large series of children affected by APL. A good proportion of these children received a treatment regimen that can now be considered the standard of care for standard-risk APL with the combination of all-trans-retinoic acid and arsenic trioxide, without chemotherapy.

In the end, the study of Cwiklinska et al. highlighted the role of pharmacogenetics in the treatment of children with acute lymphoblastic leukemia (ALL). In particular, the authors identified genetic polymorphisms of the *SLC19A1*, *MTHFR*, and *TS* genes that influence the pharmacokinetics of methotrexate (MTX), increasing the risk of developing hepatotoxicity and vomiting in children with ALL. This study adds an important piece in the comprehension of the entire pathway of the MTX metabolism, that is still far to be fully elucidated. The identification of genetic factors predisposing to the development of specific toxicities could be of great utility in the management of a personalized treatment within the therapeutic protocols.

Overall, this topic highlights that several new perspectives in pediatric acute leukemia are raising and that children suffering from leukemia will have, in a near future, more chances to be cured with innovative approaches and new incoming drugs.

AUTHOR CONTRIBUTIONS

RM wrote the manuscript. MP and DZ contributed to the manuscript preparation and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Moving Myeloid Leukemia Drug Discovery Into the Third Dimension

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The development of therapies aimed at leukemia has progressed substantially in the past years but childhood acute myeloid leukemia (AML) remains one of the most challenging cancers to treat. Genomic profiling of AML has greatly enhanced our understanding of the genetic and epigenetic landscape of this high-risk leukemia. With it comes the opportunity to develop targeted therapies that are expected to be more effective and less toxic than current treatment regimens. Nevertheless, often overlooked in leukemia drug discovery are the dynamic interactions between leukemic cells and the bone marrow environment. The interplay between leukemic cells, stromal cells and the extracellular matrix plays critical roles in the development, progression and relapse of AML as well as in drug response and the development of resistance. Here we will review pediatric leukemia with a special focus on acute myeloid disease in children, and discuss the tumor microenvironment in the context of drug resistance and leukemia stem cell survival. We will emphasize how three-dimensional (3D) cell-based drug discovery may offer hope for both the identification and advancement of more effective treatment options for patients suffering from this devastating disease.

Keywords: three-dimensional cell culture, bone marrow, leukemia, AML, tumor microenvironment

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INTRODUCTION

Leukemia encompasses a set of malignant conditions that affect blood and blood forming tissues. Normal blood cells are derived from hematopoietic progenitor cells, which go on to differentiate into cells of either myeloid or lymphoid lineage. Myeloid cells become erythrocytes, platelets, myeloblasts, and granulocytes, while lymphoid cells develop into lymphoblasts that subsequently differentiate into B-lymphocytes, T-lymphocytes or natural killer cells (1, 2). In leukemia, normal hematopoiesis is suppressed by the uncontrolled proliferation and accumulation of leukemic cells (3). Leukemias are classified by the types of cells affected and are further defined by the developmental stage of the originating cells. They are grouped into four major categories: acute myeloid (AML) and acute lymphoblastic leukemia (ALL), and chronic myeloid (CML), and chronic lymphoblastic leukemia (CLL). Myeloid leukemias are derived from myeloblasts and lymphoblastic leukemias are derived from lymphoblasts, acute conditions arise from early, immature cells and chronic conditions are derived from mature, abnormal cells. Leukemia is generally considered an uncommon condition. In the United States in 2019, there were estimated to be approximately 1.76 million new cases of cancer diagnosed in the overall population with 61,780 being leukemia (4). Approximately 9% of newly diagnosed cases of leukemia are in children and young adults. Acute leukemias are the most common type and are responsible for approximately one third of all cancers in this age group. Mixed lineage leukemias (MLL) are a subtype of acute disease and have features of both AML and ALL. Chronic leukemias are very rare in children.

Identification of early drugs used to treat leukemia was not target driven. These compounds were, instead, already in use for various other diseases and disorders and were administered to patients in the hope of providing a path to recovery. Arsenic was the first recognized leukemia therapy and was a principal component in a potassium bicarbonate based solution of arsenic trioxide, developed in the late Eighteenth century by Thomas Fowler (5) and first used as a leukemia treatment in 1865 (6). Arsenic containing compounds, with or without concomitant radiation, remained the standard therapy for leukemia up until the introduction of busulphan in 1953 (6). L-asparaginase, an enzyme that catalyzes the conversion of L-asparagine to L-aspartic acid and ammonia, has been used to treat pediatric patients with ALL since the mid-1960s (7–9). In the first account of clinical use, Dolowy et al. showed that an 8 year-old child achieved a partial response upon treatment with this enzyme (10). Hill et al., subsequently reported that patients treated with L-asparaginase had significant improvement, even with advanced disease, and achieved complete remission in one of three patients tested (11). Clinical trials followed, which further demonstrated the efficacy of such a targeted treatment as both a stand-alone therapy (12) and in combination with other pharmaceutical agents (13). But it was not until recently that it was found that a cytogenetic and molecular subgroup of AML characterized by chromosome 7 monosomy could also benefit from L-asparaginase treatment (14), a treatment that may preferentially target leukemia stem cells in the bone marrow microenvironment (15).

In addition to the development of new drug treatment strategies, radiation therapy became part of the conventional methods used to treat leukemic disease (16). Since then, treatment options for leukemia have evolved, from technological advances such as stem cell transplants, to the development of new targeted therapies based on the increased understanding of molecular events leading to leukemia, to taking advantage of a patient's own immune system in immunotherapy. In this review, we will give an overview of disease subtypes, etiology and current treatment options. We will discuss the more recent understanding of the influence of the tumor microenvironment within the bone marrow on cancer stem cell proliferation and its impact on drug resistance. Lastly, we will address the challenges this poses for traditional drug discovery efforts and how new phenotypic 3D screening methods that can more closely mimic such a tumor microenvironment may help to overcome these limitations.

ETIOLOGY OF MYELOID LEUKEMIAS

Leukemias are thought to arise from a single mutant cell, but the cancer populations are not clonal. Several mutations are possible within the spectrum of an individual patient's disease. In accordance with Knudson's two-hit hypothesis (17), leukemias are cancers that are generally caused by two oncogenic events. Knudson's hypothesis, although ground-breaking for its time, does have some limitations because it is now known that several tumor suppressor genes (TSGs) do not fit within its confines. In an effort to expand and redefine this paradigm, Paige placed these TSGs into three main categories, including those that arise from (a) monoallelic disruption (haploinsufficiency, dominant negative and gain-of function isoforms); (b) multiple gene interactions (multi-step tumorigenesis, genetic modifiers or mutators); and (c) dual function TSGs that have both tumor-suppressing and tumor-promoting properties [reviewed in (18)]. Oncogenic events include deviant expression of proto-oncogenes, and chromosomal abnormalities that result in changes in chromosome number, chromosome inversions, or creation of translocations leading to gene fusions and subsequent modification(s) of cell signaling pathways due to over or under activity of kinases and/or transcription factors (3, 19, 20). A subset of AML, referred to as t- or therapy related-AML are, as the name suggests, caused by mutations that arise as a result of treatments for other disease conditions, including benign and malignant neoplasms and immune system disorders. Prior disease management may include individual or combined treatments including chemotherapy, radiation, and immunosuppressive therapies (21, 22). Prognosis for patients with t-AML is generally very poor; supportive therapy is most often the only treatment option (23).

Irregularities at the *Mixed Lineage Leukemia* gene (*MLL*) locus present on 11q23 are frequently responsible for aggressive cancers that most often occur in the pediatric population and are due to conversion of *MLL* into an active oncogenic state (24). MLL has been shown to fuse with many partners including the Acute Lymphoblastic Leukemia 1-Fused Gene from Chromosome 4 (AF4), Acute Lymphoblastic Leukemia 1-Fused Gene from Chromosome 6 (AF6), Acute Lymphoblastic

Abbreviations: UTR, 3′-untranslated region; ABI1, abl-interactor 1; ALL, acute lymphoblastic leukemia; AF4, acute lymphoblastic leukemia 1-fused gene from chromosome 4; AF6, acute lymphoblastic leukemia 1-fused gene from chromosome 6; AF9, acute lymphoblastic leukemia 1-fused gene from chromosome 9; AF10, acute lymphoblastic leukemia-1 fused gene from chromosome 10; AML, acute myeloid leukemia; AF1p, ALL 1 [acute lymphoblastic leukemia 1]-fused gene from chromosome 1 protein; BCL2/BCL-XL, B-cell lymphoma 2/B-cell lymphoma-extra large; COG, Children's Oncology Group; CAR, chimeric antigen receptor; CLL, chronic lymphoblastic leukemia; CML, chronic myeloid leukemia; CD19, cluster of differentiation 19; CD33, cluster of differentiation 33; CD34, cluster of differentiation 34; c-Myc, c-myelocytomatosis oncogene cellular homolog; CBP, CREB [cAMP (cyclic adenosine monophosphate) response element binding] binding protein; CXCL, cysteine-x-cysteine chemokine ligand; CXCR, cysteine-x-cysteine chemokine receptor; DKK1, Dickkopf-1; DMSO, dimethyl sulfoxide; ELL, eleven-nineteen lysine-rich leukemia; ENL, eleven-nineteen-leukemia; EEN, extra eleven-nineteen leukemia; FLT3, FMS [feline McDonough sarcoma] related tyrosine kinase 3; GAS7, growth arrest specific protein 7; HSCT, hematopoietic stem cell transplantation; HSC, hematopoietic stem cell; HTS, high-throughput screening; HOXA, homeobox A; HOXB, homeobox B; ITD, internal tandem duplications; MTOR, mechanistic target of rapamycin; MLL, mixed lineage leukemia; MDM2, mouse double minute 2 homolog; c-Myb, myeloblastosis viral oncogene homolog; MEIS1, myeloid ecotropic viral integration site 1 homolog; NCI, National Cancer Institute; NSD1, nuclear receptor-binding SET [su(var)3-9, enhancer-of-zeste and trithorax] domain protein 1; NUP98, nucleoporin 98-kDa; PI3K, phosphatidylinositol 3-kinase; PDT, photodynamic therapy; p53, protein 53; P300, protein 300; AKT, protein kinase B; ROS, reactive oxygen species; pRb, retinoblastoma protein; SDT, sonodynamic therapy; starPEG, star-shaped polyethylene glycol; t-AML, therapy related-acute myeloid leukemia; 3D, three-dimensional; TSG, tumor suppressor gene; 2D, two-dimensional; TKD, tyrosine kinase domain; FDA, US Food and Drug Administration; Wnt, wingless-int.

Leukemia 1-Fused Gene from Chromosome 9 (AF9), Acute Lymphoblastic Leukemia-1 Fused Gene from Chromosome 10 (AF10), Eleven-Nineteen-Leukemia (ENL), Eleven-Nineteen Lysine-rich Leukemia (ELL), CREB [cAMP (cyclic adenosine monophosphate) Response Element Binding] Binding Protein (CBP), Protein 300 (P300), ALL 1 [Acute Lymphoblastic Leukemia 1]-Fused Gene from Chromosome 1 Protein (AF1p), Growth Arrest Specific Protein 7 (GAS7), Abl-Interactor 1 (ABI1), and Extra Eleven-Nineteen Leukemia (EEN) proteins [reviewed in (24)].

Alcalay et al. showed that some fusion proteins in AML induced a mutator phenotype, down regulating the activity of DNA base excision repair genes (25). Hence, the presence of fusion proteins impaired DNA repair mechanisms leading to further DNA damage and induction of a leukemic phenotype. The NUP98-NSD1 fusion protein occurs in 4.4% of pediatric AML and is associated with a <10% event-free 4-year survival rate (26). NUP98, or Nucleoporin 98-kDa, is located on chromosome 11p15, and is part of the nuclear pore complex, which controls movement of protein and RNA between the nucleus and the cytoplasm (27). Chromosomal rearrangements are responsible for fusion of NUP98 with several different partner genes which may, broadly, be grouped into three categories: homeodomain; nuclear nonhomeotic, which includes NSD1; and cytoplasmic [reviewed in (28)]; (29). NSD1, or Nuclear Receptor-binding SET [Su(var)3-9, Enhancer-of-zeste and Trithorax] Domain Protein 1, is located on chromosome 5q35 and is a histone methyltransferase (30). NSD1 predominantly dimethylates lysine 36, located close to the globular domain of nucleosomal histone H3 (31). NSD1 retains methyltransferase activity in the fusion, and it is this property that is essential for leukemia progression (32). Aberrant expression of NUP98-NSD1 promotes leukemogenesis by activating transcription of hematopoietic regulatory genes, principally *Homeobox A* (*HOXA*), *Homeobox B* (*HOXB*), and *Myeloid Ecotropic Viral Integration Site 1 Homolog* (*MEIS1*), which subsequently activate down-stream proto-oncogenic target gene *Myeloblastosis Viral Oncogene Homolog* (*c-Myb*) (33, 34). *HOX* expression is markedly reduced as myeloblasts differentiate into mature hematopoietic cells (24). When *HOX* expression is continually stimulated, myeloblastic cells become self-renewing and fail to differentiate, thus exhibiting a stem cell-like, immortal phenotype (24) that most often leads to cancer.

Secondary events leading to leukemogenesis include activating mutations in additional proto-oncogenes such as the NOTCH1 transmembrane receptor, implicated specifically in T-cell derived ALL (35, 36). Mutations in the receptor tyrosine kinase FMS [Feline McDonough Sarcoma] Related Tyrosine Kinase 3, FLT3] (37) are often due to internal tandem duplications, referred to as FLT3 ITD (38) or due to point mutations in the tyrosine kinase domain in the codon for an aspartate (D835) or an isoleucine (I836) residue, collectively termed FLT3 TKD (39). Loss-of-function mutations in tumor-suppressor genes such as Retinoblastoma protein, pRb, and p53 have been described as well (40) and mutations in non-coding regions of DNA have also been implicated in malignant transformation (41).

CURRENT TREATMENT OPTIONS

Treatment for leukemia generally involves a series of two to three steps: (a) induction therapy which is intended to bring the patient into remission, (b) consolidation therapy, designed to eradicate cancer cells that may have escaped front line treatment strategies, and (c) maintenance therapy, with the goal of keeping the patient in a disease remissive state. Clinically, remission is defined as a significant decrease in detectable disease, and is most often concurrent with a considerable reduction in symptomatology. With respect to cancer patients, total remission entails the inability to detect cancer in the body with current diagnostic technologies. Although this outcome is certainly encouraging, it does not mean that a patient is cured. Ideally, treatment would be both innocuous and lifelong, such that any signs of recurrence would be promptly addressed and eradicated.

Currently, for the pediatric population, management of AML involves predominantly induction and consolidation therapies. Whereas, >80% of children diagnosed with AML will achieve remission, only about half will remain disease-free for an appreciable period of time. Children who fail treatment are often referred for hematopoietic stem cell transplantation (HSCT), which can be administered after the first complete remission or subsequently, following one or more relapses of the disease [reviewed in (42)]. Biomarker guided treatments are evolving, with molecular targets including cell surface antigens, disease-associated regions of proteins, and enzymes. While treatment options vary according to risk group, preliminary therapy with cytarabine in combination with anthracycline drugs such as topoisomerase inhibitors is favored, and is often combined with purine antagonists and sometimes combined with cytokine exposure (43, 44). The cytotoxic antitumor drug-conjugated antibody gemtuzumab ozogamicin has been approved by the US Food and Drug Administration (FDA) for use in children 2 years of age and older with CD33-positive AML, but not as a first line treatment strategy. This drug is only indicated for use in children who have failed traditional treatment by either exhibiting no response or by having a recurrence of their disease. The antibody portion of the conjugate binds to Cluster of Differentiation 33 (CD33), a cell surface adhesion protein that is present on leukemic blasts and immature normal myelomonocytic lineage derived cells, but not on normal hematopoietic stem cells or on non-hematopoietic cells. After attachment to CD33, the drug-linked antibody enters the cell via endocytosis and the drug is released in the lysosomes after which it binds to DNA and causes double-stranded DNA breaks, leading to cell cycle arrest and ultimately to cell death by apoptosis (45, 46).

Chimeric antigen receptor (CAR) T-cell therapy is a type of personalized medicine in which the patient's own immune system is primed to destroy malignant cells. CAR T-cell therapy has, so far been approved by the FDA for the treatment of patients from 3 to 25 years of age with B-cell derived ALL who have failed standard treatments by either having no response or who have responded, achieved remission and then have had their cancer recur two or more times (47, 48). This therapy, termed "Tisagenlecleucel," involves (a) collection and purification of T-cells from the patient's body; (b) engineering of these cells to

express CAR; and (c) transfusion of these cells back into the patient (49). While these T-cells are being genetically altered in a process that takes about one month's time, the patient receives lymphocyte depleting chemotherapy so that when reintroduced, these engineered cells stand a better chance of recognizing and eradicating the cancer (49). Efficacy of this treatment relies on the ability of CAR expressing T-cells to recognize Cluster of Differentiation 19 (CD19), a cell surface antigen that is highly expressed on leukemic B-cells, but insignificantly present on normal B-lymphocytes (50). While CAR T-cell therapy is still in its infancy, with our steadily increasing understanding of molecular events leading to the development and progression of AML, it will only be a matter of time before CAR T-cell therapy will also become a treatment option for pediatric AML patients.

Treatments that combine chemical or cell destabilizing agents with physical disruption include photodynamic (PDT) and sonodynamic (SDT) therapies. The intent of these treatments is targeting and damaging malignant cells. As their names suggest, the energy sources for PDT and SDT are light and sound, respectively. Although both light and sound waves can be focused, sound is advantageous over light because it can penetrate deeper into the body and can be effectively aimed at hard to reach or otherwise inoperable tumors. SDT, extensively reviewed in (51), relies on administration of a sonosensitizing agent and exposure to ultrasound irradiation that is focused on the tumor. These sound waves disrupt the sensitizing compound, causing it to transform into an excited state, which then reacts with molecular oxygen and generates reactive oxygen species (ROS), which go on to disrupt mitochondria and ultimately leads to apoptosis (51). Although PDT and SDT are emerging therapies for various types of cancer, each treatment modality is applicable for eradication of leukemic cells of both myeloid and lymphoid origins.

Development of therapies aimed at leukemia has progressed substantially in the last decade. Pediatric disease targets, extensively reviewed in (52), include protein tyrosine kinases, protein serine/threonine kinases, proteases, anti-apoptotic proteins, DNA methyltransferases, histone deacetylases, and chemokine receptors. The National Cancer Institute (NCI) online database lists over 350 clinical trials for various types of leukemia (see **Supplementary Information**). Most allow pediatric patients, defined here as being <18 years of age. This population is, however, generally under-represented. The Children's Oncology Group (COG) currently has 18 clinical trials addressing various hematologic malignancies in the pediatric population. Still, most experimental therapeutics target intrinsic properties of leukemic cells. However, and in particular in myeloid diseases, there is increasing evidence that the microenvironment in the leukemic bone marrow niche contributes to disease progression, therapeutic response and evasion of therapy as well as the development of resistance to treatment (**Figure 1**). While this opens up avenues for the development of new therapeutic strategies that target the tumor microenvironment, current phenotypic screens using leukemic cells in suspension culture are poorly suited for drug discovery approaches targeting the interaction between leukemic cells and the tumor microenvironment.

AML AND THE BONE MICROENVIRONMENT—A CHANCE FOR DEVELOPING NEW THERAPEUTIC STRATEGIES

AML stem cells reside in the bone marrow niche and are generally refractory to chemotherapy, necessitating the development of new molecularly directed therapeutics. The question of AML stem cell origin is complex at best. The idea of a specific region in the bone giving rise to and influencing the maturation and ultimate fate of hematopoietic stem cells, in the normal non-malignant context, was first suggested by Schofield who, in 1978, referred to these areas as "stem cell niches" (53). There are currently two theories on this phenomenon. The first being that chemotherapy causes genetic and epigenetic changes in AML cells that lead to the emergence of those with drug resistance. The second stating that these refractory stem cells are already present (before treatment) and that chemotherapy simply kills off susceptible cells, effectively selecting for those with endogenous resistance (54). Chemotherapy does certainly select for resistant cells in the clonal population, but within the context of AML, stem cells are an important factor in both disease persistence and recurrence.

In recent years, it has become clear that leukemic cells influence the bone marrow microenvironment and, at the same time, the bone marrow microenvironment influences leukemic cells. This becomes a problem when the interactions with the bone marrow leukemic niche promote the evasion of leukemic cells from treatment. For example, the bone marrow microenvironment contains a wealth of components that safeguard leukemia cells from treatment-induced demise. Cytotoxic insult leads to drug resistance by way of one or more environmental methods, classified as soluble factor- and cell adhesion-mediated mechanisms [reviewed in (55)]. Secretion of cytokines, especially those of the Cysteine-X-Cysteine chemokine ligand (CXCL) structural motif, in the bone marrow microenvironment attracts Cysteine-X-Cysteine chemokine receptor (CXCR)-bearing leukemia cells in the bone marrow. The CXCL12/CXCR4 ligand/receptor axis is associated with AML, and patients whose cancers over express CXCR4 tend to have aggressive disease and a poor overall prognosis (56–58). On the other hand, AML cells modify the bone marrow microenvironment by producing angiogenic factors that restructure vascularity within endosteal niches and promote a phenotype that favors AML growth but hinders propagation of normal hematopoietic stem cells (HSC) (59). This results in loss of normal HSC which plays a major role in leukemia progression. Duarte and colleagues showed that when the endosteal niche is protected, either by chemical or by genetic means, efficacy of chemotherapeutic treatments is substantially increased (59). Exosome secretion has recently been implicated in modifications observed in the marrow of AML permeated bone (60). Kumar and colleagues found that exosomes were significantly increased in the bloodstream of AML patients when compared to blood from normal controls, and that up regulation of Dickkopf-1 (DKK1) was positively

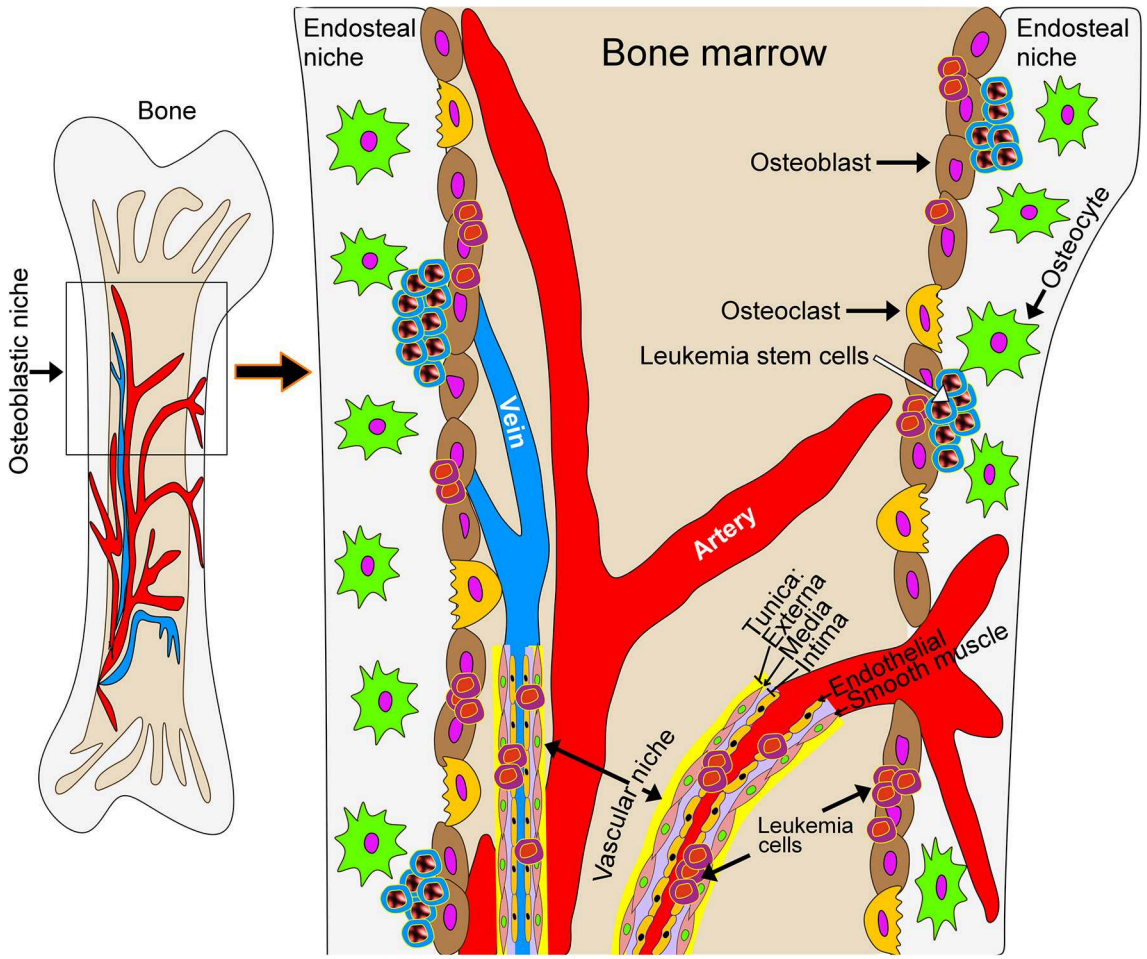

FIGURE 1 | Schematic model of the leukemic bone marrow niche. The microenvironment in the leukemic bone marrow niche contributes to disease progression, therapeutic response, evasion of therapy and the development of resistance to treatment. New treatment strategies may target the interaction of leukemic cells with stromal cells or with the extracellular matrix underlying bone tissue.

correlated with the presence of these vesicles (60). DKK1 is secreted by mesenchymal stem cells, including osteoblasts, is involved in bone and blood cell formation, and is a potent inhibitor of Wingless-Int (Wnt) pathway signaling (61). In contrast to most reported findings (62–64), this discovery suggests that reduced Wnt signaling may also play a role in AML-driven bone marrow remodeling. An ideal strategy for circumventing such problems will be to identify inhibitors that disrupt the interplay between AML and the bone marrow microenvironment. However, genetic alterations are not limited to leukemic cells but molecular alterations can also be found in bone marrow mesenchymal stromal cells of AML patients opening up the possibility of niche-directed therapies in AML (65).

Like all cancers, AML becomes fatal when patients fail to respond to therapy or when they respond initially then, after a period of time, experience recurrence with cells that have become resistant to intervention. Despite subsequent administration of dissimilar therapeutics, the phenomenon of acquired resistance often replays itself numerous times until no effective disease management strategies remain. Preventing relapse is the key to keeping the cancer at bay in a state that is compatible with life, i.e., stable disease, or to eradicating the disease completely. Since growth within the confines of the bone marrow microenvironment and/or growth in multicellular clusters afford protection to AML cells, a way to prevent these hidden cells from surviving is to identify drugs capable of reaching and destroying them. Cytotoxicity of therapeutics targeting AML cells that are rapidly dividing will fall short in eradicating those in a quiescent state, and is of particular concern with regard to leukemia stem cells (66). In both cases, a fair percentage of AML cells residing in the bone marrow niche are slowly or not actively growing. A way to discover these types of first-in-class therapeutics is to combine the best aspects of phenotypic and target-driven high-throughput screening (HTS) in drug discovery. Phenotypic because drug induced effects on cells are investigated, and target-driven in that cells are grown in a format that more closely replicates

in vivo conditions. From this perspective, co-culturing AML cells with stromal cells is a good first step in mimicking these circumstances.

A CASE FOR CO-CULTURING AML AND STROMA CELLS

Phenotypic changes resulting from cell-cell interactions are factors that need to be considered in defining how the bone marrow niche contributes to AML survival. In a recent study, Zeng et al. used a mechanism-based selection strategy to identify combinations of drugs to eliminate bone marrow microenvironment-mediated resistance in AML (67). Interestingly, this method utilized co-culturing of human leukemic cells and mouse derived stromal cells under more traditional two-dimensional (2D) culture conditions. A 2D niche-based phenotypic screen utilizing T-ALL derived mouse leukemia stem cells co-cultured with mouse stromal cells genetically altered for optimal activation of the transmembrane receptor NOTCH1 identified compounds that were selectively toxic to stem cells of lower proliferative state (68). In this study, expression of the transcription factor c-Myelocytomatosis oncogene cellular homolog (c-Myc) was implicated as a causal factor for drug resistance in leukemia cells, specifically those of the stem cell state. NOTCH1 signaling promotes self-renewal and survival of hematopoietic progenitor cells (69), and has been shown to induce c-Myc expression and to augment cell growth in leukemia (70). In an effort to elucidate the mechanism by which interactions between AML and stromal cells increases c-Myc expression, Tian et al. performed microRNA array analysis on AML cells from patients and AML cell lines cultured with and without human stromal cells and found differences in the expression levels of various microRNAs under both growth conditions (71). MicroRNAs are small RNA molecules that do not code for protein, and function in gene silencing and in post-transcriptional modifications by binding to and interacting with the 3′-untranslated region (UTR) of target genes (72). When AML cells were grown in close proximity to stromal cells, expression of microRNA-494 was down regulated, allowing c-Myc expression to be maintained at enhanced levels which confers drug resistance to AML cells. In agreement with this finding, primary AML cells taken from patients whose microRNA-494 expression was weak were found to have worse prognoses than those whose levels were at normal or enhanced levels. In another study utilizing 2D co-culturing of AML and stromal cells, all of human origin, Xia et al. showed that expression of c-Myc was significantly up-regulated in primary AML cells from patients and AML cell lines grown in the presence of human mesenchymal stromal cells (73). Expression of c-Myc conferred resistance to apoptosis induced by the type II topoisomerase inhibitor, mitoxantrone, while inhibiting c-Myc either by expression (siRNA) or functional (small molecule inhibitor) based approaches abrogated mitoxantrone resistance in these cell lines. Interestingly, in order to be protected from mitoxantrone induced apoptosis, AML cells needed to have cell-to-cell contact with stromal cells. Exposure of these cells to stromal cells produced soluble factors that, in the absence of this contact, afforded no chemotherapeutic protection.

In another study, investigation of 53 fundamental proteins in 11 cell signaling pathways showed that AML cells responded differently to treatment with Mechanistic Target of Rapamycin (MTOR) inhibitor temsirolimus, B-cell lymphoma 2/B-cell lymphoma-extra large (BCL2/BCL-XL) antagonist ABT737, and mouse double minute 2 homolog (MDM2) inhibitor Nutlin-3a based on whether or not they were grown in the presence or absence of stromal tissue (67). While initial findings were obtained from human AML cells co-cultured with mouse stromal cells, stroma-mediated differences were also found in the presence of human stromal cells. Modified drug sensitivities were due to stromal-induced changes in AML signaling, and resulted in chemo-resistant phenotypes in most cases. Importantly, the authors found that simultaneously blocking phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/MTOR and BCL2-associated cell signaling pathways was an effective means to deter stromal-mediated AML survival. This study supports the notion that directed therapeutics also have off-target effects on other proteins in other cell signaling pathways. As these stray targets have the potential to be therapeutically exploited, they are also clinically relevant.

DISEASE MODELING OF AML IN SCAFFOLD-BASED THREE-DIMENSIONAL (3D) CULTURES

Leukemias are thought to persist during and after chemotherapeutic treatments due to regional proximity to and interactions with mesenchymal stromal cells of the bone marrow niche which (a) provide physical protection, and (b) bring about drug resistance in leukemia of both stem and blast designations. However, discovering drugs to circumvent this phenomenon should consider both the complex interactions of these cells with each other and also those with their extracellular environment. Growing cells, especially cancer, in 3D format is now considered to more closely resemble phenotypic characteristics of the originating tumor, namely cell morphology, proliferation potential, proliferation rate, and response to chemical, biologic, and radiation-based therapeutics [reviewed in (74–78)], thus providing a new means in drug discovery. As the bone marrow niche is itself a 3D structure, it makes sense that more closely replicating this phenotype would be an ideal method for identifying potential therapeutics, especially in phenotypic HTS. Phenotypic 3D cell culture may be applied to many facets of drug development including drug discovery through HTS, target identification and validation, drug characterization and toxicity profiling, and disease modeling to determine efficacy and safety of investigational new therapeutics (74, 78).

In the broadest sense, 3D cell culture may be broken down into two distinct categories—those that contain scaffolds and those that do not. Scaffold-bearing models support anchorage dependent growth, while scaffold-free systems enable growth with anchorage independence. Common models of 3D cell culture [reviewed in (78)] are scaffold-based biological

and synthetic hydrogels, and scaffold-free hanging drop, low attachment microplate, and magnetic levitation methods. Replicating the *in vivo* environment of AML cells would best be served by co-culturing AML cells with mesenchymal stromal cells in a matrix-based scaffold, either of biological or synthetic origin. Advantages of biological scaffolding are that aside from the extracellular matrix proteins they contain sugars, amino acids, lipids, hormones and other soluble growth factors that are more reflective of the tumor microenvironment (79). These advantages have drawbacks, however, since natural products are fraught with innate differences leading to variability between lots and their composition depends on the tissue of origin. Synthetic scaffolds lack these intrinsic properties but are advantageous because of their reproducible uniformity. These structures consist of biologically compatible polymers and hydrogels (80, 81). Nutrients and growth factors can be added purposefully, which makes for a more chemically defined supportive growth structure. Because of their defined chemical composition and batch-to-batch consistency, synthetic scaffolds are expected to provide more reliable and reproducible results in drug screening.

In an effort to more closely mimic the AML tumor microenvironment, Houshmand et al. grew human TF-1 erythroblasts and human bone marrow mesenchymal stem cells together in a glass slide mounted microfluidic chamber in both two and three dimensions (82). For 2D, collagen was injected into the chamber. In 3D, demineralized bone matrix coated with collagen was loaded into the chamber. Cell culture medium was perfused throughout the system under both conditions. Phenotypic characteristics of TF-1 cells grown in 2D and 3D formats were investigated, and it was shown that cellular proliferation rates, percentage of cells in S, G2, and M phases, and resistance to chemotherapeutics was significantly increased in cells grown under 3D vs. 2D conditions (82), highlighting the relevance of 3D cell culture in leukemia drug discovery.

In a phenotypic study where human leukemic cell lines were co-cultured with human bone marrow-derived mesenchymal stem cells on a synthetic co-polymeric 3D scaffold, effects of chemotherapeutics on leukemic cells were investigated (83). Drug response of leukemia cells grown in 3D co-culture was compared to that of cells grown in 2D co-culture and in 2D suspension culture formats. Seeded scaffolds were shown to allow diffusion of molecules of up to 1,000 Da, including chemotherapeutics. Presence of stromal cells on scaffolding structures had a statistically significant, but realistically negligible difference on the drug absorption capabilities of these structures. Nevertheless, leukemia cells co-cultured with mesenchymal stem cells on 3D synthetic scaffolds had higher resistance to chemotherapeutics compared to cells co-cultured under 2D or singly cultured under 2D suspension culture formats. Immunohistochemical staining of cross sections of drug treated 3D co-culture models revealed decreased levels of Ki-67 protein, indicating reduced cellular proliferation that was both dose and time-dependent. Analysis of soluble factors collected from 2D and 3D co-cultures showed that these factors offered similar chemotherapeutic protection for cells grown under both conditions. Having produced equivalent results from two different culturing systems, soluble factors were not considered vital for stromal mediated chemotherapeutic protection of leukemic cells. The authors suggested that N-cadherin expression may play a role in 3D co-culture mediated chemoresistance in leukemia cells.

Along with the stromal niche, the vascular niche plays an important role in maintaining AML disease progression and chemoresistance. In a tri-culture model, human AML cells, human vascular endothelial cells and human mesenchymal stromal cells were grown together using star-shaped polyethylene glycol (starPEG)-heparin hydrogel scaffolds (84). To regulate growth conditions and to enable localized cellular remodeling, heparin components were covalently bound with cell specific adhesion ligands and growth factors, and hydrogel was permeated with matrix metalloproteinase-responsive peptides. Light and confocal microscopy revealed that after 1 week of tri-culture, most AML cells grew in mixtures of spheroids and clusters and accumulated along the vascular endothelial and mesenchymal stromal networks. AML cells were also grown in 2D and 3D co-culture with either vascular endothelial cells or mesenchymal stromal cells, and in 2D and 3D mono-culture formats. Resistance to chemotherapeutics daunorubicin and cytarabine was highest in AML cells grown under 3D tri-culture conditions. When, however, these two drugs were added in combination for a period of 5 days, viability of AML cells grown in 3D tri-culture was reduced by more than 90%. Since ∼10% of the AML cell population survived this initial combination treatment, cells were grown in drug-free medium for 14 additional days. At the end of this recovery period, cell viability was not measurable and was thus, considered 0%, suggesting that a 3D cell culture system may serve more accurately as a model for drug resistance and combination therapy.

To replicate the hematopoietic stem cell bone marrow niche, a macroporous polyethylene glycol hydrogel was infused with a biologically active compound added to promote integrin receptor mediated cell attachment (RGDSK-PEG_6-acrylate), co-seeded with human hematopoietic progenitor cells and human mesenchymal stem cells, and incorporated into a perfusion bioreactor system (85). It was thought that a perfusion system would be more representative of *in vivo* conditions, where nutrients, gases and secreted cellular factors are in continuous motion throughout the bone marrow. Flow rates are, however, adjustable in this system. In the dynamic setting, perfusion occurs. This represents activated conditions and promotes differentiation of hematopoietic stem cells. In static mode, perfusion does not take place. This corresponds to steady-state growth and favors maintenance of hematopoietic stem cells. When compared to treatment with DMSO vehicle control, hematopoietic progenitor cells exposed to chemotherapeutic compound 5-fluorouracil for 5 days under perfusion conditions had an ∼22% death rate, while those under static conditions were more sensitive, dying at a rate of ∼36% over this time period. Testing for the cell surface marker Cluster of Differentiation 34 (CD34), the presence of which identifies cells as being of hematopoietic progenitor subtype, indicated that CD34 positive cells were significantly less sensitive to 5-fluorouracil under static conditions. Under dynamic growth conditions, chemo sensitivity of CD34 positive and negative cell lines was roughly equivalent.

These findings are reflective of those so often experienced by leukemia cancer patients, and cancer patients in general, in that off target effects of chemotherapeutics on non-cancerous tissues cause significant losses in the functionality of normal cells. This study provides another example of the utility of 3D cell culture systems, particularly in the toxicity testing of pharmaceutical compounds.

CONCLUSIONS

Interaction with bone marrow stromal cells and the extracellular matrix is vital to AML cancer cell survival in that it brings about changes in cell signaling that favor drug resistance and provides a safe haven for leukemia stem cells. Traditional cell propagation methods involve growing cells on flat polystyrene surfaces that have been modified to become hydrophilic and negatively charged when serum containing medium is added which, in turn, allows for cell attachment (86). Growing cells in such 2D cultures presents a host of problems because it does not reflect how cells grow in their natural environment. Inhibitors that are toxic to cells grown in this way have historically suffered significant losses in efficacy when applied to cells grown under more disease-relevant conditions (87). Studies indicate that leukemia cells co-cultured with stromal cells (mostly derived from normal donors) in 3D fair generally better after drug treatment than cells co-cultured in 2D or singly cultured in suspension growth systems. However, while many of these studies use cells of human origin, so far there is a lack of cell systems that use AML and stromal cells from the same patient. Such autologous cell systems would be desirable as stromal cells may also undergo genomic alterations in AML patients. Replicating the AML microenvironment in 3D cultures using patient-specific cell systems in combination with high-throughput synergy screening strategies may offer better hope for identifying combinations of drugs for effective treatment of this devastating disease. Thus, if the 3D leukemia/mesenchymal co-culturing method could be miniaturized and formatted for HTS, more relevant leukemia targeting compounds would likely be identified, and the concept of hindering bone marrow niche-induced chemoresistance may ultimately be realized.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Molecular Approaches to Treating Pediatric Leukemias

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Over the past decades, striking progress has been made in the treatment of pediatric leukemia, approaching 90% overall survival in children with acute lymphoblastic leukemia (ALL) and 75% in children with acute myeloid leukemia (AML). This has mainly been achieved through multiagent chemotherapy including CNS prophylaxis and risk-adapted therapy within collaborative clinical trials. However, prognosis in children with refractory or relapsed leukemia remains poor and has not significantly improved despite great efforts. Hence, more effective and less toxic therapies are urgently needed. Our understanding of disease biology, molecular drivers, drug resistance and, thus, the possibility to identify children at high-risk for treatment failure has significantly improved in recent years. Moreover, several new drugs targeting key molecular pathways involved in leukemia development, cell growth, and proliferation have been developed and approved. These striking achievements are linked to the great hope to further improve survival in children with refractory and relapsed leukemia. This review gives an overview on current molecularly targeted therapies in children with leukemia, including kinase, and proteasome inhibitors, epigenetic and enzyme targeting, as well as apoptosis regulators among others.

Keywords: leukemia, children, targeted therapy, precision medicine, molecular approaches

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INTRODUCTION

Leukemia is the most common type of cancer in childhood, accounting for 25–30% of cancers in children and adolescents aged 0–18 years (National Cancer Institute SEER Cancer Stat Facts Annual Report to the Nation 2019, German Childhood Cancer Registry Annual Report 2018). Over the past decades, survival rates have steadily increased and now exceed 90% for acute lymphoblastic leukemia (ALL) and 75% for acute myeloid leukemia (AML) in developed countries (1–5). This success has mainly been achieved through remarkable progress in antileukemic treatment, risk-directed therapy, randomized clinical trials performed by collaborative study groups, supportive care, second-line treatment, and advances in the knowledge of leukemic cell biology including genomic variations (2, 3, 5, 6).

The achievement of improved cure rates and survival into adulthood for most children with leukemia necessitates reduction of acute and long-term toxicity to minimize reduced quality of life, long-term morbidity, and premature death without compromising survival. Yet, even nowadays, leukemia remains the leading cause of death from cancer in children and adolescents in many developed countries. Especially, outcome of refractory/relapsed (r/r) leukemia remains poor (5, 7, 8). Indeed, it becomes more and more difficult to achieve further improvement of survival. This is demonstrated by survival rates of ALL, which seemed to reach a plateau,

and the constant non-response/relapse rates despite intensified first-line therapy in AML (1, 5). In addition, the treatment paradigm of even further intensification of traditional multiagent chemotherapy including stem cell transplantation that allowed long-term disease-free survival in childhood leukemia reaches the point of inflection at which as many children decease due to r/r leukemia—and thus chemoresistance—as well as treatment-related toxicity. This underscores the urgent need to identify more effective and less toxic first line and salvage regimens for these patients.

To this end, the ever-expanding knowledge on leukemia biology is vital in identifying novel therapeutic targets by disclosing the heterogeneity of childhood leukemia, by unveiling the molecular drivers and by understanding the mechanisms of drug resistance. These developments may ultimately brake with the practice paradigms of "one-size-fits-all" therapy and guide the development of precision/personalized treatment including immunotherapy and targeted (genomic) therapy to offer the "right drug for the right patient at the right time" even in children (9). As such, treatment of chronic myeloid leukemia (CML) with imatinib (targeting BCR-ABL) is a prime example for precision oncology.

In recent years, several novel subtypes of AML and ALL with various prognostic impact have been identified. These are mainly characterized by genetic alterations that perturb multiple key cellular pathways including hematopoietic development, signaling or proliferation, and epigenetic regulation (10). These alterations partly include actionable genes and may thus serve as therapeutic targets. This progress is chaperoned by a brisk pace in genomic and immunological drug development. To date, several new drugs that may target these alterations have been approved by the European Medicines Agency (EMA) or the United States Food and Drug Administration (US FDA) or are still under development. However, it is hard to keep up with these rapid achievements in busy daily routine.

Therefore, we herein aimed to give an overview on the most important new drug developments in the treatment of pediatric leukemia. The review focuses on molecular targeted therapies excluding immunotherapy/antibody and CAR-T cell approaches (**Figure 1**, **Table 1**).

KINASE INHIBITORS ACROSS SEVERAL SIGNALING PATHWAYS

More than 500 kinases are encoded in the human genome that are involved in the signal transduction process of the proteome via reversible phosphorylation, thus activating protein function (11, 12). Protein kinases play a major role in cellular regulation including differentiation, survival, proliferation, metabolism, migrating, and signaling, as well as cell-cell interactions. Dysregulated kinases—mainly serine/threonine and tyrosine kinases—are of significant importance in carcinogenesis and metastatic spread (13). Unsurprisingly, previous studies demonstrated that kinases are the most frequently mutated proteins in tumors (14, 15). Moreover, in most malignancies various tyrosine kinases are mutated or overexpressed (16).

The kinome has thereby become an attractive target for the treatment of various human malignancies (11, 12). The first tyrosine kinase inhibitor (TKI), imatinib, was approved for the treatment of CML already in 2001. Generally, kinase inhibitors are employed in those malignancies, which carry specific genetic alterations. To date, 43 orally administered single and multiple kinase inhibitors have been approved and are used in the treatment of a variety of cancers (17). Many more kinase inhibitors are in advanced phase clinical trials and even more in the preclinical stage of drug development.

Most drugs approved to date have limited selectivity thus targeting multiple kinases (18, 19). For example, imatinib shows activity against ABL, BCR-ABL, PDGFR, and c-KIT (20). BCR-ABL kinase selectivity was enhanced with the second-generation TKI nilotinib. The recently approved TKIs bosutinib and ponatinib are designed as dual (SRC-ABL) and multi-kinase (FGFR, PDGFR, SRC, RET, KIT, and FLT1) inhibitors, respectively. Due to potential resistance mechanisms, pharmacokinetics, selectivity and tumor environment, single- and multi-kinase inhibitors have both advantages and disadvantages. In addition, attention should be paid to the various acute and long-term side effects of TKIs including gastrointestinal, cardiovascular, pulmonary, dermatologic, and–particularly in children–endocrine toxicities (21, 22).

Dasatinib has been evaluated in a phase I trial in children and adolescents with imatinib-resistant/-intolerant Philadelphia chromosome (Ph)+ CML, r/r Ph+ ALL and relapsed Ph+ AML (NCT00306202) (23). Based on this, a phase II trial in children with newly diagnosed CML and Ph+ leukemias resistant or intolerant to imatinib (NCT00777036) has been conducted but results have not been published yet. A phase I/II trial for the treatment of r/r leukemias with ponatinib has recently been registered and is not yet recruiting (NCT03934372). The St. Jude trial Total Therapy XVII for Newly Diagnosed Patients With Acute Lymphoblastic Leukemia and Lymphoma (NCT03117751) will give dasatinib to all patients with ABL-class fusions (24).

Other studies evaluate the safety and efficacy of TKI therapy after allogeneic hematopoietic stem cell transplantation (NCT01883219, NCT03624530 both also recruiting adolescents aged 14 years and older) but results are also pending.

JAK/STAT Inhibitors

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is the signaling mechanism for cytokines and growth factors and thus plays a key role in cytokine dependent inflammation and immunity. It also affects gene expression via epigenetic modifications (25, 26). JAK/STAT inhibition was expected to suppress the pro-inflammatory tumor-microenvironment and by doing so to provide a strategy for the prevention of tumor progression (27).

Janus kinases (JAK1, 2 and 3) are frequently mutated in a subset of AMLs and high-risk ALLs (28). However, preclinical data of the JAK1/2 inhibitor AZD1480 in patient-derived xenografts (PDXs) from pediatric ALL showed somewhat disappointing results (29). Currently, a phase II trial of the

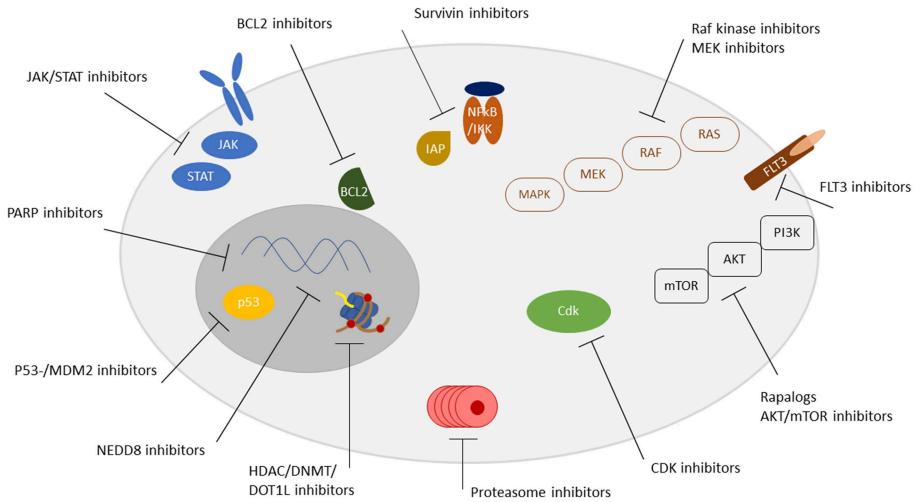

FIGURE 1 | Overview of targetable pathways. Receptors, signaling pathways, and localization of targetable structures are indicated.

TABLE 1 | Overview of druggable pathways and genetic targets.

Pathway/mechanism of action	(Genetic) target	Drug name	Adult trials	Pediatric trials	FLT3-ITD	KMT2A-r	Ph+	IDH1/2
Kinase inhibition	JAK-STAT pathway	Ruxolitinib	+	+			+	
	FLT3 inhibitor	Midostaurin, quizartinib, lestaurtinib	+	+	+	+		
	MEK inhibitor	Trametinib, selumetinib	+	+				
	Multi-kinase inhibitors	Imatinib, ponatinib, dasatinib, sorafenib	+	+	+		+	
Proteasome/ubiquitin system	Proteasome	Bortezomib, carfilzomib, ixazomib	+	+		+		
	MDM2	Idasanutlin, milademetan, ALRN-6924	+	+	+	(+)	(+)	
	NEDD8	Pevonedistat	+	+				
Epigenetic targeting	HDAC	Vorinostat, panobinostat	+	+				
	DNMT	Azacitidine, decitabine	+	+				
	DOT1L	Pinometostat	+	+		(+)		
Apoptosis	TP53	APR-246	+	No				
	MCL1	S64315	+	No				
	BCL2	navitoclax, venetoclax	+	+		(+)	(+)	+
	survivin	EZN-3042, LY2181308	No	No				
Other approaches	IDH1	Ivosidenib	+	+				+
	CDK4/6	Palbociclib, ribociclib	+	+		+	(+)	
	PARP	Olaparib, veliparib	+	No	+			+
	mTOR	Everolimus, temsirolimus, sirolimus	+	+				
	Menin	MI-463, MI-503, MI-1481, MI-525	No	No		(+)		
	CBFβ-SMMHC	AI-10-49	No	No				

Drug names are indicated. +, studies currently recruiting (adult trials or pediatric trials, respectively). FLT3-ITD, KMT2A-r, Ph+, IDH1: cases, in which these leukemia specific defects can be targeted by any of the mentioned drugs are designated by "+".

JAK1/2 inhibitor ruxolitinib in combination with standard multi-agent chemotherapy for the treatment of B-precursor ALL in children is running and evaluates the safety and efficacy of this combination (NCT02723994). An already completed phase I/II study of ruxolitinib included patients aged 14 years and older with r/r AML and ALL (NCT01251965). In these heavily pretreated patients, ruxolitinib was reasonably well tolerated with infections being the most frequently reported non-hematologic grade 3 and 4 toxicity (30). Just recently, data from preclinical models provided evidence, that JAK1/2 inhibitors may also be active in pediatric acute megakaryoblastic leukemia (31, 32).

FLT3 Inhibitors

FMS-like tyrosine kinase 3 (*FLT3*) encodes a class III receptor tyrosine kinase controlling survival, proliferation, and hematopoietic cell differentiation. In up to 20% of pediatric patients with AML, particularly in cytogenetically normal AML, *FLT3* is mutated and confers a poor prognosis (33, 34).

Several non-specific TKIs (e.g., sorafenib, sunitinib) target FLT3 and have been approved for the treatment of a variety of solid malignancies (35). However, they are commonly associated with significant side-effects and toxicity.

The first-in-class FLT3 inhibitor—midostaurin—has been approved for the treatment of adult patients with FLT3-mutated AML. A phase I/II study with single-agent midostaurin (PKC412) in children with r/r acute leukemia including *KMT2A*-rearranged ALL and *FLT3*-mutated AML was terminated early due to insufficient recruitment (NCT00866281). In this heavily pretreated cohort, midostaurin showed limited activity in *FLT3*-mutated AML, whereas in r/r *KMT2A*-rearranged ALL, which is often associated with high-level expression of mutation-negative FLT3 (36), no clinical activity of midostaurin was demonstrated (37). Currently, a phase II trial evaluating midostaurin combined with standard chemotherapy and as single agent post-consolidation therapy is recruiting children with untreated FLT3-mutated AML (NCT03591510).

Another FLT3-inhibitor, quizartinib (AC220), is currently investigated in a phase I/II trial in children with *FLT3*-mutated r/r AML in combination with re-induction chemotherapy and as single-agent maintenance therapy (NCT03793478).

Further phase I/II studies evaluating the safety and efficacy of FLT3-inhibitors (e.g., lestaurtinib) as single-agent therapy or in combination with standard chemotherapy in children with newly diagnosed AML, *KMT2A*-rearranged ALL and r/r AML have already been completed but results have not been published yet (e.g., NCT00469859, NCT00557193).

MEK Inhibitors

Mitogen-activated protein kinase/ERK kinase (MEK 1/2) inhibitors were the first selective inhibitors of the Ras/Raf/MEK/ERK pathway (also known as MAPK pathway), the latter playing a critical role in the regulation of cell proliferation, differentiation, and survival (38). *MAPK* pathway activating mutations are a hallmark of pediatric low-grade glioma (39) and are highly prevalent in relapsed ALL. Moreover, in relapsed ALL, these mutations are associated with high-risk features and dismal prognosis (40, 41).

To date, only two studies are registered that investigate MEK inhibitors in children with leukemia. In a phase II trial, trametinib is evaluated in children with r/r juvenile myelomonocytic leukemia (JMML) (NCT03190915). Interestingly, following promising preclinical data on a combination of the MEK inhibitor selumetinib and dexamethasone in RAS pathway mutated ALL primagraft cells (42), this combination is now investigated in a phase I/II trial (Seludex trial) in children with r/r RAS pathway mutated ALL (NCT03705507).

To conclude, beside imatinib in the treatment of Ph+ CML and ALL, so far, there is no proof that the use of TKIs—either as single-agent or combination treatment—improves survival in pediatric AML and ALL. Most likely, single small subgroups of children with ALL and AML may benefit from the addition of selected TKIs to conventional chemotherapy and as post-remission/-transplant maintenance, respectively. However, substantial acute toxicity and long-term side effects including hematologic, gastrointestinal, cardiovascular, dermatologic, and endocrine toxicities with varying severity depending on the TKI used need to be considered.

UBIQUITIN-PROTEASOME SYSTEM

Proteasome Inhibitors

The proteasome is a large, multi-subunit protein complex, which is responsible for the degradation of most cellular proteins in physiological conditions, thereby playing a vital role in most cellular processes including cell survival and signaling. As cancer cells have an elevated protein turnover, it was hypothesized early on that they might be sensitive to proteasome inhibition, which indeed turned out to be the case. The first proteasome inhibitor to enter clinical trials was bortezomib, a reversible inhibitor of the 26S subunit (43). Single-agent bortezomib treatment was mostly not efficient. However, the combination with various chemotherapeutic agents proved to be highly beneficial (44). Currently, there are three proteasome inhibitors approved by the FDA, namely bortezomib, carfilzomib, and ixazomib, the latter being the first orally available drug.

The exact molecular consequences of proteasome inhibition via bortezomib are still unsolved, but multiple pathways seem to be involved. One of those results in the stabilization of I-κB, a suppressor of NF-κB signaling, another in the accumulation of the two tumor suppressors $p27^{KIP1}$ and p53 (45, 46).

The TACL study in children with relapsed ALL demonstrated that the combination of bortezomib with vincristine, dexamethasone, doxorubicin, and pegylated asparaginase is highly active in B-precursor ALL but not in T-ALL (47). In using bortezomib, particular attention is needed to infectious complications and peripheral neuropathy. There are currently seven pediatric trials recruiting patients (all ALL) employing bortezomib. All use it in combination with standard chemotherapy backbones. These are AIEOP-BFM ALL 2017 (NCT03643276), International Study for Treatment of High Risk Childhood Relapsed ALL 2010 (NCT03590171), Total Therapy for Infants With Acute Lymphoblastic Leukemia (ALL) I (NCT02553460), Total Therapy XVII for Newly Diagnosed Patients With Acute Lymphoblastic Leukemia and Lymphoma (NCT03117751), ALL-MB 2015 (NCT03390387), a relapse study run by St. Jude Children's Research Hospital (NCT03515200), a relapse study run by the M.D. Anderson Cancer Center (NCT03136146), and a high throughput-guided approach coupled to targeted therapy (NCT02551718, this study also tests carfilzomib). One pediatric AML trial evaluates the combination of bortezomib with sorafenib (NCT01371981).

Carfilzomib, a proteasome inhibitor with fewer off-target effects and supposedly higher degree of inhibition, is tested in one additional pediatric study in r/r ALL (NCT02303821).

Two pediatric ALL studies test the orally available ixazomib (NCT03817320, NCT03888534) in a r/r setting.

MDM2 Inhibitors

TP53 represents the gene most frequently mutated in human tumors (in some entities up to 80% of cases have heterozygous mutations); mutations are commonly located in the core p53 DNA-binding domain. Wild-type p53 plays central roles in the transcriptional regulation of genes involved in cell-cycle arrest, DNA repair, apoptosis, and senescence. Tumors with wild-type p53 frequently found other ways to block p53 function, such as MDM2 overexpression, which results in p53 inactivation and degradation (48).

Currently, there are two major therapeutic strategies for restoring p53 function in tumor cells, namely increasing the levels of wild-type p53 by preventing its MDM2-mediated degradation. The second approach is to restore p53 transcriptional activity through targeting p53-mutant proteins (see below, section "Targeting Mutant TP53") (48).

The first small molecule MDM2 inhibitors were nutlin derivatives, which bind the p53-binding cleft of the MDM2 protein. Following exposure to nutlin, cancer cells expressing wild-type p53 (*TP53* mutated cells are intrinsically resistant to this approach) undergo cell cycle arrest and/or apoptosis (48). Interestingly, pre-treatment MDM2 expression can be correlated to clinical response, enabling its use as a biomarker (49).

Available evidence on the efficacy of MDM2 inhibitors in preclinical models of AML as well as clinical studies were recently reviewed (49). There is currently a phase I pediatric trial ongoing, which tests the combination of ALRN-6924 (a dual MDM2/MDMX inhibitor) with cytarabine (NCT03654716) in r/r AML.

Evidence of the therapeutic benefits of MDM2 inhibitors in ALL was also recently reviewed (50). Despite several preclinical and early clinical observations of the efficacy in various poor risk subtypes including Ph+ (51) and *KMT2A*-rearranged ALL (52), there is currently no clinical trial recruiting ALL patients.

NEDD8 Inhibitor

Neddylation is a posttranslational modification, through which the ubiquitin-like protein NEDD8 is added to lysine residues of substrate proteins. Neddylation is triggered by a cascade of NEDD8-activating enzymes, regulating well known tumor suppressors and oncoproteins, such as VHL, p53, and MDM2 (53).

As such, it does not come as a surprise that neddylation is frequently highly activated in several malignancies and thus presents an attractive therapeutic target. The first NEDD8-activating enzyme (NAE) inhibitor was MLN4924, which functions by blocking the first step of the neddylation cascade. MLN4924 (also known as pevonedistat) has significant tumor-inhibiting effects mainly by triggering apoptosis, senescence and autophagy (54). More recently, there was also preclinical evidence for possibly more potent inhibitors such as TAS4464 (55).

MLN4924 was recently tested in the treatment of AML as NEDD8 regulates the cullin subunits of Cullin-RING ligases (CRLs). These represent the largest family of E3 ubiquitin ligases controlling degradation of about one-fifth of proteasome-regulated proteins and are vital for AML cell survival, among others. Increased neddylation of substrates including cullins promotes degradation of tumor suppressors (e.g., p21 and p27) and facilitates tumorigenesis (54). MLN4924 was indeed shown to have tumor growth inhibiting properties in AML cells *in vitro* and in xenograft assays, later also in clinical studies (56, 57). Currently, there are two studies recruiting pediatric patients, one adding pevonedistat in r/r ALL to a standard backbone ALL chemotherapy regimen (NCT03349281) and the other adding pevonedistat/azacitidine/fludarabine/cytarabine in r/r AML (NCT03813147).

To sum up, bortezomib, one of several inhibitors of the ubiquitin-proteasome system, has already entered phase III clinical trials in children with r/r acute leukemias. However, single-agent treatment was not efficient and whether its combination with conventional chemotherapy improves survival is currently being evaluated. Like any other molecular targeted drug, in combinatorial approaches it comes with considerable side effects including deaths due to infections.

EPIGENETIC TARGETING

HDAC Inhibitors

Histone deacetylases (HDACs) are a key component of the epigenetic machinery regulating gene expression. Deacetylation results in a closed chromatin structure and consequently in suppressed transcription of many genes including tumor suppressor genes. HDACs are overexpressed and mutated in tumors, which has led to the hypothesis that they may act as oncogenes (58). In humans, there are 18 HDAC proteins, which are grouped into four classes. HDAC inhibitors block proliferation, induce cell cycle arrest and apoptosis and lead to differentiation. Despite the fact, that HDACs play roles in a vast number of cellular processes (and thus targeting them might come with many off-target effects), they represent desirable therapeutic targets and many HDAC inhibitors have been developed (59). So far, the four pan-HDAC inhibitors vorinostat, romidepsin, bellinostat, and panobinostat have been approved by the FDA. They come with a very similar toxicity profile including gastrointestinal, neurological, and (transient) hematologic toxicities, fatigue and (asymptomatic) ECG changes (60).

Many (pre-)clinical data exist on the use of HDAC inhibitors in leukemias (61). One study showed that pretreatment with vorinostat and/or decitabine induced chemosensitivity in primary pediatric ALL samples (62). Likewise, decitabine and vorinostat followed by re-induction chemotherapy demonstrated a clinical benefit in patients with r/r ALL (patient cohort with a median age of 16 years) (63). In pediatric AML, the combination of azacitidine and panobinostat induced remission in a mouse xenograft model (64). In adult patients, the combination of decitabine plus vorinostat was well tolerated and resulted in a higher response rate (65).

The currently recruiting study NCT02553460 run by the St. Jude Children's Research Hospital tests the addition of bortezomib and vorinostat in infant ALL. Additionally, vorinostat is administered to those childhood T-ALL patients, who showed a poor early response to treatment without a targetable lesion (NCT03117751; Total Therapy XVII study). In pediatric AML, the addition of vorinostat and decitabine to FLAG is also currently evaluated (NCT03263936).

DNMT Inhibitors

DNA methylation is another major epigenetic modification that impacts nearly all cellular processes. DNA methyltransferases (DNMTs) are overexpressed in several cancer types contributing to tumorigenesis. DNMT3A is mutated in up to 22% of AML patients (66) (its catalytic activity being disrupted by the mutation), but mutations in other genes affecting DNA methylation such as TET1/TET2 have also frequently been described (58).

Azacitidine and decitabine are nucleoside analogous, which are incorporated into DNA resulting in depletion of DNMTs, hypomethylation of DNA, and induction of DNA damage (67). Azacitidine has been successfully used in pediatric MDS (68), common toxicities were hematologic and gastrointestinal.

A phase I clinical trial demonstrated that azacitidine followed by intensive chemotherapy can be used safely to treat children with r/r AML and showed promising activity (69). In T-ALL, first results suggested that decitabine enhances chemosensitivity of both cell lines and patient-derived samples (70).

Treatment with HDAC inhibitors results in increased DNMT1 acetylation and decreased total DNMT1 protein (71). As it has been known for some time that the combination of DNMT and HDAC inhibitors may result in even superior therapeutic outcomes (72), these two drug classes are now frequently combined (for those studies see the section above on HDAC inhibitors). Azacitidine and decitabine are currently compared in a randomized trial in pediatric patients newly diagnosed with AML (NCT03164057). Another trial evaluates the novel approach of treating a molecular AML relapse after first complete remission in pediatric patients (NCT02450877).

DOT1L Methyltransferase Inhibitor

Disruptor of telomeric-silencing 1-like (DOT1L) is an enzyme, which methylates H3K79. The abnormal expression of KMT2A fusion target genes is associated with high levels of H3K79 methylation at these gene loci (58, 73). Loss-of-function mouse models, as well as small molecular inhibitors of DOT1L, reported that *KMT2A*-rearranged leukemias are DOT1L dependent for proliferation (74).

A recently completed study in adults with advanced *MLL* rearranged acute leukemias showed that the addition of pinometostat, a small molecule inhibitor of DOT1L, was safe with tolerable toxicities including fatigue, nausea, constipation, and febrile neutropenia. Although it has to be noted that this was a phase I study, modest clinical activity could be shown (75). Pinometostat has also been evaluated in a phase I study in children with KMT2A-rearranged r/r leukemias (NCT02141828) but results have not been published yet.

APOPTOSIS REGULATORS

Targeting Mutant TP53

The general principles of targeting p53 were already detailed above (see section "MDM2 Inhibitors"). As explained, the second approach (besides inhibiting MDM2) involves restoring p53 transcriptional activity (48). Despite being originally considered as undruggable, a small molecule screen nearly 20 years ago identified APR-246 via its ability to induce apoptosis in human tumor cells through restoration of the transactivation function of mutant p53 (76). Since that time, several reports have shown anti-leukemic activity of APR-246 *in vitro* both in ALL (77) and AML (78, 79). A first-in-human trial in refractory hematologic malignancies concluded that APR-246 could be administered safely and induced p53-dependent biologic effects in tumor cells *in vivo* (80).

Currently, there are only studies recruiting, which test APR-246 plus azacitidine in adults with TP53 mutant MDS/MPN/CMML/AML plus or minus allogeneic stem cell transplantation. Unpublished results of the latter (abstract Blood 2018 132:3091) show that this combination is well tolerated. Responses have been achieved in all evaluable pts (82% CR) accompanied by deep molecular and durable remissions. In April 2019, a fast track and orphan drug designation was granted by the FDA for APR-246 in *TP53*-mutated MDS.

In summary, more studies with molecules targeting mutant p53 (APR-246 being the only one, which has been employed in hematologic malignancies) are needed, as it is not yet clear, which of the nearly 1,500 different missense mutations they are able to target (48) but first results are encouraging.

BCL2 Inhibitors

The oncogenic protein B-cell lymphoma 2 (BCL2) was discovered in B-cell leukemias and follicular lymphomas 40 years ago. BCL2 induces transformation by blocking apoptosis. Later, many structurally related proteins were identified, which are clustered into three groups: (i) multidomain anti-apoptotic proteins such as BCL2, BCL-XL and MCL1, (ii) multidomain pro-apoptotic effector proteins such as BAX or BAK and (iii) BH3-only group of pro-apoptotic proteins (including BIM, BID, PUMA, BAD, BIK, NOXA) (49).

Members of the BCL-2 gene family play vital roles in apoptosis by controlling pro-apoptotic and anti-apoptotic intracellular signals (81). Inhibitors targeting both MCL1 and BCL2 have been developed.

Navitoclax, a first generation BCL-2 inhibitor targeting BCL-2 and BCL-XL, showed clinical activity, however, its use was restricted by the occurrence of neutropenia. Shortly after, the selective BCL-2 inhibitor venetoclax was developed and approved by FDA/EMA for a subset of CLL patients. In acute leukemias, venetoclax has shown efficacy both in AML and in ALL. In using venetoclax, attention is needed to tumor lysis syndrome, other side effects include hematologic and gastrointestinal toxicity, respiratory infections, and fatigue (82).

Of special interest in the pediatric setting was a report, which showed that patient-derived ALL cells carrying the *KMT2A-AF4* fusion had high BCL-2 levels (via H3K79 methylation through

DOT1L) and were highly sensitive to treatment with venetoclax, also in a xenograft setting (83).

Moreover, the combination of dasatinib and venetoclax resulted in superior antileukemic efficacy compared to either agent alone in Ph+ ALL xenografts (84). Another report showed that the combination of venetoclax and the JNK inhibitor SP600125 exhibited synergistic cytotoxicity against imatinib-resistant Ph+ ALL cells (85).

Several recruiting studies test the combination of venetoclax with cytarabine and daunorubicine liposome (NCT03826992), with cytarabine with or without idarubicin (NCT03194932), and with navitoclax and chemotherapy (NCT0318126) in children with r r/r ALL, AML and acute leukemia of ambiguous lineage, respectively, to name just a few. In addition, one study run by the MD Anderson Cancer Center including children is testing venetoclax in combination with chemotherapy including nelarabine in previously untreated patients with T-ALL and lymphoblastic lymphoma (NCT00501826). Noteworthy, a phase I/II study has just recently been registered but is not yet recruiting which will evaluate the MDM2 antagonist idasanutlin in combination with either chemotherapy or venetoclax in children and young adults with r/r acute leukemias (NCT04029688).

Survivin Inhibitors

Survivin (*BIRC5*) is a member of the inhibitor-of-apoptosis proteins (IAPs) family. It is an oncofetal protein, which is not expressed in differentiated normal tissue. Furthermore, survivin overexpression has been correlated with resistant and refractory disease in many different malignancies. IAPs regulate caspase activity, cell division, and cell survival pathways as well as being involved in DNA repair and drug resistance (86).

An early report showed that an imatinib-resistant Ph+ chronic myelogenous leukemia (CML) cell line had high survivin expression levels. Down-regulating survivin expression induced cell-growth arrest and subsequent cell death (87). Later, high survivin expression levels were also reported in ALL and downregulation of survivin via the antisense oligonucleotide EZN-3042 in combination with chemotherapy resulted in deep molecular remission of disease in a xenograft model (88). Later, this drug was employed in a trial with pediatric r/r ALL. However, the combination of EZN-3042 with intensive reinduction chemotherapy led to intolerable toxicity (grade 3 gamma-glutamyl transferase elevation and gastrointestinal bleeding) and the trial was terminated (89).

Another survivin antisense oligonucleotide, LY2181308, was tested in an adult phase I refractory/relapsed AML study, both as a monotherapy and in combination with cytarabine and idarubicine. In this case, the drug was well tolerated and showed some clinical benefit, which will need to be verified in future clinical trials (90).

In summary, despite the growing knowledge on survivin and despite its important roles in oncogenesis, the development of survivin inhibitors or survivin-related molecular therapies has been slow (86). Currently, there are no trials listed employing survivin inhibitors.

OTHER TARGETED APPROACHES

Targeting Metabolic Enzymes

Two isoforms of isocitrate dehydrogenase (IDH), *IDH1* and *IDH2*, are among the most commonly mutated genes in AML occurring in about 20% of all newly diagnosed patients. They are key enzymes in the metabolism of a cell and also function in the regulation of oxidative stress. The heterozygous, mutually exclusive mutations occur at hotspot positions ($IDH1^{R132}$, $IDH2^{R140}$, $IDH2^{R172}$) and lead to a neomorphic enzyme activity resulting in the generation of very high levels of 2-hydroxyglutarate. This oncometabolite causes epigenetic changes and impairs cell differentiation. Preclinical data indicated early on that targeted inhibition of both IDH1 and IDH2 blocked colony formation of AML cells from *IDH1*-mutated patients and induced differentiation (91, 92).

As there is great excitement regarding this class of inhibitors, many studies testing these substances both as monotherapies as well as in addition to standard chemotherapy backbones are being conducted, however, not yet in a pediatric setting, where *IDH1/2* mutations are rare. Only ivosidenib, a small-molecule IDH1 inhibitor approved by the FDA in 2018, is offered in an expanded access program in r/r AML with an $IDH1^{R132}$ mutation to children $\geq$12 years of age (NCT03245424).

Cell Cycle Regulation

Loss of cell cycle control resulting in unrestrained growth is generally considered a hallmark of cancer and aberrations in the cyclin-dependent kinase-retinoblastoma (CDK-Rb) pathway are common in multiple malignancies. Consequently, inhibition of this pathway is an attractive therapeutic strategy. The G1 cyclin-dependent kinases 4 and 6 (CDK4 and 6) phosphorylate—in a complex with cyclin D—retinoblastoma protein (Rb), which leads to cell cycle progression and cell growth (93).

More than 10 years ago, it was reported that the D-cyclin-CDK4/6 complex was a downstream effector of FLT3-ITD signaling. Inhibiting CDK4/6 caused sustained cell-cycle arrest (94). Another study showed that the CDK4/CDK6 kinase inhibitor palbociclib (PD 0332991) sensitized AML cells to cytarabine, opening the possibility of a combination therapy (95).

Ribociclib, another CDK4/CDK6 kinase inhibitor, enhanced glucocorticoid sensitivity in primary cultures derived from bone marrow of pediatric B-precursor ALL patients (96). A recent study showed that palbociclib suppressed dissemination of Ph+ ALL and prolonged survival in a xenograft model (97).

Of special interest in the pediatric setting was the observation, that the KMT2A fusion proteins activate CDK6, thus driving proliferation in *KMT2A*-rearranged infant ALL. Treating *KMT2A*-rearranged leukemia cell lines with palbociclib resulted in a G1 arrest in ALL (98).

The high interest in this class of drugs is reflected by a total of three studies recruiting pediatric patients, namely one, which evaluates ribociclib/everolimus/dexamethasone in relapsed ALL (NCT03740334), and two combining palbociclib with mostly standard chemotherapy backbones in r/r ALL (NCT03515200, NCT03792256).

The various CDK4/6 inhibitors show similar side effects including high-grade hematologic toxicities, gastrointestinal and hepatobiliary toxicities, and QTc prolongation.

PARP Inhibitors

Several mechanisms seem to be responsible for the action of poly (ADP-ribose) polymerase (PARP) inhibitors, one is synthetic lethality. The PARP inhibitor blocks base excision repair leading to a double strand break. As tumor cells frequently have defects in homologous repair genes—such as *BRCA1*, *BRCA2*, *ATM*, or Fanconi anemia pathway mutations (which on their own are advantageous and result in growth advantages through increased genomic instability)—it will be unable to repair the double strand defect and will undergo apoptosis (99). This makes the concept of PARP inhibitors attractive, as they target cancer cells based on their genetic deficiencies while sparing normal cells, which have backup mechanisms for repairing DNA strand breaks.

Preclinical evidence of the potency of PARP inhibitors in leukemia included anti-proliferative effects in T-ALL cell lines (100) and re-sensitizing adriamycin-resistant leukemia cells (101). Recently, it was hypothesized, that *TET2*, which is frequently mutated in hematologic malignancies, maintains genomic stability via promotion of DNA damage repair and that loss of *TET2* might sensitize myeloid leukemia cells to PARP inhibitors (102).

As mentioned above, FLT3-ITDs, which can be found in up to 23% of AML patients, confer a poor prognosis. It was shown that treatment with a FLT3 inhibitor caused downregulation of DNA repair proteins. The combination with a PARP inhibitor significantly delayed disease onset and reduced leukemia-initiating cells in a FLT3-ITD-positive primary AML xenograft mouse model (103).

Many PARP inhibitors have been developed, of which two (olaparib and veliparib) have been tested in acute leukemias. A previous trial reported that veliparib/temozolomide was well tolerated and showed activity in advanced AML (104). Several current trials are exploring PARP inhibitors, however only in the adult setting.

mTOR Inhibitors

The PI3K/Akt/mTOR pathway is a key regulatory pathway, which controls cell growth, survival, and cellular metabolism. The discovery of high mutational frequencies in multiple malignancies of both genes in the pathway itself but also in upstream, membrane-associated genes, early on sparked interest in targeting this pathway therapeutically (105). Upon mutation, this pathway becomes constitutively active in several malignancies, including ALL and AML (106). In some solid malignancies such as breast cancer and renal cell carcinoma, mTOR inhibitors are already firmly established in therapeutic regimens (107).

Early studies in leukemia showed that allosteric mTOR inhibitors resulted in decreased growth and induced apoptosis in ALL cell lines as well as xenograft models (108). Mammalian target of rapamycin (mTOR) exists in two complexes, mTORC1 and mTORC2. The rapamycin analogs (rapalogs) everolimus, temsirolimus and sirolimus, which target mTORC1 through binding to the protein FKBP12 (109), were the first mTOR inhibitors to enter clinical trials.

Several studies have been performed so far with either rapalogs as monotherapy or as an addition to established therapies. However, the addition of temsirolimus to UKALL R3 in children with second or greater relapse of ALL resulted in excessive toxicity including mucositis, ulceration, hypertension with reversible leukoencephalopathy, liver toxicity and sepsis. Moreover, despite the fact, that an inhibition of PI3K signaling was detected in all patients at an early timepoint, there was no correlation with clinical responses at the end of re-induction therapy (110). Many more studies have been conducted in leukemias [for an overview see Fransecky et al. (105)] but mostly with only modest clinical responses.

A recent phase I trial tested co-administering everolimus with a four-drug reinduction in children with ALL and came to the conclusion that this treatment was well tolerated and was associated with favorable CR2 rates (111). However, only 22 patients were enrolled.

Currently, there are several studies recruiting, which are exploring temsirolimus (NCT01614197), everolimus (NCT03740334) and other rapalogs and some, for which recruitment was terminated but for which results are still pending.

In summary, given the initially convincing preclinical data on mTOR inhibition in acute leukemias, the results of nearly all clinical trials have not lived up to the expectations. Yet, there is still much interest in targeting this pathway and novel inhibitors are still being developed for use in future studies (105).

Menin Inhibitors

Menin, encoded by the *MEN1* gene, is considered an oncogenic cofactor of KMT2A fusion proteins, as it functions as an adaptor between KMT2A and LEDGF. It was shown that its presence is required for transformation of *KMT2A*-rearranged leukemia (112, 113).

As it represents an attractive therapeutic target, a whole series of small molecule inhibitors [i.e., MI-463 and MI-503 (114)] were developed, which block the KMT2A-binding site on Menin in a fusion-partner independent manner. This led to a downregulation of KMT2A-fusion targets, differentiation of leukemic blasts, and prolonged survival of mouse models of *KMT2A*-rearranged leukemia (114, 115). Recently, two more menin inhibitors were reported [MI-1481 (116) and M-525 (117)]. One group reported that the combination of a menin inhibitor with a DOT1L inhibitor proved beneficial as it facilitated enhanced induction of differentiation and apoptosis in a mouse model injected with *KMT2A-AF9* leukemic cells (118).

However, evidence on menin inhibitors has so far only been preclinical, as none of those compounds has entered clinical trials yet.

Targeting the CBFβ-SMMHC Rearrangement

AML is characterized by recurrent chromosomal rearrangements encoding for fusion proteins that both initiate and maintain leukemic cell growth. One such rearrangement is inv (16)

(p13q22) resulting in the CBFβ-SMMHC fusion protein. This transcription factor fusion outcompetes wild-type CBFβ for binding to the transcription factor RUNX1, neutralizes RUNX1-mediated repression of MYC expression, and induces AML. A small molecule protein-protein interaction inhibitor, AI-10-49, was developed, which selectively binds to CBFβ-SMMHC and disrupts its binding to RUNX1 (119, 120). Treatment of primary inv (16) AML patient blasts with AI-10-49 restored RUNX1 transcriptional activity and delayed leukemia progression in mice (121). However, AI-10-49 has not entered clinical trials so far.

CONCLUSION AND OUTLOOK

The concept of contemporary randomized clinical trial (RCT) designs providing risk-adapted therapy has significantly contributed to the success story of acute leukemia in childhood. To date, the gold standard in leukemia treatment is multiagent chemotherapy, in some cases including hematopoietic stem cell transplantation, within traditional RCTs. Recent advances in cancer genomics, knowledge on stem cell biology, and experimental modeling of leukemia provide new insights into leukemogenesis, subtypes of leukemia, drug resistance, host pharmacogenetics, and potential therapeutic approaches as well as targeted therapies. In addition, a wealth of new cancer drugs has been approved by the EMA and US FDA and most likely much more will be approved in the near future.

A number of the recently approved new cancer drugs have already been evaluated in children with leukemia. However, except for rare examples such as imatinib, groundbreaking improvements in survival have not been achieved. Instead, most of them failed to demonstrate sufficient single-agent activity. For instance, employing 53 approved new cancer drugs (across several cancer types not only hematologic malignancies), only a mean total increase in overall survival of 3.4 months over the so far available treatments was seen in adults (122). Thus, the great hopes accompanied by precision medicine have -at least in adults- not been fulfilled so far. Yet, the impact of targeted therapies on improving survival in children with cancer still remains to elucidated by the range of pediatric precision oncology trials which are now available.

Most likely, optimal (personalized) treatment strategies in children and adolescents with acute leukemias will have to integrate traditional chemotherapy, immunotherapy, and molecularly targeted drugs as well as combinations of those to obtain synergistic effects. Noteworthy, currently, all different types of immunotherapies are prioritized, in, at least, ALL.

AUTHOR CONTRIBUTIONS

MK and JH wrote the manuscript. J-HK revised the manuscript critically for important intellectual content. All authors contributed to manuscript revision, read and approved the submitted version.

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REVIEW
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Pediatric Acute Myeloid Leukemia (AML): From Genes to Models Toward Targeted Therapeutic Intervention

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This review aims to provide an overview of the current knowledge of the genetic lesions driving pediatric acute myeloid leukemia (AML), emerging biological concepts, and strategies for therapeutic intervention. Hereby, we focus on lesions that preferentially or exclusively occur in pediatric patients and molecular markers of aggressive disease with often poor outcome including fusion oncogenes that involve epigenetic regulators like KMT2A, NUP98, or CBFA2T3, respectively. Functional studies were able to demonstrate cooperation with signaling mutations leading to constitutive activation of FLT3 or the RAS signal transduction pathways. We discuss the issues faced to faithfully model pediatric acute leukemia in mice. Emerging experimental evidence suggests that the disease phenotype is dependent on the appropriate expression and activity of the driver fusion oncogenes during a particular window of opportunity during fetal development. We also highlight biochemical studies that deciphered some molecular mechanisms of malignant transformation by KMT2A, NUP98, and CBFA2T3 fusions, which, in some instances, allowed the development of small molecules with potent anti-leukemic activities in preclinical models (e.g., inhibitors of the KMT2A–MENIN interaction). Finally, we discuss other potential therapeutic strategies that not only target driver fusion-controlled signals but also interfere with the transformed cell state either by exploiting the primed apoptosis or vulnerable metabolic states or by increasing tumor cell recognition and elimination by the immune system.

Keywords: pediatric AML, genomic landscape, mouse models, fusion oncogene, therapeutic targeting, UKBB

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GENOMIC LANDSCAPE OF PEDIATRIC AML

From Cytogenetics to Next-Generation Sequencing

Molecular hematology–oncology starting in the 1970s of the last century was heavily influenced by the pioneering work of Janet Rowley and others that used conventional cytogenetics followed by the upcoming recombinant DNA technology to show that, in addition to other structural lesions, balanced chromosomal translocations frequently lead to expression of fusion genes (1). Following these developments, the classification of leukemia evolved from a morphology-based classification to the progressive, and still ongoing, inclusion of genetic-based criteria (2, 3). During the last decade, high-throughput sequencing technologies (often referred to as next-generation sequencing, NGS) have facilitated the establishment of almost complete maps of the genomic landscape of

leukemic cells in acute myeloid leukemia (AML) patients (4). In landmark studies by Timothy Ley and members of the Cancer Genome Atlas Research Network, the genome of a single AML patient was obtained in 2008. They later sequenced the whole genome of 24 selected AML cases but also the exomes of the progeny of hematopoietic stem and progenitor cells (HSPCs) taken from seven healthy individuals of different age (5, 6). Subsequently, they characterized the genomes of 200 clinically annotated adult cases of *de novo* AML either by whole-genome sequencing or exome sequencing along with RNA, miRNA sequencing, and DNA methylation analysis (7). Together with previous genetic and functional studies, several important observations can be highlighted. Firstly, the mutational rate of AML cells is lower than for most other cancers. Secondly, almost all samples had at least one mutation in genes of nine different categories [transcription factor fusions, nucleophosmin (NPM1), tumor suppressors, DNA-methylation-related genes, signaling mediators, chromatin modifiers, myeloid transcription factors, cohesin genes and spliceosome complex]. Thirdly, recurrent patterns of co-existence suggested functional cooperation as previously reported for transcription factor fusions/mutations [often referred to as "class II mutations"] and signaling mutations in tyrosine kinases or RAS-type GTPase (RAS) [often referred to as "class I mutations"] but also novel mutations targeting epigenetic regulators such as DNA methyltransferase 3a (DNMT3A) and isocitrate dehydrogenase (IDH)1/2 became apparent. Together with functional studies, these associations suggest that as little as two mutations in different categories might be sufficient to initiate leukemogenesis. Finally, the data obtained from healthy individuals suggested that the HSC compartment accumulates about 10–15 single-nucleotide variants every year.

Over a decade later, the Children's Oncology Group (COG)–National Cancer Institute (NCI) TARGET AML initiative was able to characterize the genomic landscape of almost 1,000 pediatric AML patients by whole genome sequencing of samples from 197 and targeted sequencing of tumor cells from 800 patients (8). This extensive effort revealed similarities but also important differences between adult and pediatric AML. First, the overall somatic mutation frequency in pediatric AML is lower than that in adult patients. Notably, the mutational burden increases with age, with fusions and focal copy number aberrations being more common in younger patients, whereas smaller sequence variants are more frequent in older individuals. Second, pediatric AML patients with fusions involving transcriptional regulators like lysine methyltransferase 2A (KMT2A), CBFA2/RUNX1 translocation partner 3 (CBFA2T3), or motor neuron and pancreas homeobox 1 (MNX1) tend to have few additional mutations and were associated with a particularly poor outcome. Third, distinct combinations of co-occurring alterations, such as the nucleoporin 98 (NUP98)–nuclear receptor binding SET domain protein 1 (NSD1) fusion and mutation of fms-related tyrosine kinase 3 (FLT3) or WT1 transcription factor (WT1), were observed, significantly affecting disease outcome. Fourth, alterations in signaling mediators such as N- or K-RAS and the receptor tyrosine kinases KIT and FLT3 appeared to be more prevalent than in adult patients. In contrast, mutations in DNMT3A, IDH1/2, NPM1, or tumor protein p53 (TP53) were less common in pediatric AML. Fourth, some "novel" pediatric-specific chromosomal copy number changes were found, including focal deletions in genes like muscleblind like splicing regulator 1 (MBNL1), zinc finger E-box binding homeobox 2 (ZEB2), E74-like ETS transcription factor 1 (ELF1), or interleukin 9 receptor (IL9R). Collectively, the TARGET AML initiative provided a comprehensive dataset of genetic alterations in pediatric AML that confirmed and extended previous observations indicating that similar to adult patients, pediatric AML is the product of a low number of cooperating mutations frequently involving transcriptional regulators affecting differentiation and self-renewal properties and mutations of signaling mediators (9) (**Figure 1**). Here, we focused on hallmarks of aggressive pediatric AML fusion oncogenes, including KMT2A, CBFA2T3, and NUP98 fusions.

Fusion Genes Associated With Aggressive Pediatric AML

The TARGET-AML study suggested that the association of pediatric AML with different fusion oncogenes strongly correlates with age of the patient (8) (**Figure 1**). Whereas, fusions involving KMT2A, CBFA2T3, or MNX1 are molecular hallmarks of AML affecting infants and early childhood (<3 years), those affecting the core binding factor (RUNX1 and CBFB) or the retinoid acid receptor (RARA) occur at any age but peak in children (3–14 years) or even in young adults (15–39 years). In addition, two particular NUP98 fusions, NUP98–lysine demethylase 5A (KDM5A, a.k.a. JARID1A, or RBP2) and NUP98–NSD1, are molecular hallmarks of cytogenetically silent infant or childhood AML, respectively.

The KMT2A (better known as mixed lineage leukemia, MLL) gene on the long arm of chromosome 11 (11q23) encodes a SET-domain histone methyltransferase that is important for the maintenance of the hematopoietic stem cells (10). KMT2A is the target of chromosomal translocations in adult and pediatric acute leukemia, mostly leading to fusions of the N-terminus of KMT2A with a large number of different partners, of which AF4/FMR2 family member 1 (AFF1, a.k.a. AF4), MLLT3 super elongation complex subunit (MLLT3, a.k.a. AF9), MLLT1 super elongation complex subunit (MLLT1, a.k.a. ENL), and MLLT10 histone lysine methyltransferase DOT1L cofactor (MLLT10, a.k.a. AF10) are the most prevalent (11). Although t(4;11)(q21;q23) leading to a KMT2A–AFF1 fusion is a molecular marker of infant acute lymphoblastic leukemia (ALL), it can occur at any age, and is rarely also found in AML. In contrast, t(9;11)(p22;q23) and ins(10;11)(p12;q23q13) leading to expression of KMT2A–MLLT3 and KMT2A–MLLT10 fusions appear more prevalent in pediatric than in adult AML. Interestingly, KMT2A–MLLT3^{+} disease in infants presents more often as ALL than AML, whereas the phenotype changes into a typical myelo-monocytic AML M5 with increasing age of the patient. The difficulty to classify leukemia with KMT2A fusions, including lymphoid diseases, is also based on the fact that leukemic blasts retain a substantial amount of lineage

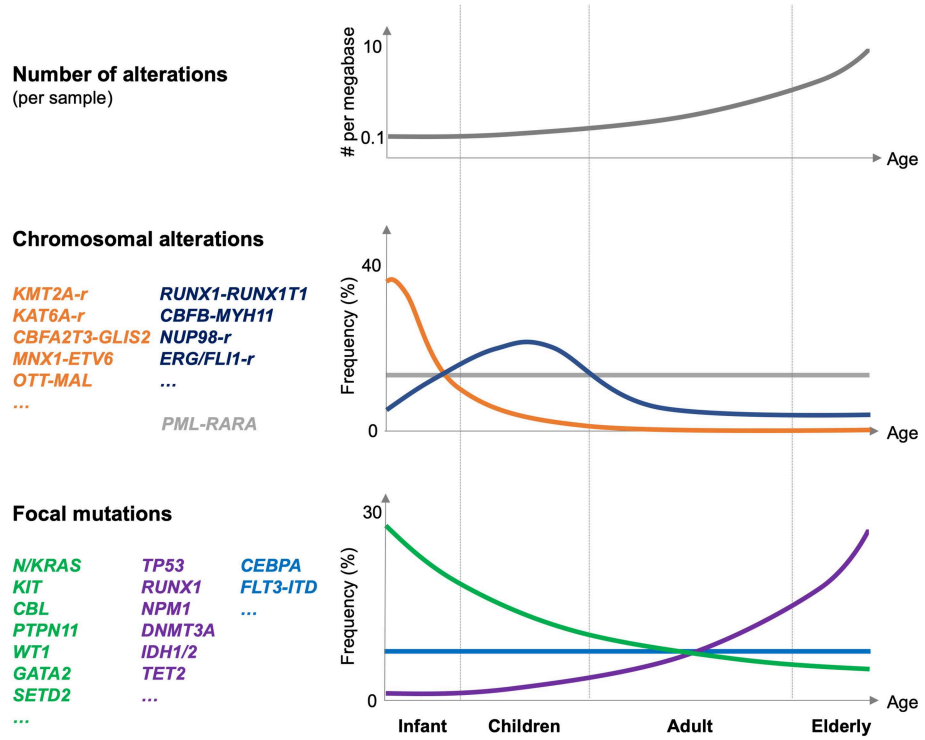

FIGURE 1 | Genetic alterations in pediatric AML. Schematic illustration of the number of genetic alterations (per AML sample, **top panel**), frequency, and type of chromosomal alteration **(middle panel)**, and frequency and type of focal mutations in pediatric AML related to different age groups (infant, children, adults, and elderly; **lower panel**). Frequencies are indicated globally for the entire group of indicated mutations. The figure is mostly based on Bolouri et al. (8).

infidelity and/or plasticity highlighted by frequent co-expression of myeloid markers and relapse of KMT2A$^+$ B-ALL as AML (12). Functionally, it is currently thought that KMT2A fusions transform HSPCs by recruitment of a large super elongation protein complex (SEC) that activates transcription of target genes via directly influencing elongation by the RNA polymerase II (RNA-pol II). In addition, KMT2A fusion proteins also recruit the DOT1L histone 3 lysine 79 (H3K79me) methyltransferase that positively regulates expression of critical target genes (13) (**Figure 2**).

The importance of aberrant transcriptional control is furthermore highlighted by fusions between KMT2A and lysine acetyltransferase 6A (KAT6A a.k.a. Monocytic leukemia zinc finger protein, MOZ, or MYST3) or 6B [KAT6B a.k.a. Moz-related factor (MORF) or MYST4] to the histone acetyl transferases EP300 or CREBBP in some rare cases of pediatric myelodysplastic syndromes (MDS) and AML (14–16). In contrast to adult patients where MYST (MOZ/Ybf2/Sas2/TIP60) fusions are often associated with therapy-related AML, in pediatric patients, these fusions are found in congenital and perinatal leukemia. Interestingly, some infants with KAT2A-CREBBP$^+$ AML were reported to go into spontaneous remission; however, the underlying biology remains poorly understood (17, 18).

The CBFA2T3 (a.k.a. ETO2 or MTG16) gene on the long arm of chromosome 16, encoding a transcriptional co-repressor, is targeted by two recurrent AML-associated chromosomal rearrangements, the t(16;21)(q24;q22) and the cytogenetically silent inv(16) (p13q24) leading to expression of RUNX1–CBFA2T3 and CBFA2T3–GLIS2 fusions, respectively. Whereas, the first is more prevalent in therapy-related adult AML and rarely found in pediatric patients, the second appears to be an exclusive pediatric lesion (19, 20). NGS strategies allowed the identification of the CBFA2T3–GLIS2 fusion from tumor cells of pediatric patients with *de novo* acute megakaryoblastic leukemia (non-DS AMKL) (21, 22). CBFA2T3–GLIS2 is the most prevalent chromosomal aberration of this disease entity followed by KMT2A, RBM15 (RNA-binding motif protein 15)-MRTFA (Myocardin related transcription factor A) (a.k.a. OTT-MAL), NUP98–KDM5A and other rare events (e.g., GATA2–HOXA9, MN1–FLI1, or NIPBL–HOXA9) (23). CBFA2T3–GLIS2 is not restricted to AMKL but can also be found in cytogenetically normal (CN) pediatric AML with different phenotypes (M0, M1, M2, M4, and M5, according to the FAB classification). Notably, AMKL patients are significantly younger than those with other AML phenotypes (24). Mechanistically, CBFA2T3–GLIS2 binds DNA, through CBFA2T3-associated transcription factors or directly through GLIS2 (GLIS family zinc finger 2) zinc-finger domains at enhancers and regulatory elements and leads to altered transcription and activity of key transcription factors like the upregulation of the ETS transcription factor ERG and a strong downregulation of GATA1 (25). Genetic

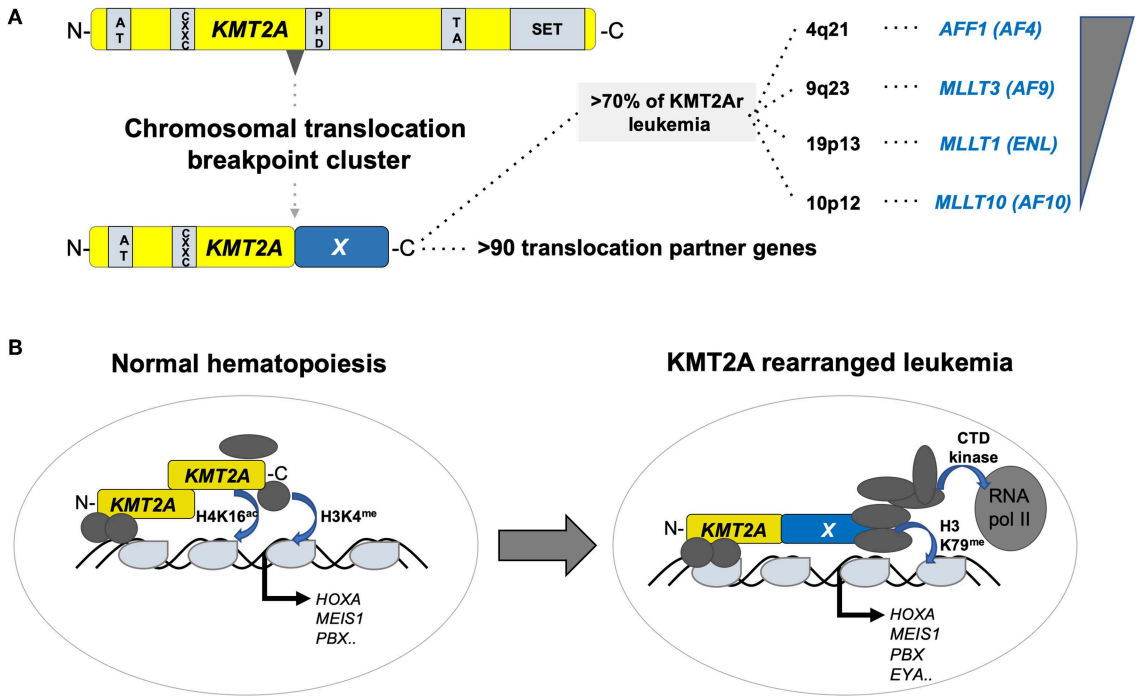

FIGURE 2 | Structure and function of KMT2A fusion oncogenes associated with pediatric leukemia. **(A)** Schematic outline of the KMT2A (a.k.a. MLL1) protein organization containing AT-rich, CXXC, PHD-fingers, transactivation (TA), and a SET methyltransferase domain with recurrent chromosomal breakpoint clusters leading to fusions to >90 different partners, of which >70% are formed by AFF1 (AF4) on 4q21, MLLT3 (AF9) on 9q23, MLLT1 (ENL) on 19p13, and MLLT10 (AF10). **(B)** Simplified illustration of the function of KMT2A in normal hematopoiesis (left) and upon rearrangement in acute leukemia (right). In normal hematopoiesis, KMT2A is proteolytically cleaved into an N- and C-terminal fragment that associated with co-regulators leading to activation of its targets (including, e.g., the *HOX-A* gene cluster, *MEIS1* or *PBX1*) through H4K16 acetylation and H3K4 methylation. In leukemia, KMT2A fusion proteins recruit a super elongation multi-protein complex and the DOT1L H3K79 methyltransferase to activate its target genes including the *HOX-A* gene cluster, *MEIS1*, *PBX1*, and *EYA*1.

hijacking of ERG and GATA1 activities represents a common theme among pediatric AMKL as constitutive trisomy 21 (a.k.a. Down's syndrome) AMKL disease progression is characterized by independent genetic alterations also impacting *ERG* (carried by chromosome 21) and *GATA1* (**Figure 3**) (26, 27). Notably, additional mutations in cohesin components (∼50% of patients), CTCF (∼20% of patients), epigenetic regulators (∼45% of patients), and signaling pathway intermediates (∼45% of patients) may also re-enforce an ERG/GATA activity imbalance (28, 29). CBFA2T3–GLIS2 is also associated with aberrant expression of GLI-family target genes, including BMP factors, motivating evaluation of GLI inhibitors' efficacy and specificity for this fusion (21, 22, 30).

The NUP98 gene on the short arm of chromosome 11 (11p15) encodes a structural component of the nuclear pore but the protein can also function as a transcriptional regulator (31). Similar to KMT2A, NUP98 is targeted by numerous chromosomal translocations or inversions in various but mostly myeloid hematological malignancies, leading to the expression of chimeric proteins containing the N-terminus of NUP98 fused to a large variety of different partners including several homeobox proteins (32). The best studied is t(7;11)(p15;p15), leading to a NUP98–HOXA9 fusion in MDS, chronic myeloid leukemia (CML) in blast crisis, and AML of any age. In contrast, the cytogenetically cryptic t(5;11)(q35;p15) and t(11;15)(p15;q35) translocations, leading to the expression of NUP98–NSD1 or NUP98–KDM5A fusions, respectively, are preferentially found in pediatric AML. NUP98–NSD1 contains the GLFG repeats of NUP98 fused to several PHD finger domains and the SET methyltransferase domain of NSD1 (33). NUP98–NSD1 is one of the most prevalent aberration in pediatric CN-AML, often presenting with a myelomonocytic phenotype (M4/M5; FAB) associated with poor outcome. Interestingly, in the majority of patients, tumor cells also harbor an internal tandem duplication in FLT3 (FLT3-ITD) and/or mutation in WT1 (34). NUP98–KDM5A was identified from a patient with megakaryoblastic leukemia and later shown to be present in about 10% of non-DS-AMKL (35, 36). NUP98–KDM5A contains the GLFG repeats of NUP98 fused to the C-terminal PHD finger domain of the KDM5A histone demethylase. Similar to KMT2A fusions or CBFA2T3–GLIS2, the presence of NUP98–KDM5A confers a poor clinical outcome (23). The mechanism of transformation by NUP98 fusions might involve the N-terminus containing GFLG repeats recruiting a large WDR82-SET1A/B-COMPASS (WSC) protein complex to promote trimethylation of lysine 4 of histone 3 (H3K4me)

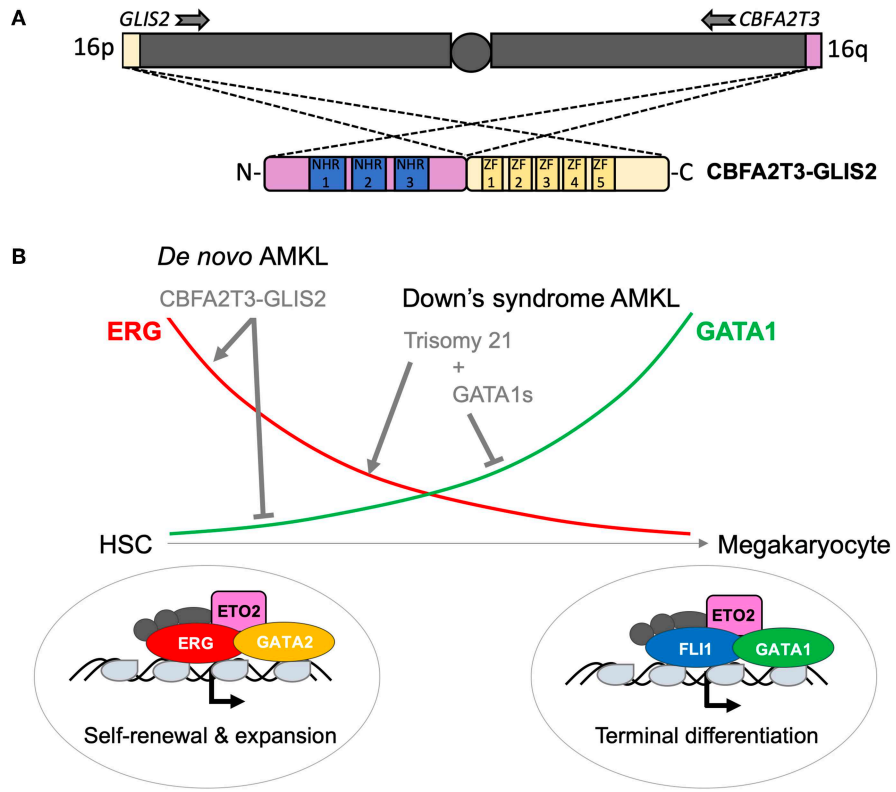

FIGURE 3 | Structure and function of the CBFA2T3–GLIS2 fusion associated with pediatric AMKL. **(A)** Schematic outline of the karyotypically silent inv(16) leading to fusion between the telomerically located GLIS2 (16p13) and CBFA2T3 (16q24) genes, respectively. All known fusions contain the Nervy homology domains (NHR) 1–3 of the transcriptional co-repressor CBFA2T3 and the five zinc fingers (ZF) of the GLIS2 transcription factor. **(B)** Schematic representation of the change in key transcription factors activity during the normal differentiation of hematopoietic stem cells (HSC) toward mature platelet-producing megakaryocytes. ERG and GATA2 activity are higher in HSC and progressively replaced by FLI1 and GATA1 in megakaryocytes. A schematic representation of the consequences of genetic alterations found in pediatric AMKL is also shown. It is currently thought that, while AMKL associated with Down's syndrome target ERG and GATA1 through independent genetic alterations, the CBFA2T3–GLIS2 fusion is able alone to maintain both high ERG and low GATA1 activity contributing to the blockage of differentiation and aberrant self-renewal capacities of AMKL leukemic blasts.

favoring active transcription (37). On the other hand, the fusion partners appear also to contribute to alter target gene expression such as the HOX-A gene cluster by, e.g., H3K36 methylation (NUP98–NSD1) or by acting as a boundary factor that prevents spreading of repressive polycomb factors (NUP98–KDM5A) (38, 39) (**Figure 4**).

The MNX1 (a.k.a. HLXB9) gene on the long arm of chromosome 7 (7q36) encodes a homeobox transcription factor that is essential for pancreatic organogenesis, and moto-neuron differentiation, and is involved in a t(7;12)(q36;p13) translocation that is a hallmark of infant AML with poor outcome (8, 40). Although initial reports suggested that this translocation would lead to a chimeric fusion protein containing HLH and ETS domains of ETV6 joined to regulatory sequences and first exon of MNX1 lacking the homeodomain, it appeared that overexpression of MNX1 might be the primary consequence (41). The role of MNX1 in the hematopoietic system remains unclear. However, functional studies suggested that the protein might regulate cell–cell interaction and adhesion of leukemic cells and that aberrant expression of MNX1 leads to differentiation block in megakaryocyte-erythroid progenitor cells (42, 43).

Signaling Mutations in Pediatric AML

Mutations in signaling mediators such as receptor tyrosine kinases (e.g., FLT3 and KIT) and RAS-related molecules (e.g., N-RAS, K-RAS, PTPN11, or NF1) are found in adult and pediatric AML (8). These mutations generally lead to constitutive activation of interconnected signaling cascades that activate downstream effectors, such as signal transducers and activators of transcription (STAT), ELK, MYC, c-JUN, or NF-kB involved in transcriptional regulation of cell proliferation and survival.

The RAS proteins are a family of highly homologous low-molecular-weight proteins that bind GTP, located at the inner face of the plasma membrane. In the normal situation, the activity of RAS is controlled by hydrolysis of bound GTP by GTPase activating proteins (GAPs) and the replacement of bound GDP with fresh GTP, which is catalyzed by the family of guanine nucleotide exchange factors (GEFs) (44). About

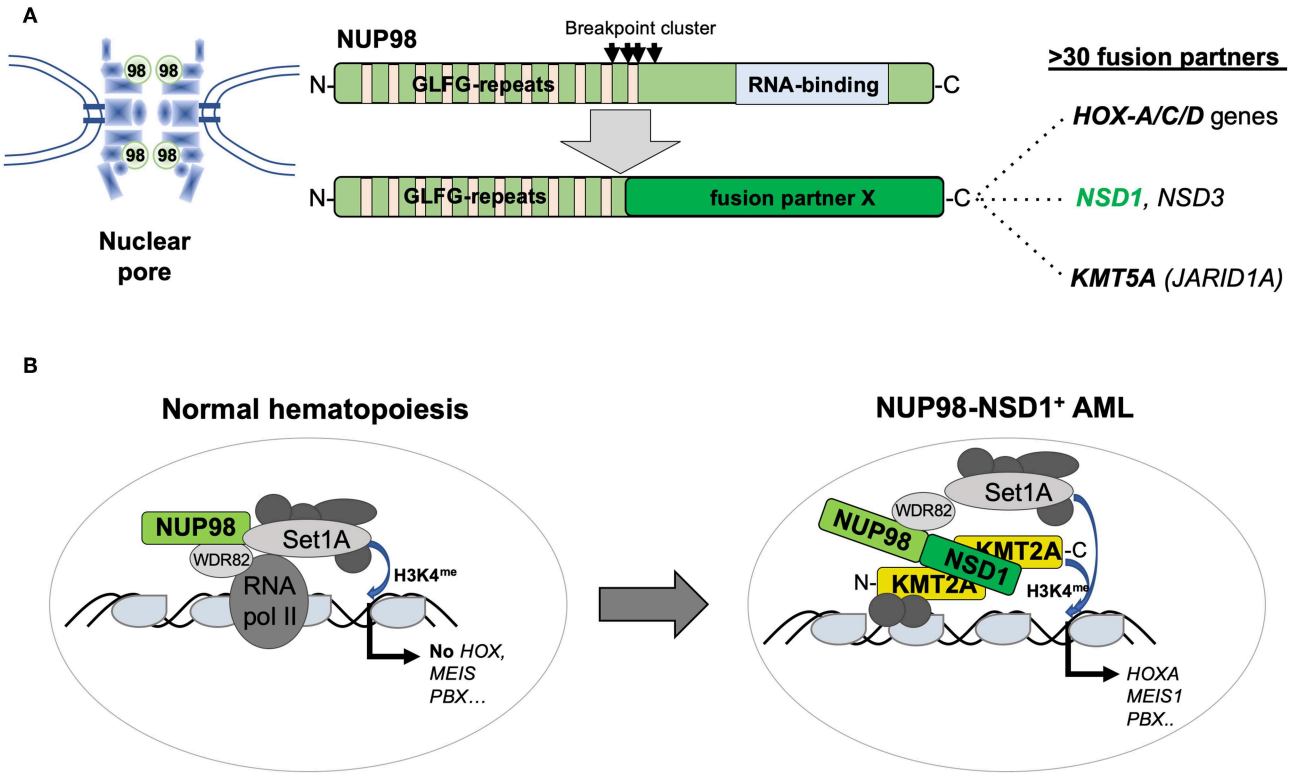

FIGURE 4 | Structure and function of AML-associated NUP98 fusions. **(A)** Schematic outline of the NUP98 pore protein organization containing multiple N-terminal GLFG repeats and a C-terminal RNA binding domain lost by fusion to >30 different fusion partners including homeodomain proteins (*HOXA-C-D* gene cluster) and non-homeodomain proteins including NSD1, NSD3, or KDM5A (JARID1A) [modified from Masetti et al. (30)]. **(B)** Recent work (36) suggested that, in normal cells, in addition to its role as a pore protein, NUP98 is part of a transcriptional co-activator protein complex containing WDR82 and SET1A that regulate their targets (that are not the classical KMT2A targets) by setting H3K4me marks. The NUP98–NSD1 interacts with KMT2A and re-localizes the NUP98-associated WSC activity resulting in activation of classical KMT2A targets including the *HOX-A* gene cluster, *MEIS1* or *PBX1*.

20% of human cancers have activating point mutations of RAS most frequently affecting K-RAS, less N-RAS, and rarely H-RAS (45). The TARGET study reported N/K-RAS mutations in over 40% of investigated pediatric AML cases. Notably, the highest prevalence of RAS mutations was observed in infant patients that also harbored KMT2A fusions (8). Most cancer-associated RAS mutations affect codons 12, 13, and 61 and all compromise the GTPase activity of RAS, preventing GAPs from promoting hydrolysis of GTP on RAS and leading to the accumulation of RAS in the GTP-bound active form. G12D, G12V, G13D, and Q61H are the most prevalent RAS mutations in pediatric AML. Although the prognostic value of RAS mutations is an ongoing matter of debates, like other signaling mutations, they appear to affect the outcome by changing clonal expansion in AML (46).

The FLT3 protein is a class III receptor tyrosine kinase (RTK) family that contains an extra-cellular domain made up of five immunoglobulin-like regions, a single transmembrane region, an intracellular juxtamembrane domain (JMD), and two kinase domains at the carboxyl terminus. Inactivation studies in mice have shown that FLT3 signaling is central to the development of HSPC, B-cells, dendritic cell progenitors, and natural killer cells (47). Binding of FLT3-ligand (FL) leads to dimerization of FLT3 and autophosphorylation of tyrosine residues in the kinase domains, resulting in activation of multiple signaling cascades including RAS/RAF, PI3K/AKT, or STAT5, resulting in increased proliferation and survival. FLT3 is highly expressed in many hematological malignancies and often co-expressed with its ligand FL, suggesting autocrine signaling (48, 49). FLT3 is targeted by two categories of activating mutations, internal tandem duplication (ITD, variable in length) and tyrosine kinase domain (TKD) mutations. FLT3-ITD results in FL-independent dimerization, constitutive phosphorylation, and activation of downstream mediators. FLT3-ITD is found in about 10–20% of newly diagnosed pediatric AML patients and was reported to be an independent prognostic factor for poor outcome particularly for patients with high ITD allelic ratios and/or loss of the wild-type FLT3 allele leading to copy number-neutral ITD homozygosity (48). FLT3-TKD mutations mostly affecting aspartic acid D835 are less common (5–10%) than ITD and seem not to carry the same prognostic significance. Nevertheless, TKD mutation may also occur secondary at relapse of ITD^+ patients that are treated with FLT3 inhibitors. Notably, FLT3-TKD mutations are particularly prevalent in pediatric leukemia patients with KMT2A fusions (50).

Mutations in Epigenetic Regulators

In contrast to adult AML, mutations in regulators of DNA methylation and histone modification including Ten-Eleven Translocation2 (TET2), IDH1 or IDH2, Enhancer of Zeste Homolog 2 (EZH2), DNMT3A, and Additional Sex Combs like-1 (ASXL1) are much less prevalent, affecting only about 1–2% of pediatric patients (51). Nevertheless, IDH1 or IDH2 mutations in codons 132 and 140, respectively, were found in over 10% of a cohort of CN pediatric AML mostly in combination with alterations of KMT2A, NUP98, and FLT3-ITD or RAS (52). DNMT methyltransferases (DNMT1, DNMT3A/B), the TET family of enzymes (TET1–3), and IDH1/2 are functionally interconnected (53). DNMTs methylate DNA cytosine residues that can be oxidized from 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) by TET enzymes in an iron- and α-ketoglutarate (α-KG)-dependent manner. The presence of an IDH1/2 mutation results in the production of 2-hydroxyglutarate (2-HG), which is structurally very similar to α-KG and competes with α-KG to inhibit α-KG–dependent enzymatic processes (53). Mouse models have shown that inactivation of TET2 and DNMT3A or targeted mutagenesis of IDH1/2 mostly induces preleukemic states and leads to AML development upon cooperation with additional mutations (54–56). Even though rare in pediatric patients, it is important to search for alterations of these factors, as novel approaches for therapeutic targeting (as outlined below) showed very promising results in adult AML.

MODELING GENETIC LESIONS IN PEDIATRIC AML

Development of recombinant DNA technologies allowed the cloning and characterization of a large number of genetic alterations identified in tumor cells from AML patients. To address their potential for induction and maintenance of a transformed phenotype, the cDNA of a gene of interest carrying a mutation or eventually an entire fusion gene is transferred into hematopoietic cells (cell lines or primary cells) mostly by recombinant retroviruses. Based on the limited access to primary material of a genetically rather heterogeneous disease as pediatric AML, the majority of functional studies are performed in animals, mostly mice. *In vitro* experiments measure the impact of an AML-associated mutation on proliferation and survival but also self-renewal and differentiation of bone marrow (BM)-derived HSPC. The latter two are often determined by measuring the serial replating capacity in growth factor-containing semisolid medium such as methylcellulose (MC). Expression of many KMT2A, NUP98, CBF, or RARA fusions support serial HSPC replating in MC associated with variable blockage of normal maturation. This assay also allows structure-function studies to dissect critical domains of a given gene product. To determine the transforming potential *in vivo*, researchers aim to express the respective cDNA/mini gene in HSPC of their animal model. In one widely used approach, mouse BM-derived cells are virally transduced to overexpress the respective genetic lesion and transplanted into irradiated syngenic recipients [often referred to as BM transplant (BMT) or reconstitution model] (57). Despite the drawbacks of often higher-than-physiological expression, viral integration, and transduction bias of multipotent progenitor cells, this assay allows relatively fast and easy-to-obtain insights into the leukemogenic potential of a given lesion. This strategy was used to show functional cooperation of transcription factor fusions (e.g., KMT2A–MLLT3, KMT2A–MLLT1, NUP98–HOXA9, RUNX1–RUNX1T1, PML–RARA, and others) with signaling mutations like FLT3-ITD, N-/K-RAS, or activating KIT mutations (58, 59). Nowadays, putative leukemogenic driver oncogenes can also be knocked into the mouse genome, resulting in expression regulated from its native promoter/enhancers. In addition, several chemically inducible transgenic mouse lines have been generated that identify the role of a mutation not only for induction but also for maintenance of a leukemic phenotype. Most likely, genome editing using Crispr/Cas9 will facilitate targeted recombination and eventually boost the development of novel AML animal models (60).

As the hematopoietic system of the mouse is not identical to the situation in humans, researchers developed transgenic mouse lines with a humanized hematopoiesis expressing several human hematopoietic regulatory genes in combination with defects of a normal immune response such as the MISTRG strain (61). These humanized mice not only allow to expand patient-derived AML cells but also to study the biology of the disease (62). Immunodeficient mice also allow to model the disease by transplanting human HSPCs engineered to virally overexpress a leukemia-associated fusion oncogene (63) or, more recently, obtained from the hematopoietic differentiation of induced pluripotent stem cells (iPSCs) derived from AML patient blasts (64, 65). Due to space constraints, we focus on some recent findings in mouse models related to those genetic lesions that are recurrently found in pediatric AML.

Modeling KMT2A Fusion-Driven Pediatric AML

KMT2A fusions, such as KMT2A–MLLT3, are among the best-studied AML-associated alterations. Transgenic knock-in as well as BMT models resulted in a myelo-monocytic AML that closely phenocopied the human disease (66). Strikingly, transduction of lineage-marker-depleted, Kit^+ $Sca1^+$(LSK) cells, common myeloid progenitor (CMP), or granulocyte-macrophage progenitor (GMP) cells with virus expressing KMT2A–MLLT3 or KMT2A–MLLT1 followed by transplantation resulted in a very similar disease (67). To dissect the activity of KMT2A–MLLT3 and KMT2A–MLLT1 in different cells of the hematopoietic hierarchy, we developed DOX-regulated transgenic mouse lines. As expected, we observed that KMT2A–MLLT3 is able to transform hematopoietic stem cells (HSC), but also more committed progenitor (CMP and GMP) cells. However, activation in long-term hematopoietic stem cells (LT-HSC) resulted in some animals in a particularly aggressive AML characterized by high expression levels of the transcription factors MECOM (MDS1 and EVI1 complex locus, a.k.a. EVI1) and ERG, which also characterize human AML with poor outcome (68). In contrast, using the same strategy, we found

that activation of KMT2A–MLLT1 preferentially transformed HSC, while CMP were transformed less efficiently and GMP were not transformed at all (69). This finding in transgenic mice might also reflect the situation in patients, as KMT2A–MLLT1 leukemic cells in patients often express lymphoid markers and are diagnosed as ALL or mixed lineage leukemia, whereas KMT2A–MLLT3 mostly present with AML-M5 (11). These observations in adult mice suggest that cells of the hematopoietic hierarchy have a differential sensitivity for a given leukemogenic fusion gene. In attempts to model pediatric KMT2A-rearranged AML, Chen et al. transplanted fetal liver-derived HSPC from a KMT2A–MLLT3 transgenic (knock-in) mice into wild-type mice and observed induction of a leukemic phenotype often expressing lymphoid surface markers after long latency. In contrast, transplantation of adult BM-derived cells led to the typical AML-M5 phenotype (70). Although differential grafting potential of highly cycling fetal liver-derived cells compared to adult BM cells might have influenced these experiments, pediatric but not adult KMT2A–MLLT3^{+} leukemia often express lymphoid markers, strongly suggesting that activation at a particular developmental stage significantly influences disease biology. However, so far, we are not aware of any animal model that appropriately phenocopies KMT2A fusion-driven pediatric AML.

Modeling NUP98 Fusion-Driven Pediatric AML

Multiple transgenic mouse models have shown that expression of several NUP98 fusions in the hematopoietic system results in various malignancies (32). Functional cooperation between NUP98–HOXA9 and BCR–ABL fusions became a widely used model to study CML in blast crisis (71, 72). Transgenic NUP98–HOXD13 mice, which develop myelodysplasia eventually progressing to AML, are often used to study molecular mechanisms of MDS (73). However, the transforming potential of the preferentially or exclusively pediatric NUP98 fusions (NUP98–KDM5A and NUP98–NSD1) is less clear. Transplantation of adult BM-derived HSPC retrovirally expressing the NUP98–KDM5A fusion resulted in a fully penetrant AML phenotype after 50–100 days, indicating a strong leukemogenic potential. Tumor cells expressed surface markers of early myeloid progenitor cells, but expression of megakaryoblastic markers observed in pediatric cases was not reported (40). Transplantation of adult BM-derived HSPC retrovirally expressing the NUP98–NSD1 fusion was reported to induce an AML phenotype in mice after a long latency with tumor cells expressing myeloid but also early stem cell-related (FLT3, CD34, and KIT) surface markers (38). However, using the same retroviral vector but a slightly different experimental strategy, we only observed development of AML upon co-transduction of NUP98–NSD1 together with FLT3-ITD, a mutation that is found in the majority of the patients (74). Even though these models indicate that NUP98–NSD1 has some transforming potential, alone it seems not sufficient to induce the disease. In addition, most of NUP98–NSD1^{+} AML occurring in children and younger adults present mostly with a myelomonocytic AML for which we currently do not have an appropriate model.

Modeling Pediatric AMKL Fusion Oncogenes

CBFA2T3–GLIS2, RBM15–MRTFA, NUP98–KDM5A, and KMT2A fusions are found in about 60–70% of non-DS-related pediatric AMKL. Additional rare fusions like GATA2–HOXA9, MN1–FLI1, NIPBL–HOXB9, or NUP98–BPTF were cloned from tumor cells from pediatric AMKL patients (75). Earlier work identified some JAK3 activating mutations in cell lines derived primarily from DS-AMKL, which induced a transient myeloproliferative disease with megakaryoblastic elements when retrovirally expressed in hematopoietic cells from Balb/c mice (76). Knock-in of the MRTFA cDNA at the endogenous Rbm15 locus led to *bona fide* Rbm15–MRTFA fusion expression in mice, altered clonogenic potential of fetal liver-derived hematopoietic cells, and AMKL with a low penetrance. Retroviral co-expression of the thrombopoietin receptor (MPL) carrying an activating mutation (W515L) induced leukemia with morphologic characteristics of AMKL; however, such a combination is rarely seen in patients (77). Using a similar approach to combine a transgenic model of Trisomy 21 (Ts1Rhr) with GATA1s and MPL^{W515L}, Malinge et al. demonstrated that all three alterations were required to induce AMKL in mice (78, 79). Retroviral expression of CBFA2T3–GLIS2, GATA2–HOXA9, MN1–FLI1, and NIPBL–HOXB9 allowed serial replating of adult mouse BM-derived hematopoietic cells in MC associated with expression of megakaryocytic markers on some CBFA2T3–GLIS2- and MN1–FLI1-expressing cells. Consistently with the high homology between ERG and FLI1, transplantation of these MN1–FLI1-transduced cells was sufficient for the development of murine leukemia presenting clear features of AMKL (80, 81). However, GATA2–HOXA9- and NIPBL–HOXB9-transduced cells led to penetrant AML phenotypes but with limited megakaryocytic features, and CBFA2T3–GLIS2-transduced cells did not induce any disease (81). Of note, expression of RBM15–MRTFA was also not able to induce leukemia development using a retroviral transduction/BM transplant approach, suggesting that this approach is not suitable to model the leukemogenic activity of all fusion oncogenes (77). Very recent work suggested that transplantation of fetal liver hematopoietic cells retrovirally expressing the CBFA2T3–GLIS2 fusion in lethally irradiated recipients is able to induce an AMKL phenotype co-expressing the CD41 and CD61 megakaryocytic markers but with a limited penetrance (82). Together, these results indicate that better models are required to more faithfully recapitulate pediatric AMKL.

Challenges to Accurately Model Pediatric Acute Leukemia in Mice

The genetic heterogeneity, as well as the rareness of pediatric AML that limits access to primary cells, urges for the development of models that closely phenocopy the biology of the human disease. Establishment of appropriate models for pediatric leukemia is a challenging task illustrated by the efforts

to develop a mouse model for KMT2A–AFF1$^+$ infant B cell ALL. The observation that KMT2A fusion$^+$ infant leukemias have on average only about two non-silent mutations strongly suggests that the fusion might be sufficient for inducing the disease or that only very few cooperating hits are necessary (8, 83). Expression of a knocked-in KMT2A–AFF1 fusion ORF developed mostly B-cell lymphomas after a long latency in mice (84). An ALL phenotype developing after a relatively long latency was observed in mice carrying a conditional KMT2A–AFF1 allele, or by retroviral co-expression of KMT2A–AFF1 and the reciprocal AFF1–KMT2A fusion (85). More recently, Jim Mulloy et al. demonstrated that transplantation of human CD34$^+$ HSPC retrovirally expressing a human–mouse chimeric KMT2A-Aff1 fusion developed pro-B-ALL after a latency of 100–250 days. In contrast, expression of this chimeric fusion in mouse HSPCs resulted in AML, whereas only low titers could be generated of viruses containing the fully human fusion ORF. Although this study was the first that indeed produced a KMT2A–AFF1-driven pro-B-ALL, the disease did not develop in "infant" or newborn mice (86). In another attempt to model KMT2A–AFF1 infant leukemia, Barrett et al. induced the fusion between developmental E12 and E14 to all definitive hematopoietic cells formed during embryonic development using a conditional invertor mouse strain controlled by the VE-Cadherin-cre recombinase. Expression of KMT2A–AFF1 at this early stage increased engraftment and self-renewal of fetal liver cells and provided the cells with a high clonogenic B-lymphoid potential; however, no early progression to B-ALL was observed (87). Interestingly, Menendez et al. earlier found the KMTA2A–AFF1 fusion in BM mesenchymal stoma cells in affected patients, suggesting an early pre-hematopoietic precursor cell origin of the fusion (88). Of note, one cannot exclude that these observations are explained by species-related differences, inappropriate expression levels in cells at a particularly sensitive developmental stage, or the lack of a potential cooperative lesions.

Experiments based on transplantation of fetal liver- or BM-derived cells retrovirally expressing the NUP98–HOXA9 fusion into adult recipients revealed that the age of the cell of origin determines not only the latency period for disease development but also the lineage phenotype and changes of the BM niche (89). In the attempt to model Trisomy 21-associated AMKL, retroviral expression of ERG in murine adult BM leads to 100% of T-cell leukemia, while expression in E12.5 fetal liver cells generated erythro-megakaryocytic leukemia in 40–60% of recipient mice (90). Also, GATA1s and Trisomy 21 have been shown to induce stage-specific alterations of fetal hematopoiesis in both murine transgenic models and humans (91, 92). Proof of concept that neonatal hematological malignancies can also be induced in mice was provided by experiments that modeled juvenile myelomonocytic leukemia (JMML). Hereby, either fetal expression of KrasG12D controlled by Flt3-cre recombinase or constitutive co-deletion of Cbl/Cbl-b resulted in aggressive neonatal myeloproliferative disorders that were lethal within 2–3 weeks after birth (93, 94).

It is very likely that the type of hematopoietic stem or progenitor in which the mutation/fusion first appears is of importance for both development and phenotype of pediatric AML. Although under considerable debates, the normal hematopoietic hierarchy is constituted of a continuum of progenitors presenting different self-renewal and differentiation potential (95, 96). Notably, there is increasing evidence that the fetal and adult hematopoietic hierarchies significantly differ in structure and composition (97). Although not yet demonstrated in murine models of pediatric AML, several AML oncogenes (including KMT2A–MLLT3) studied in an adult context are able to transform both HSC and more committed progenitors (e.g., GMP) while others are not able to do so (67, 98–101). Also, as indicated above, the phenotype of KMT2A–MLLT3$^+$ and KMT2A–MLLT1$^+$ leukemia was dependent on the stage of the hematopoietic hierarchy in which the driver mutation was expressed (68, 69). Recently, some studies have reported the successful derivation of iPSCs from human AML cells, including from blasts presenting MLL fusions, suggesting that this approach can generate human-based preclinical models (64, 65, 102, 103). Although the frequency of successful iPSC reprograming from AML blasts is likely low, iPSC-derived hematopoiesis followed by transplantation into immunodeficient models may represent a future opportunity to investigate specific stages of the human fetal hematopoietic development that are difficult to access using primary human samples (104).

Collectively, these studies indicated several factors to be taken into account for appropriately modeling pediatric AML in mice: (1) driver (and eventually also cooperating) mutations most likely need to be active *in utero*; (2) a driver and cooperating lesion might not occur and/or be active at the same developmental stage; (3) a given genetic lesion (e.g., fusion gene) has its optimal expression level; and (4) particular developmental cell stages or identity are likely more permissive to transformation than others (**Figure 5**).

MOLECULAR TARGETING OF PEDIATRIC AML

Functional cooperation studies in cellular and animal models suggest that we can group AML-associated mutations into drivers that are essential for induction and maintenance of the disease, and cooperating mutations that support expansion of the malignant clone or may facilitate transformation by mostly metabolic modifications (101). As a consequence, inactivation or degradation of the driver might represent the most promising approach for long-term cure of the disease. However, in cases without a clearly defined driver, inhibition of other cooperating lesions (such as constitutive active protein kinases) or interfering with more general dependencies of transformed hematopoietic cells might provide an alternative strategy (**Figure 6**). Here, we discuss selected targeting strategies for pediatric AML that are either effective in the clinic, being explored in ongoing trials or just demonstrated as proof of concept in preclinical models.

Targeting the Driver Mutation (e.g., Fusion Oncogene)

Positive proof of concept that long-term cure can be achieved in AML patients through inactivation of the driver mutation comes from acute promyelocytic leukemia (APL) that is, in the vast majority of the cases, driven by the PML–RARA fusion resulting from t(15;17)(q22;q21). Although, the TARGET

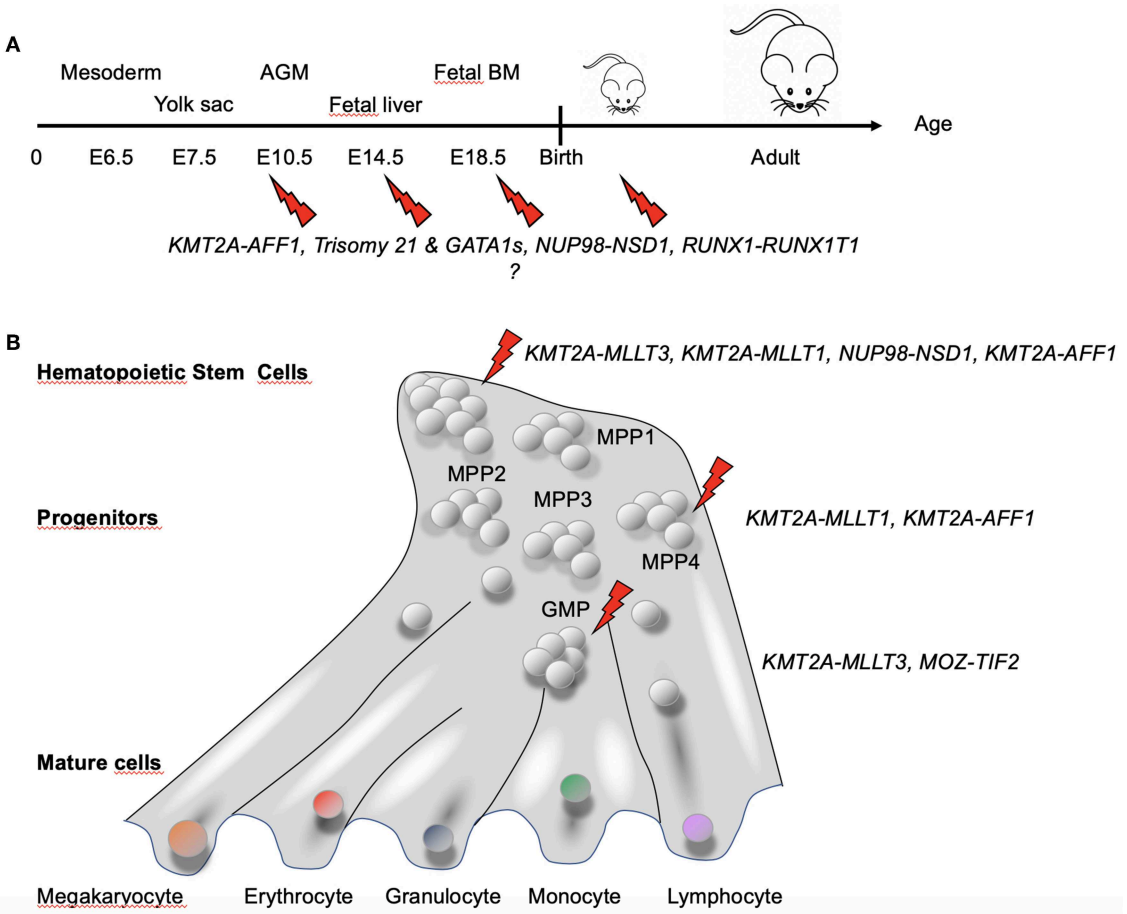

FIGURE 5 | Hematopoietic developmental stage and hierarchy-dependent susceptibility for transformation by AML-associated fusion oncogenes. **(A)** Increasing evidence suggests that pediatric AML-associated oncogenes have a particular window of opportunity during development to transform hematopoietic cells. GATA1s and Trisomy 21 have been shown to induce stage-specific alteration of fetal hematopoiesis (86). For KMT2A–AFF1$^+$ leukemia, the fusion was found not only in hematopoietic precursors but also in BM stroma cells (83), suggesting that the fusion might target a very early precursor cell that maintain some mesenchymal properties. **(B)** Differential susceptibility of cells of the hematopoietic hierarchy for transformation by fusion oncogenes associated with pediatric AML [adapted from Rodriguez-Fraticelli et al. (95)]. Together, these observations suggest that both the developmental stage and the type of cell in the hematopoietic hierarchy in which a genetic alteration occurs determines whether a leukemia will develop and the associated disease phenotype and aggressiveness.

pediatric AML study did not report PML–RARA$^+$ APL patients, who are often considered separately for therapeutic reasons, they make up to of 5–10% of pediatric AML patients in the United States (105). Notably, about 10% of the pediatric cases clinically presenting as APL seem not to carry any RARA fusion (106). Several transgenic mouse models have shown that the PML–RARA fusion is essential but most likely not sufficient to induce the disease as it cooperated with other mutations such as FLT3-ITD (107). Pioneer work by Hughes de Thé and Zhu Chen demonstrated that pharmacological doses of all trans retinoid acid (ATRA) or arsenic trioxide (As_2O_3) induce different molecular mechanisms that ultimately lead to proteasome-dependent degradation of the PML–RARA fusion protein (108). A phase III randomized multicenter trial demonstrated clinical efficacy and superiority of the combination of ATRA with As_2O_3 over ATRA and chemotherapy in adult patients (109). Pediatric APL patients treated with ATRA and anthracyclines or As_2O_3 reached an estimated overall 5- and 8-year survival of >95% (106). Collectively, these observations strongly suggest that targeted degradation of a driver fusion oncogene is the strategy of choice for long-term cure of a significant fraction of AML patients.

Multiple mouse models demonstrated that several KMT2A fusions are strong leukemogenic oncogenes that drive the disease in the presence of few, if any, cooperating mutations (66, 110). We were able to show that induction and maintenance of a transformed state of murine hematopoietic cells by the KMT2A–MLLT3 or KMT2A–MLLT1 is dependent on the fusion dose and is fully reversible (68, 69), indicating that targeted reduction of the fusion protein might be sufficient to induce differentiation and dissolve the leukemic phenotype. Biochemical studies suggested that leukemogenic KMT2A fusions form large protein complexes that bind to and activate KMT2A targets in an uncontrolled manner (111). Recent experimental work suggests that KMT2A fusion-mediated transformation could be impaired by stabilization of the non-rearranged protein, which naturally

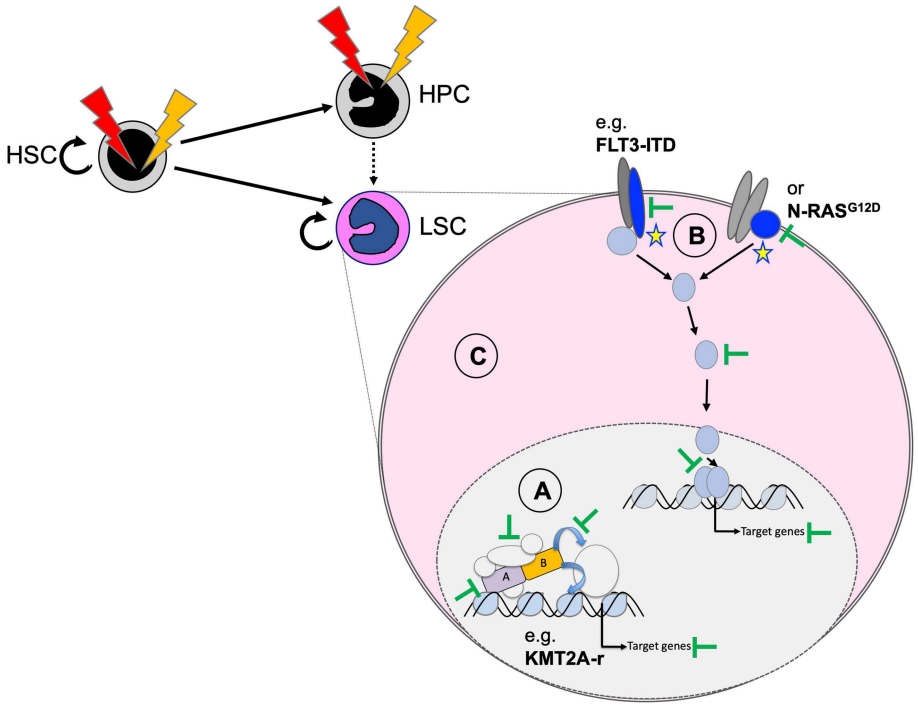

FIGURE 6 | Potential therapeutic targeting of functionally cooperating molecular mechanisms. Personalized efficient AML therapies will most likely be based on a combination of strategies that target the driver mutation **(A)**, cooperating processes **(B)**, and general characteristics of the transformed state **(C)**. Targeting the driver can be achieved by degradation of the fusion oncogene (e.g., as shown for PML–RARA by ATRA and arsenic) and blocking critical protein–protein interactions (e.g., MENIN–KMT2A interaction for chromosome binding of KMT2A fusions), related enzymatic activities, or essential downstream targets. Cooperating mutations, including constitutive active protein tyrosine kinases or RAS proteins, can be targeted with highly selective and potent small-molecule inhibitors (e.g., FLT3 inhibitors). The transformed state can be impaired by blocking survival by primed BH3-apoptosis regulators (e.g., BCL2, MCL1) or by targeting altered metabolism-related regulators (e.g., mutated IDH1/2).

undergoes proteasomal degradation regulated by casein-kinase II and IRAK4-dependent phosphorylation events (112, 113). The transforming potential of KMT2A fusions depends on several protein–protein interactions as well as enzymatic activities that have the potential for therapeutic interference. Interaction of the N-terminus of KMT2A with MENIN and the adapter protein LEDGF is essential to bind to critical targets and productive transformation by KMT2A fusion genes (114). Structure-function studies identified critical interphases, and small molecules were developed that disrupted binding of MENIN to KMT2A and impaired KMT2A fusion transformation (115–117). Constant optimization allowed the generation of MENIN protein–protein interaction (PPI) inhibitors that impair the viability of KMT2A fusion-driven cells at a nanomolar concentration (118). Notably, MENIN PPI inhibitors were shown to have potent anti-cancer activity not only in KMT2A fusion-driven leukemia but also in prostate tumors (119, 120). Novel, more potent and stable MENIN PPIs with increased selectivity for KMT2A fusions have been presented at ASH 2018 with potent anti-leukemic activity in patient-derived xenotransplant (PDX) models of AML (121).

KMT2A fusion complexes recruit the DOT1L histone H3 lysine 79 (H3K79) methyltransferase that positively regulates expression of the target genes most likely by preventing association of the sirtuin-1 (SIRT1) deacetylase complex resulting in loss of H3K9 and H4K16 acetylation, and reduced SUV39-mediated methylation on H3K9m3 (122). The maintenance of a KMT2A fusion-induced transformed state showed high dependence of DOT1L-mediated H3K79 methylation and small-molecule DOT1L inhibitors showed promising anti-tumor activity *in vitro* and *in vivo* in preclinical models (123). However, although application of a DOT1L inhibitor (Pinometostat, a.k.a. EPZ-5676) reduced H3K79 methylation, only modest clinical activity was observed in adult patients with KMT2A rearranged leukemia (124).

In addition to MENIN/LEDGF or DOT1L, targeted interference with other components of the KMT2A fusion complexes (e.g., WDR5, BRD4, CDK9) have been explored in various preclinical models (125). Interestingly, AML cells carrying CEBPA mutations leading to expression of the short oncogenic CEBP/α p30 isoform appear sensitive to pharmacological targeting of the KMT2A complex (126). However, so far, no strategy has been reported that allows selective degradation of KMT2A fusion proteins to replicate the success in APL.

Studies with Drosophila cells showed that NUP98 acts as a transcriptional activator physically interacting with non-specific lethal (NSL) and Trithorax (KMT2A) protein complexes (127).

More recent work suggested that NUP98 and some NUP98 fusion proteins (NUP98–HOXA9, NUP98–NSD1, and NUP98–HOXD13) physically interact with proteins of the human NSL and KMT2A complexes such as WDR5 or MOF most likely through the GLFG repeats (37, 128). Notably, conditional genetic ablation of KMT2A significantly reduced the leukemogenic activity of the NUP98–HOXA9 fusion *in vivo*. In addition, KMT2A-dependent gene expression signatures from murine NUP98–HOXA9 transformed cells resembled human NUP98–NSD1-derived profiles, suggesting at least overlapping pathways. These observations suggest that therapeutic targeting of KMT2A would also be effective against NUP98 fusion-driven AML. However, more translational studies found significant differences in gene expression signatures of pediatric AML cases harboring either KMT2A fusions or NUP98–NSD1. Indeed, whereas the first group is often characterized by increased expression of EVI1, the latter is associated with increased expression of another PRDM family member PRDM16 (a.k.a. MEL1) (32, 129, 130). Nevertheless, the activity of leukemogenic NUP98 fusions might be impaired by blocking the activities of distinct partner genes. Leukemic blasts immortalized by retroviral expression of the NUP98–NSD1 fusion showed elevated levels of H3K36me marks on putative target genes including the *Hox-A* gene clusters leading to the idea that small molecules blocking the NSD1 SET methyltransferase domain might have anti-leukemic activity (38, 131). Interestingly, disulfiram (DSF), known for its aldehyde dehydrogenase blocking activity, was found to induce apoptosis of murine myeloblasts transformed by PHD-containing NUP98–KDM5A and NUP98–PHF23 fusions. Although the detailed molecular mechanism remains unclear, it appeared that DSF blocked the interaction of the fusion proteins with the promoters of critical downstream targets like *HoxA7-10*, *Meis1*, and *HoxB5* (132).

Although the use of high-dose cytarabine has improved the outcome of core binding factor (CBF) AML, we still lack efficient strategies to selectively inactivate the RUNX1–RUNX1 partner transcriptional co-repressor 1 (RUNX1T1, a.k.a. CBFA2T1 or ETO) or CBFB–MYH11 driver fusions. Earlier work found that oligomerization of RUNX1–RUNX1T1 through the nervy homology 2 (NHR2) domain of RUNX1T1 was essential for its activity as a transcriptional corepressor and mediator of self-renewal to BM cells (133). Interfering with tetramerization by peptides and by small molecules reduced the oncogenic activity of the RUNX1–RUNXT1 fusion in preclinical models (134, 135). RUNX1–RUNXT1 seems to form stable complexes containing hematopoietic co-factors including E-proteins, of which the interaction, e.g., between the NHR2 domain of RUNX1T1, with a novel binding motif in E proteins seems critical (136). The oncogenic potential of the RUNX1–RUNXT1 fusion was also shown to depend on EP300-medicated acetylation of distinct lysine (K24, K43) lysine residues; hence, blocking EP300 activity impaired leukemic transformation (137). Pioneer work by Illendula et al. provided a novel concept to molecularly target CBFB–MYH11$^+$ AML. They developed a small molecule (AI-10-49) that selectively binds the CBFB–MYH11 fusion protein, resulting in RUNX1-mediated repression of the potent oncogenic driver MYC (138, 139). CBFA2T3 is highly homologous to RUNX1T1 containing nervy homology domains (NHR1–3) that mediate oligomerization of the AMKL-associated CBFA2T3–GLIS2 fusion. Notably, overexpression of a small NHR2 peptide (NC128) was able to significantly reduce the leukemia development of a CBFA2T3–GLIS2$^+$ human AMKL cell line in immunodeficient mice (25). Collectively, the oncogenic activity of CBF-related fusions can be impaired by interfering with oligomerization (RUNX1–RUNX1T1 and CBFA2T3–GLIS2) and post-translational modification (RUNX1–RUNXT1) or by impairing binding to RUNX1 (CBFB–MYH11); however, no such strategies were so far successfully translated into the clinic.

Targeting Cooperating Mutations (e.g., Tyrosine Kinase Mutation)

Several small molecules have been established that block the uncontrolled activity of FLT3-ITD, ranging from pan-kinase inhibitors like Sunitinib, to promiscuous inhibitors of multiple tyrosine kinases including Sorafenib, Midostaurin (a.k.a. PKC412), or Lestaurtinib (a.k.a. CEP-701), to very selective compounds such as Quizartinib (a.k.a. AC220), Tandutinib (a.k.a. MLN518), Crenolanib, or Gliternitinib (a.k.a. ASP2215) (140). Based on an international randomized controlled study showing that the combination of Midostaurin and chemotherapy improved the outcome of adult AML patients, the drug recently became FDA-approved for therapy of *de novo* FLT3-mutated AML (141). Very recently, Gliternitinib was also FDA-approved for relapsed/refractory AML with FLT3 mutations based on results from the ADMIRAL trial (142). Several FLT3 inhibitors have been explored in small clinical trials in pediatric AML patients, and partial or complete responses were reported not only in KMT2A-rearranged ALL (Midostaurin, Lestauritinib), but also in refractory/relapse AML (Sorafenib) (143). Promising results have been reported with the combination of the selective FLT3 inhibitor Quizartinib with chemotherapy in children with relapse or refractory AML or KMT2A-rearranged ALL (144). However, more selective FLT3 inhibition has been linked to resistance mutations in FLT3-ITD$^+$ AML particularly affecting the gatekeeper (F69L) or activation loop (D835/I836) residues (145). Future prospective controlled clinical studies will be necessary to show the profit and risks of selective FLT3 inhibition in pediatric AML.

Based on *in vitro* and *in vivo* cooperation in AML, targeted inhibition of constitutive active mutant RAS should be of therapeutic benefit. Although long viewed to be undruggable, recent observations suggest that pharmacological inhibition of RAS could be achieved (146, 147). Earlier targeting attempts focused on interfering with RAS posttranslational modification and farnesylation of the CAAX motif necessary for localization of the protein to the cellular membrane. Small-molecule farnesyltransferase inhibitors exhibited anti-leukemic activity in H-RAS but not in K-RAS mutant AML. However, the addition of Tipifarnib (Zarnestra), a selective non-peptidomimetic competitive farnesyltransferase inhibitor, to low-dose cytarabine did not improve outcome in older AML patients (148). More recent work provided proof of concept of targeted interference

with distinct RAS mutations: compounds were identified that block K-RAS^{G12C} by forming covalent disulfide bridges with the cysteine. Other compounds were found to block GDP-bound K-RAS^{G12C} and selectively impair cancer growth. Impairing RAS activity by blocking the interaction with downstream mediators, genetic depletion by anti-sense oligos, or interfering with RAS dimerization also suggested that pharmacological targeting could be achieved. However, clinical translation of targeting mutated RAS in cancer has not yet been achieved.

In contrast to adult AML, mutations in the genes encoding for IDH1 and IDH2 are rare in pediatric AML (51, 52). However, small molecules were generated that potently and selectively inhibit mutant IDH1 or IDH2 through binding in an allosteric manner at the interface of the dimerized enzymes (149, 150). IDH1$^{R132H/C}$- or IDH2^{R140Q}-selective inhibitors were shown to induce differentiation of primary AML cells *in vitro* and in PDX models, leading to a statistically significant survival benefit (151). Phase I/II clinical trials with Ivosidenib (targeting IDH1-R132) and Evasidenib (targeting IDH2^{R140Q}) in refractory or relapsed adult AML patients show overall response rates >40% with about 20% complete remission over several months underlining the potential for these compounds for personalized therapeutic strategies (152, 153). However, some patients developed clinical resistance to Evasidenib by secondary mutations in trans, in the IDH2 allele without the neomorphic R140Q mutation (154). Despite these limitations, the fact that IDH1/2 mutations can be selectively blocked by clinically effective small-molecule inhibitors urges for clinical trials in pediatric AML patients.

Targeting Hallmarks of Transformed Cells and the Immune System

It is well-known that malignant transformation leads to various cellular dependencies that may offer targets for therapeutic intervention (155). A very promising emerging strategy is to interfere with the cells' capability to evade programmed cell death known as apoptosis. In a simplified view of this complex regulatory pathway, apoptotic cell death is prevented by pro-survival BCL2-like proteins (e.g., BCL2, BCL2L1: a.k.a. BCL-XL, MCL1) by keeping in check the cell death effector proteins BAX and BAK that are activated by BH3-only proteins (e.g., BCL2L11: a.k.a. BIM; BBC3: a.k.a. PUMA, BAD, BID; PMAIP1: a.k.a. NOXA) (156). Small molecules (e.g., Venetoclax, a.k.a. ABT-199) that mimic the function of BH3-only proteins ("BH3-mimetics") have been developed that are inducing apoptosis not only in lymphoid neoplasms like CLL or B-cell non-Hodgkin's lymphoma but also in myeloid neoplasms including AML (157, 158). Selective BCL2 inhibition by Venetoclax induces rapid cell death in AML cells with an IC_{50} as low as 10 nmol/L (159). A phase II study revealed that Venetoclax monotherapy has potent anti-leukemic activity in high-risk relapsed/refractory adult AML patients (160). Small-molecule MCL1 inhibitors (e.g., VU661013, AMG176) were shown to be synergistic and rescued Venetoclax resistance of AML cells (161, 162). In addition, Venetoclax showed synergistic therapeutic activity in combination with other drugs including low-dose cytarabine, JAK1/2 inhibitors, or DNMT1 inhibitors (decitabine, azacytidine) (163–165). Moreover, inhibition of BCL2 was found to enhance the anti-leukemic activity of FLT3 inhibitors (Midostaurin, Gliternitinib) in preclinical AML models (166). Very recent work suggested that TP53, the apoptotic signaling network, and the mitochondrial functionality are the drivers of Venetoclax sensitivity in AML cells (167). These observations in adult AML patients initiated some studies to explore Venetoclax in pediatric patients with relapse/refractory malignancies including acute leukemia (NCT03236857) (168).

Intensive research is currently ongoing that aims to therapeutically target the capacity of pediatric leukemia cells to escape destruction by the immune system (169). Some studies reported some significant therapeutic responses in AML treated with antibody-drug conjugates (ADCs) targeting surface molecules like Gemtuzumab, a calicheamicin-conjugated antibody against CD33 and the response seems to correlate with a splicing polymorphism affecting the antibodies' binding site (170). Potent anti-leukemic activity has been reported for bispecific T-cell engaging antibodies (BiTEs) targeting T-cell CD3 and CD19 (Blinatumomab) on B-cell ALL cells leading to FDA approval to treat pediatric B-ALL (171). Several BiTEs targeting some AML-associated surface proteins (CD33, CD123, and CD371) that have shown potent experimental activities are currently undergoing clinical trials (172). The immunotherapy revolution in pediatric hematologic cancers is mostly marked by the development of chimeric antigen receptor T-cells (CAR-T). Remarkable success was reported by targeting CD19 on relapsed/refractory B-ALL patients (173). CAR-T approaches to target AML-associated antigens (CD33 and CD123) have been explored in preclinical models, but it appeared that yet to be defined more tumor cell-selective epitopes might be necessary to reach the efficacy observed in B-ALL (174). Very recent experimental studies suggested improved anti CD33 CAR-T therapy for AML by genome editing-mediated ablation of CD33 in HSC (175, 176). Finally, the success of checkpoint inhibitors mostly antibodies targeting immune suppressive antigens such as PD-1, PD-L1, or CTLA-4 in some solid tumors associated with a high mutational burden such as malignant melanoma initiated intensive study for their potential in hematological malignancies including AML (177). PD-1 and/or PD-L1 are expressed in AML cells, and their blockade coupled with depletion of regulatory T-cells showed potent anti-leukemic activity in preclinical models (178). Several monoclonal antibodies (e.g., Nivolumab, Prembrolizumab, Durvalumab, and Ipilimumab) are currently studied for their anti-leukemic potential in refractor/relapse AML patients; however, checkpoint inhibitors alone seem to be much less effective in AML than in solid cancers (179).

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Both authors have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Targeted Therapies for Pediatric AML: Gaps and Perspective

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Acute myeloid leukemia (AML) is a hematopoietic disorder characterized by numerous cytogenetic and molecular aberrations that accounts for ∼25% of childhood leukemia diagnoses. The outcome of children with AML has increased remarkably over the past 30 years, with current survival rates up to 70%, mainly due to intensification of standard chemotherapy and improvements in risk classification, supportive care, and minimal residual disease monitoring. However, childhood AML prognosis remains unfavorable and relapse rates are still around 30%. Therefore, novel therapeutic approaches are needed to increase the cure rate. In AML, the presence of gene mutations and rearrangements prompted the identification of effective targeted molecular strategies, including kinase inhibitors, cell pathway inhibitors, and epigenetic modulators. This review will discuss several new drugs that recently received US Food and Drug Administration approval for AML treatment and promising strategies to treat childhood AML, including FLT3 inhibitors, epigenetic modulators, and Hedgehog pathway inhibitors.

Keywords: Pediatric AML, targeted therapy, FLT-3 inhibitors, Hedgehog pathway inhibitors, DOT1L inhibitors

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INTRODUCTION

Acute myeloid leukemia (AML) is a hematological malignancy characterized by the clonal expansion of myeloid precursors, which acquire genetic abnormalities in cellular components involved in self-renewal, proliferation, and differentiation. Pediatric AML accounts for ∼25% of pediatric leukemias and although relative low frequent, it represents a clinical challenge, due to its poor prognosis. Over the last 20 years, considerable improvements in overall survival (OS) were achieved, mainly due to intensified treatment strategy, enhancements in supportive care and progresses in risk-adapted patient stratification. Despite that, OS does not exceed 70%, and relapse rates range between 25 and 35% (1), highlighting an urgent need for novel treatments. AML has an age-related profile, with regard to either incidence rate or genetic features. Indeed, the incidence of pediatric AML peaks in infants (children aged 0–1 years), and it is rare in children up to 18 years and further increases in persons between 18 and 60 years of age (2). In addition, genetic abnormalities that occur in infants distinguish a peculiar subgroup of patients (3).

In recent years, with advances in technology, there have been tremendous progresses in defining the molecular landscape of AML, and novel AML entities were included in the most recent World Health Organization (WHO) classifications (4), including AML with *NPM1* or *CEBPA* mutations. However, the occurrence of cytogenetic abnormalities as well as genetic mutations identifying specific WHO entities (e.g., *NPM1*, *FLT3*, *CEBPA* mutations) is lower in pediatric than in adult AML, and a high percentage of pediatric patients (>40%) fall in the "AML not otherwise specified" (AML-NOS) category, thus limiting the applicability of WHO classification in children with AML (5). Furthermore, thanks to the recent sequencing approaches, major insights into

pediatric AML genetic alterations, distinct from those of adult AML, were achieved. Importantly, these findings greatly affected patient risk stratification and provided new therapeutic targets (6). In this regard, in 2018, Bolouri et al. published the results of the TARGET project, reporting a comprehensive analysis of the molecular aberrations occurring in a large cohort of pediatric AML (7). The main features of pediatric AML emerged from this study were a low overall mutation rate, likewise adult AML, but a landscape of somatic aberrations different from that observed in adult patients, and including structural changes, aberrant DNA methylation, and novel pediatric-specific mutations in genes characteristically mutated in AML. More specifically, the most common mutated genes in pediatric AML included *RAS*, *KIT*, and *FLT3*, and novel pediatric-specific *FLT3* mutations were identified. Conversely, *DNMT3A*, *IDH1*, and *IDH2* gene mutations were nearly absent in pediatric AML. Novel focal deletions were identified in *IL9R*, *MBNL1*, and *ZEB2* genes, and further deletions affected *ELF1* expression. A variety of fusion genes were detected, many of which were primarily or exclusively associated to pediatric AML, for instance, *CBFA2T3-GLIS2* and *NUP98-NSD1*. Also, multiple epigenetic regulators, particularly *KMT2A* and *WT1*, were affected by both structural and mutational anomalies. Interestingly, the associated epigenetic changes induced transcriptional silencing of activating ligands for natural killer (NK) cells or genes that converge on Wnt–β-catenin signaling, both representing potential therapeutic targets. The most remarkable information emerged from the TARGET study were the age-related distribution of genomic anomalies and the interactions among mutations that have clinical consequences, thus demonstrating the importance to improve the identification of genomic alterations to better stratify pediatric AML patients as well as to develop novel targeted therapies. Indeed, at present, all the information regarding the molecular landscape of AML marginally resulted in novel therapeutic strategies, and in the last decades, with a few exceptions, there was a general stagnation in standard chemotherapeutic approaches. Fortunately, this scenario is gradually changing (8). In this review, we provided an overview of several therapeutic approaches to target specific genetic lesion of pediatric AML, with special attention on drugs that recently received US Food and Drug Administration (FDA) approval for AML treatment together with promising strategies to treat definite subgroup of pediatric AML. A summary of selected inhibitors discussed in the present review and currently investigated in pediatric AML is provided in **Table 1**.

TARGETING GENE MUTATIONS: FOCUS ON *FLT3* AND *KIT*

AML development is a multistep process that requires the cooperation of at least two genetic abnormalities, classified as type I (that confer a proliferation advantage on hematopoietic cells) and type II alterations (that impair hematopoietic differentiation) (9). These anomalies include both karyotypic alterations and gene mutations, with the latter frequently occurring in cytogenetically normal AML. Although AML is a cancer with a very low rate of somatic alterations, because of the constant identification of novel recurrent gene mutations, nowadays more than 90% of pediatric AML are identified to have at least one genomic alteration (10), among which those affecting *FLT3* and *KIT* genes are very common in children, with more than 20% and 10% frequency, respectively, according to the TARGET study (7).

FLT3 is a transmembrane type III receptor tyrosine kinase that is activated by the specific FLT3 ligand and, subsequently, regulates hematopoiesis through phosphorylation of downstream targets, including STAT5, and activation of critical oncogenic pathways such as Ras/Raf/MAPK and PI3K/Akt/mTOR (11). Activating mutations of FLT3 include both internal tandem duplication (FLT3-ITD) and point mutations of the activation loop domain (FLT3-TKD), with a prevalence of ∼15 and 7%, respectively, in pediatric AML (12). Ligand-independent FLT3 activation leads to a decreased maturation and an increased proliferation of myeloid progenitors. Importantly, FLT3 mutations are prognostically relevant in pediatric AML, and the presence of ITD particularly with an high allelic ratio (AR) of $\geq$0.5 have a prognostic impact and are significant predictive factors for an adverse outcome (12–14). Therefore, FLT3 mutated pediatric AML patients are considered high risk and, nowadays, they are offered allogenic hematopoietic stem cell transplantation (HSCT) in first complete remission (15). The use of HSCT can override the negative prognostic impact of FLT3 mutations, as demonstrated by similar probability of 8-year event free survival (EFS) in both FLT3-ITD and wild-type subgroups (15). However, there are potentially severe side effects correlated to this procedure, and there is still a consistent proportion of patients not eligible for HSCT, thus supporting the relevance to improve current treatments for FLT3 mutated patients. In addition, FLT3 mutations, even if not detectable at diagnosis, can subsequently appear at relapse because of clonal selection, and may further affect prognosis (16). Given the high number of both adult and pediatric AML patients harboring FLT3 mutations (7, 17) and their poor outcome, many efforts have been made to develop FLT3 targeted inhibitors, and a variety of compounds have entered clinical trials for both adult and pediatric patients (**Table 1**). The first generation of FLT3 inhibitors, which entered clinical trials since the early 2000s, were not FLT3 specific but targeted multiple kinases. In pediatric AML, the most extensively studied first-generation FLT3 inhibitor is Sorafenib, which was investigated as single agent or in combination with other drugs in several formal clinical trials enrolling both *de novo* or refractory/relapsed AML. In pediatric AML, the MTD of Sorafenib was defined as 150 mg/m^2 (18, 19). Sorafenib showed a significant antileukemic activity in relapsed or refractory pediatric AML, inducing a reduction by more than 50% of bone marrow blasts and, in combination with clofarabine and cytarabine, it achieved a complete remission in 8 out 12 patients, including both wild type and mutated FLT3 (18). As a single agent, the activity of Sorafenib was observed in 2/8 pediatric refractory AML, both with FLT3-ITD (19). Importantly, in these trials, remission achievement allowed to proceed with allergenic HSCT. These findings resulted in further investigation of Sorafenib in newly diagnosed or FLT3 mutant

TABLE 1 | Targeted inhibitors in clinical trials for pediatric AML.

Target	Drug	Intervention	Condition	Phase	Age group	Clinical trial identifier	Status
FLT3	Sorafenib	Sorafenib in combination with chemotherapy	*De novo* AML	III	Up to 29 years (child, adult)	NCT01371981	Completed
		Sorafenib in combination with idarubicin and Ara-C	Diagnosis AML and high-risk MDS	I–II	15–60 years (child, adult)	NCT00542971	Completed
		BTK inhibitor with chemotherapy with/without Sorafenib	Refractory/relapsed FLT3 mutant AML	II–III	14–60 years (child, adult)	NCT03642236	Recruiting
		Sorafenib in combination with cytarabine and clofarabine	Refractory/relapsed hematologic malignancies	I	Up to 31 years (Child, Adult)	NCT00908167	Completed
		Palbociclib and Sorafenib, Decitabine, or Dexamethasone	Recurrent or refractory leukemia	I	15 years and older (child, adult)	NCT03132454	Recruiting
		Sorafenib	Refractory/relapsed solid tumors or leukemia	I–II	2–21 years (child, adult)	NCT01445080	Completed
	Lestaurtinib	Lestaurtinib in combination with cytarabine and idarubicin	Refractory/relapsed FLT3 mutant AML	I–II	1–30 years (child, adult)	NCT00469859	Completed
	Midostaurin	Midostaurin in combination with standard chemotherapy	*De novo* FLT3 mutant AML	II	3 months to 17 years (child)	NCT03591510	Recruiting
		Midostaurin	Relapsed/refractory acute leukemias (MLL-rearranged ALL ad FLT3 mutated AML)	I–II	3 months to 18 years (child, adult)	NCT00866281	Completed
	Quizartinib	Quizartinib in combination with re-induction chemotherapy and as a single-agent maintenance	Refractory/relapsed FLT3 mutant AML	I–II	1 month to 21 years (Child, Adult)	NCT03793478	Recruiting
	Crenolanib	Crenolanib in combination with Sorafenib	Refractory/relapsed FLT3 mutant AML	I	1 year to 39 years (Child, Adult)	NCT02270788	Completed
	Gilteritinib	Gilteritinib in sequential combination with chemotherapy	Refractory/relapsed FLT3 mutant AML	I–II	6 months to <18 years of age (and young adults)	2215-CL-0603	Planned
		Gilteritinib in sequential combination with chemotherapy	Newly diagnosed FLT3 mutant AML	II	6 months to <18 years of age (and young adults)	2215-CL-0604	Planned
DOT1L	Pinometostat	Pinometostat	Relapsed/refractory leukemias with *MLL* rearrangements	I	3 months to 18 years (child, adult)	NCT02141828	Completed
		Pinometostat with standard chemotherapy	Newly diagnosed AML with *MLL* Rearrangement	I–II	14 years and older (child, adult)	NCT03724084	Recruiting
KIT	Dasatinib	Dasatinib in consolidation therapy in CBF-AML	*De novo* AML	N.A.	6 months to 16 years (child)	NCT03173612	Recruiting
		Dasatinib in combination with chemotherapy	Relapsed t(8;21) AML With KIT^{D816} mutation	I	Child, adult, older adult	NCT03560908	Recruiting
CD33	Lintuzumab	Lintuzumab	Relapsed/refractory AML	I	16 years and older (child, adult, older adult)	NCT00002890	Completed
		Actinium-225 labeled to lintuzumab	Relapsed/refractory AML	I	Child, adult, older adult	NCT00672165	Completed

BTK, Bruton's tyrosine kinase; MLL, mixed-lineage leukemia; FLT3, fms related tyrosine kinase 3; DOT1L, disrupter of telomeric silencing 1-like histone methyltransferase; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia.

AML (NCT01371981, NCT03642236). Other first-generation FLT3 inhibitors evaluated for pediatric AML treatment include Sunitinib (20), Lestaurtinib (NCT00469859), and Midostaurin (NCT00866281, NCT03591510). The latter is extremely important because in 2017, the FDA approved Midostaurin in combination with chemotherapy for newly diagnosed FLT3-mutated adult AML based on data of a multi-institutional, randomized phase 3 trial (RATIFY, NCT00651261). This trial showed that addition of Midostaurin to standard chemotherapy significantly prolonged overall and event-free survival among adult patients with AML and an FLT3 mutation (21). In children, Midostaurin is being evaluated as a single agent or in combination with chemotherapy in refractory/relapsed or newly diagnosed AML, respectively (NCT00866281, NCT03591510), and preliminary data indicated that single-agent Midostaurin, although adequately tolerated, has only limited clinical activity (22). However, since Midostaurin was the first drug approved in an AML genetic subgroup characterized by a specific gene mutation, this represents a starting point for novel therapy employing additional targeted agents, including second- and third-generation FLT3 inhibitors in ongoing clinical trials. Indeed, newer generation FLT3 inhibitors have a great specificity against FLT3, and consequently, they are more potent in inhibiting FLT3 with hopefully fewer off-target side effects. Among such compounds, Quizartinib, evaluated in relapsed childhood AML in combination with salvage chemotherapy, demonstrated a favorable toxicity profile and an encouraging response, consisting in complete FLT3 inhibition in all patients, and 4/17 and 10/17 complete remissions or stable disease, respectively (23). Currently, Quizartinib is being evaluated in a phase 1/2 study both in combination with re-induction chemotherapy and as a single-agent maintenance therapy in relapsed/refractory pediatric AML with FLT3-ITD mutations (NCT03793478). Crenolanib, a tyrosine kinase inhibitor developed as a selective and potent PDGFRα/β inhibitor, has also high affinity for FLT3, including both FLT3-ITD and FLT3-TKD mutations (24), and a phase 1 pilot study is currently assessing its toxicity profile in combination with Sorafenib in relapsed or refractory pediatric AML with mutated FLT3 (NCT02270788). Gilteritinib is a potent and selective FLT3 inhibitor with activity against both FLT3-ITD and FLT3-TKD mutations that demonstrated clinical efficacy in subjects with both wild-type or mutated FLT3 in phase 1/2 clinical trials enrolling adult patients with relapsed or refractory AML (25). In 2018, the FDA approved Gilteritinib as monotherapy to treat adult patients with relapsed/refractory AML and FLT3 mutations based on interim analysis of the ADMIRAL phase 3 trial (NCT02421939), which proved the superiority of Gilteritinib as compared to salvage chemotherapy in adults with relapsed and/or refractory FLT3 mutated AML. Indeed, the initial results of this trial reported a median OS significantly longer in the Gilteritinib arm than in the salvage chemotherapy arm (9.3 vs. 5.6 months), with 21% and 11% of complete remissions (CR) achieved in the two arms, respectively (26). Noteworthy, Gilteritinib is the first FLT3 inhibitor to be approved as monotherapy for AML patients. In 2016, the pediatric development program for Gilteritinib started, and in 2018, EMA approved several modifications to the pediatric investigation plan that now include two clinical studies evaluating Gilteritinib used in sequential combination with chemotherapy in pediatric patients from 6 months to <18 years of age with FLT3-ITD positive relapse/refractory AML (2215-CL-0603) or newly diagnosed AML (2215-CL-0604). Collectively, these trials provided important data regarding the efficacy of FLT3 inhibitors for AML treatment, and their application, particularly in combination with traditional chemotherapeutic agents as well as novel agents, would represent an important shift in the outcome of pediatric AML patients.

An additional gene frequently mutated in pediatric AML is *KIT* (7), a proto-oncogene that encodes a transmembrane glycoprotein type III receptor tyrosine kinase (RTK). The stem cell factor (SCF) promotes KIT dimerization and auto-phosphorylation that in turn lead to activation of complex downstream signaling pathways, including Ras/Erk, PI3K/Akt/mTOR, PLC-γ, Src kinase, and JAK/STAT signaling pathways, all essential to proliferation, differentiation, and survival of hematopoietic stem cells (27). In pediatric AML patients, *KIT* mutations occur in the extracellular portion of the receptor (exon 8), in the transmembrane domain (exons 10), in the juxtamembrane domain (exon 11), and in the activation loop of the tyrosine kinase domain (exon 17). These mutations affect RTK activity, due to ligand-independent activation of KIT, and tyrosine kinase inhibitors with activity against mutated KIT may represent effective therapeutic approaches. KIT mutations frequently associate with specific AML subtype, including core binding factor (CBF) AML. CBF-AML is characterized by the presence of aberrancies at CBF genes, and comprises t(8;21) and inv (16)/t(16;16), resulting in the *RUNX1-RUNX1T1* and *CBFB-MYH11* gene fusions, respectively. Both alterations affect CBF transcriptional complex, that is involved in the regulation of normal hematopoiesis, thus inducing leukemic transformation by blocking differentiation and promoting the self-renewal of stem cells and early progenitors (28). Collectively, CBF AML accounts about 20% of pediatric AML and is considered favorable (10). Accordingly, these patients receive a regimen of treatment based on four courses of chemotherapy (usually at lower dosages compared to other risk groups) not comprising HSCT (29). However, in some recent studies, the subgroup of t(8;21) AML patients showed a cumulative incidence of relapse of ∼30%, similarly to high-risk patients (29–31). Although most of these patients have been subsequently rescued by an allograft, this resulting in an 8-year OS approaching 83%, the event free survival (EFS) remains unsatisfactory (29). These observation prompted to better investigate the impact of *KIT* mutation on prognosis of t(8;21) AML. Indeed, according to the multistep model of leukemogenesis, RUNX1-RUNX1T1 alone is not sufficient for leukemogenesis and requires co-operation with additional genetic hits, such as *KIT* mutations (32). In an interesting retrospective analysis of children with CBF-AML, Manara et al. found several differences between t(8;21) and inv(16)/t(16;16) AML, with a higher occurrence of *KIT* mutation in *RUNX1-RUNX1T1*- compared to *CBFB-MYH11*-rearranged patients (33). More importantly, t(8;21) AML with *KIT* mutations had a significantly worse prognosis than patients harboring only the translocation, suggesting that

KIT mutations might contribute to the outcome and might be considered for risk stratification and therapeutically targetable markers in this subgroup of CBF-AML patients (33). Given the high frequency of *KIT* mutations and consequent elevated expression of this gene in AML with t(8;21), the addition to chemotherapy of the multikinase inhibitor Dasatinib has been evaluated in adult patients, and the results showed a favorable outcome (34). A recent phase 1 study is evaluating the clinical efficacy and tolerability of combination therapy of Dasatinib with multi-agent chemotherapy in relapsed child and adult AML patients with t(8;21) translocation and KIT^{D816} mutation (NCT03560908) (**Table 1**).

TARGETING DEREGULATED SIGNALING PATHWAYS

In AML, uncontrolled proliferation and increased survival of leukemic cells can be sustained by deregulation of signal transduction pathways, whose components represent potential actionable targets.

Signaling regulated by Ras proteins are among the best characterized but most intricate signal transduction pathways in cell biology. Indeed, there are three members belonging to the Ras family (HRAS, KRAS, and NRAS), all found to be activated by mutations in human tumors, that play essential roles in controlling cellular functions involved in tumorigenesis including cell growth, division, differentiation, cell cycle regulation, cell migration, and angiogenesis (35). In addition, Ras proteins operate through two distinct pathways, the mitogen-activated protein kinases (MAPK) and phosphoinositide-3 kinase (PI3K) pathways (35). Collectively, mutations in both *NRAS* and *KRAS* genes account for more than 30% of pediatric AML patients, with a prominent age-related profile, and further RAS-related mutations, that affect RAS downstream components, may occur (7). On the other hand, PI3K/Akt/mTOR pathway deregulation occurs in a large proportion of AML patients. Its constitutive activation results from a variety of mechanisms besides Ras activating-mutations, including activating mutation in RTK (e.g., FLT3 and KIT mutations), mutations and/or over-expression in key signaling components (PI3K subunits, Akt, mTOR), alterations in the activity of the negative regulators PTEN and SHIP phosphatases, and deregulation in molecules that interact with this pathway (36). A plethora of compounds targeting Ras/MAPK and PI3K/Akt/mTOR signaling components were developed and tested in adult AML patients (37, 38); however, at present, only few of those inhibitors are investigated in pediatric AML patients (39). Since this review focuses on novel and promising targeted therapies potentially available for study in pediatric AML in the near future, an exhaustive review of all the Ras/MAPK and PI3K/Akt/mTOR signaling inhibitors is beyond our scope, and only few examples will be reported. Among the Ras/MAPK pathway inhibitors, Trametinib, a highly specific and potent MEK1/2 inhibitor, is currently investigated in children with Juvenile myelomonocytic leukemia (JMML) in a phase 2 trial (NCT03190915). Various clinical trials investigated FLT3 and KIT inhibitors, as above discussed, which in turn may down-modulate PI3K/Akt/mTOR signaling, and showed encouraging results. The PI3K/Akt/mTOR pathway inhibition can be achieved by targeting the key signaling components. In pediatric AML, both Akt (MK2206) and mTOR (RAD001) inhibitors were investigated in phase 1 clinical trials (NCT01231919 and NCT00081874, respectively). However, PI3K/Akt/mTOR inhibitors may induce significant toxicities, particularly in association with chemotherapy (39), without objective responses (40), thus limiting the clinical development of therapeutic approaches based on their application.

An interesting pathway deregulated in AML and recently investigated in adult patients is the Hedgehog (Hh) pathway, an evolutionary conserved process that regulates embryonic development and organ morphogenesis (41). Hh pathway has also been implicated in hematopoiesis, although its requirements depend on developmental stage (primitive or definitive hematopoiesis), cell maturation stage, and cell physiologic state, and a wide range of *in vitro* and *in vivo* studies demonstrated that targeting specific pathway components severely impairs proper hematopoiesis (42). Classical Hh pathway can be activated by one of three different ligands (Sonic Hedgehog, SHH; Indian Hedgehog, IHH; Desert Hedgehog, DHH) that bind the transmembrane receptor Patched (PTCH) that functions as a negative regulator of the pathway through inhibition of Smoothened (Smo). As a result of ligand binding, Smo is activated and induces a signaling cascade that culminate in the activation and nucleus translocation of GLI transcription factors that in turn regulate target gene expression (41). Due to its physiological role, it is not surprising that aberrant activation of Hh pathway is commonly observed in human cancers, including myeloid malignancies. Currently, the Smo inhibitors Sonidegib, Vismodegib, and Glasdegib are evaluated with multiple intervention approaches in phase 1, 2, and 3 clinical trials, enrolling adult patients with myeloid malignancies at different stages, including AML (43). In a phase 1 study, Glasdegib, administered as a single agent, demonstrated a biological activity in 16/28 adult AML patients by inducing one CR, four partial remissions, four minor responses, and seven stable diseases (44). A more recent randomized phase 2 clinical trial evaluated low-dose cytarabine plus/minus Glasdegib in newly diagnosed AML or high-risk myelodysplastic syndrome adult patients. Addition of Glasdegib increased median OS from 4.9 to 8.8 months, with complete remission rates of 17% for the Glasdegib arm vs. 2% for the standard arm, and a general favorable benefit–risk profile (45). Based on these results, in 2018, FDA approved Glasdegib in combination with low-dose cytarabine, for newly diagnosed AML patients who are 75 years old or older or who have comorbidities that preclude intensive induction chemotherapy. Several Smo inhibitors, including Sonidegib and Vismodegib, have been evaluated in pediatric patients affected by medulloblastoma (MB), the most common malignant brain tumor in children, demonstrating efficient HH pathway inhibition and anti-tumor activity (46). Although no evidence has still been reported in childhood AML, both results obtained in adult AML and pediatric MB patients prompted an extensive investigation of Hh inhibitors in the pediatric setting (47). With regard to Smo inhibitors, it should be taken into

account the induction of permanent defects in bone growth, a toxicity profile that has been observed in both preclinical models and patients enrolled in clinical trials (46, 48). As a result, in 2016, the European Medicines Agency (EMA) modified the pediatric investigation plan of Glasdegib in pediatric patients with MB, with a waiver applied to the pediatric population from birth to <4 months of age. However, this drug-related toxicity was observed only with several compounds, including Glasdegib and Sonidegib (46, 48), thus supporting further investigation of additional Smo inhibitors especially in combinatorial drug regimens.

TARGETING FUSION PROTEINS

One of the most recurrent translocation in pediatric AML is $t_{(15, 17)}$, which results in the fusion transcript *PML-RARA* and identifies a specific AML subtype, acute promyelocytic leukemia (APL), accounting for ∼12% of pediatric AML (10). Among pediatric leukemias, APL is the first case where an effective targeted therapy was used, consisting of all-trans retinoic acid (ATRA) and arsenic trioxide (ATO), and this successful treatment radically changed APL from a detrimental to a curable malignancy, with complete remission rate over 90% (49, 50). These successful results constantly encourage the efforts in identifying effective targeted therapies against leukemia driver mutations.

The most common genetic events occurring in pediatric AML are the rearrangements of the *MLL* gene, accounting for 18% of patients (10), and in infants, the frequency is much higher, reaching 50% in several studies (3). MLL is a nuclear protein critical for hematopoietic development that normally regulates gene expression by catalyzing methylation of lysine 4 on histone 3 (H3K4). *MLL* rearranges with more than 80 different partner genes, and the resulting fusion proteins deregulate expression of MLL target genes. The most frequently overexpressed genes in *MLL*-rearranged leukemias are *HOX* cluster genes and the HOX cofactor *MEIS1*, which are normally expressed at highest levels only in the stem cells and early lineage progenitor cells, whereas they are down-regulated during differentiation (51). Although with several differences depending on fusion partner, the overall prognosis of *MLL*-rearranged AML is unfavorable (51). Great advancements in our knowledge of relevant mechanisms that determine the oncogenicity of MLL fusions have been made in recent years. The histone methyltransferase disrupter of telomeric silencing 1-like (DOT1L) is a methyltransferase that catalyzes methylation of lysine 79 on histone H3 (H3K79) and this epigenetic mark associates with active gene transcription (52). DOT1L acts in a multiprotein complex that also includes partners of MLL in the formation of MLL-fusion proteins (53). Therefore, in *MLL*-rearranged leukemias, DOT1L is recruited on MLL target sites, and its mislocated enzymatic activity promotes the leukemic gene expression program (54–56). It is not surprising that considerable efforts are being made to identify effective inhibitors to target DOT1L. Impressive results have been obtained in preclinical studies investigating pharmacologic inhibition of DOT1L (57), and the DOT1L inhibitor Pinometostat entered a phase 1 clinical trial to treat pediatric patients with relapsed/refractory leukemias bearing a rearrangement of the *MLL* gene (NCT02141828) (**Table 1**). However, despite Pinometostat biological activity and acceptable safety profile, no objective responses were observed (58). Currently, a phase 1/2 clinical trial evaluating Pinometostat in combination with standard chemotherapy to treat both child and adult patients with newly diagnosed *MLL*-rearranged leukemia is ongoing (NCT03724084) (**Table 1**).

An attractive therapeutic target for MLL-rearranged leukemias is represented by the menin–MLL interaction. Menin is a tumor suppressor protein that interacts with both wild type and rearranged MLL proteins and is required for the proper recruitment of MLL to the target genes. Because MLL-fusion proteins are difficult to target directly, pharmacological inhibition of the menin–MLL interaction is a promising therapeutic approach, since the leukemogenic activity of MLL fusion proteins is dependent on this interaction. In *in vivo* preclinical studies, the first orally bioavailable small-molecule inhibitors of the menin–MLL interaction, MI-463 and MI-503, resulted in growth inhibition and survival benefit in mouse models of MLL leukemia (59). In addition, further preclinical studies demonstrated that combining DOT1L and menin inhibition enhances the treatment efficacy in *MLL*-rearranged leukemia models (60). Interestingly, the synergistic effect of DOT1L and menin inhibitors has also been observed in AML models harboring *NPM1* gene mutations (61). Currently, two phase 1/2 clinical trials are evaluating the menin inhibitors KO-539 (NCT04067336) and SNDX-5613 (NCT04065399) in adult patients with relapsed/refractory AML or *MLL*-rearranged/*NPM1*-mutated AML, respectively.

CBFA2T3-GLIS2 is a recently identified fusion transcript resulting from a cryptic inversion of chromosome 16 and specific to pediatric AML (62, 63). This chimeric oncogene identifies a peculiar subgroup of extremely aggressive pediatric AML with an incidence ranging between 9 and 30% among the whole cytogenetically normal (CN) AML (64) or the non-Down syndrome acute megakaryoblastic leukemia (non-DS-AMKL) subgroups (62), respectively. CBFA2T3 is a CBFA2T-family member that belongs to the RUNX1T1 complex and acts as a transcriptional co-repressor via its association with DNA-binding transcription factors, other corepressors, and histone-modifying enzymes, including the chimeric protein RUNX1-RUNX1T1 resulting from t(8;21) (64). GLIS2 (GLI-similar 2) is a member of the Kruppel-like zinc finger transcription factor group, which is closely related to the GLI family proteins, the transcription factors activated by the Hedgehog signal transduction cascade that regulate cell proliferation and self-renewal (64). Based on the homology between GLIS2 and GLI proteins, our group explored the possibility to target CBFA2T3-GLIS2 employing GANT61, a GLI1, and GLI2 inhibitor. Remarkably, *in vitro* treatment with GANT61 induced apoptosis in *CBFA2T3-GLIS2*-positive AML cells and reduced the expression of GLIS2-specific signature genes (65). Even if preliminary, these results are encouraging and prompt to extend the investigation of GLI inhibitors as a promising strategy to treat CBFA2T3-GLIS2 AML. Due to the

discovery that induction of polyploidization and differentiation mediated by Aurora kinase A (AURKA) provides a therapeutic strategy for AMKL (66), Thiollier et al. investigated the efficacy of the AURKA inhibitor Alisertib (MLN8237) in a xenograft model of human AMKL expressing the CBFA2T3-GLIS2 fusion, demonstrating that Alisertib efficiently inhibits leukemic blast proliferation and increases survival, because of induction of terminal differentiation and apoptosis (67). However, a phase 2 clinical trial documented a poor response rate in children and adolescents affected by solid tumors or leukemia and receiving Alisertib as a single agent (68).

TARGETING EPIGENETIC REGULATORS

A common feature of AML is an altered epigenetic pattern resulting from both somatic mutations in epigenetic regulators or specific translocations that interfere with the normal epigenetic program (69). As a whole, mutations in epigenetic regulators are frequent in adult AML while they are uncommon in pediatric AML. For example, mutations in the $NADP^+$-dependent isocitrate dehydrogenase genes 1 and 2 (*IDH1* and *IDH2*) were found in up to 33% adult AML and only in 3–4% of pediatric AML (70–72). Other epigenetic regulators mutated in pediatric AML, albeit rarely, are *TET2*, *DNMT3A*, and *ASXL1* (73). A relevant finding that emerged from these studies is the frequent co-occurrence of mutations in epigenetic regulators with other genetic anomalies in signaling transduction pathways and hematopoietic transcription factor genes, suggesting that mutations in epigenetic regulators cooperate in leukemogenesis. The last point also implies the possibility to combine treatments that target different class of mutations to eradicate the leukemic clone. In this regard, targeting the methyltransferase DOT1L is particularly intriguing for several reasons. Firstly, DOT1L is the only known methyltrasferase that catalyze mono-, di-, and tri-methylation of H3K79, and only recently a histone demethylase that can catalyze the removal of di- and tri-methyl groups from the H3K79 lysine residue has been identified (74); therefore, most likely DOT1L plays an essential role in AML cells. Next, DOT1L has diverse functions in mammalian cells, since methylation of H3K79 is primarily linked to active gene transcription and transcription elongation, but DOT1L also acts in DNA damage response and cell cycle regulation (53). Then, DOT1L associates with several complexes, including the elongation complexes EAP (75), SEC (76), and AEP (77), which, in turn, contain other proteins with oncogenic/leukemogenic activity. Finally, although the involvement of DOT1L in leukemia was initially linked to its mislocation due to MLL fusion proteins, some of the most recent works demonstrated a role of DOT1L also in non MLL-rearranged leukemias. For example, in preclinical studies, pharmacologic inhibition of DOT1L has been reported to impair proliferation, to induce cell differentiation, or to impact gene expression of primary AML cells harboring IDH1/2 mutations (78) as well as AML cell lines and a xenograft model with partial tandem duplication (PTD) of MLL (*MLL*-PTD) (79). DOT1L inhibition has been explored as a therapeutic target for the treatment of *DNMT3A* mutant AML, wherein reversed $DNMT3A^{mut}$ induced gene activation and resulted in apoptosis and cell differentiation induction in both *in vitro* and *in vivo* AML models (80, 81). Finally, DOT1L inhibition has also been assessed in *NPM1* mutant AML and resulted synergistic with menin–MLL inhibitors in suppressing *HOX*, *MEIS1*, and *FLT3* gene expression and inducing AML cell differentiation (61). Overall, these studies demonstrated a remarkable role of DOT1L in AML cells irrespective of MLL fusion proteins, and based on these preclinical results, further investigation of combination treatments employing DOT1L inhibitors is warranted.

A further recently identified epigenetic target is the Bromodomain and Extra-Terminal Domain (BET) family of proteins, which includes BRD2, BRD3, BRD4, and BRDT proteins. This is the most prominent group of epigenetic reader proteins that regulates gene transcription by interacting with acetylated histones, thus facilitating transcriptional activation. Different small-molecule BRD inhibitors were developed and tested in cancers characterized by altered histone acetylation and aberrant gene transcription, including AML wherein epigenetic alterations are common (82). BET inhibitors demonstrated an anti-leukemic activity in preclinical models as both single agents or in combination with other drugs. In addition, their application is currently investigated in several clinical trials enrolling adult AML patients (82, 83), encouraging their application for pediatric AML treatment.

IMMUNOTHERAPY

A promising treatment to fight cancer is immunotherapy, an approach that exploits components of the immune system. There are several types of immunotherapy, including monoclonal antibodies, T-cell therapy, cancer vaccines, and other non-specific immunotherapies (84). All these therapeutic strategies have been employed to treat different types of cancer, including AML (85). In pediatric AML, the most relevant immunotherapy-based approach is represented by targeted therapy directed against surface antigens, in particular CD33 (sialic acid-binding immunoglobulin lectin, SIGLEC) and CD123 (IL3Rα), that are highly expressed, albeit not exclusively, on AML cells. A wide range of clinical trials were conducted to investigate the efficacy of gentuzumab ozogamycin (GO, Mylotarg) treatment in pediatric AML. GO is a monoclonal antibody to CD33 conjugated with the cytotoxic antibiotic calicheamicin that specifically induces cell death in CD33-expressing cells. GO originally received accelerated approval in 2000 as a stand-alone treatment for older patients with CD33-positive AML who had experienced a relapse, but it was withdrawn from the market after subsequent confirmatory trials failed to verify clinical benefit and demonstrated safety concerns, including a high number of early deaths. Nevertheless, in 2017, FDA approved GO for the treatment of adults with newly CD33-positive AML and patients aged 2 years and older with CD33-positive AML who have experienced a relapse or who are refractory (86). The newest FDA approval includes a lower dosing regimen, which induces less adverse events and is active for induction of remission,

without curative intent (86). GO as monotherapy demonstrated a limited efficacy in children with relapsed/refractory AML (87) and did not delay the time to relapse when administered in postconsolidation therapy (88). However, GO was effective in reducing MRD levels in pediatric AML patients, when administered in combination with chemotherapy (89), and this is particularly beneficial in conditioning regimen prior to HSCT (90). The use of GO was also explored in consolidation targeted immunotherapy following HSCT (91). CD33-expressing AML cells may also be targeted by the unconjugated antibody Lintuzumab that was investigated in relapsed/refractory pediatric AML in phase 1 clinical trials (NCT00002890, NCT00672165). An exhaustive review describing all the other immunotherapies investigated in pediatric AML was recently published (92).

CONCLUSIONS

After decades of therapeutic advances for AML based almost exclusively on optimizing older drugs, since 2017, the field of novel therapies for AML has been rapidly developing. A major point to take into account is that lots of novel target inhibitors might be associated with each other or with conventional chemotherapy to increase treatment efficacy. Indeed, in AML, the genomic lesions often cooperate (**Figure 1**). For example, one of the most significantly up-regulated gene in *MLL*-rearranged leukemias is *FLT3*. Further, *NPM1*-mutated AML has aberrant *HOX* expression and frequently concomitant *FLT3* mutations. Also, the presence of fusion proteins interferes with normal cellular functions, and the efficacy of fusion protein targeting might be increased with additional drugs. In conclusion, such

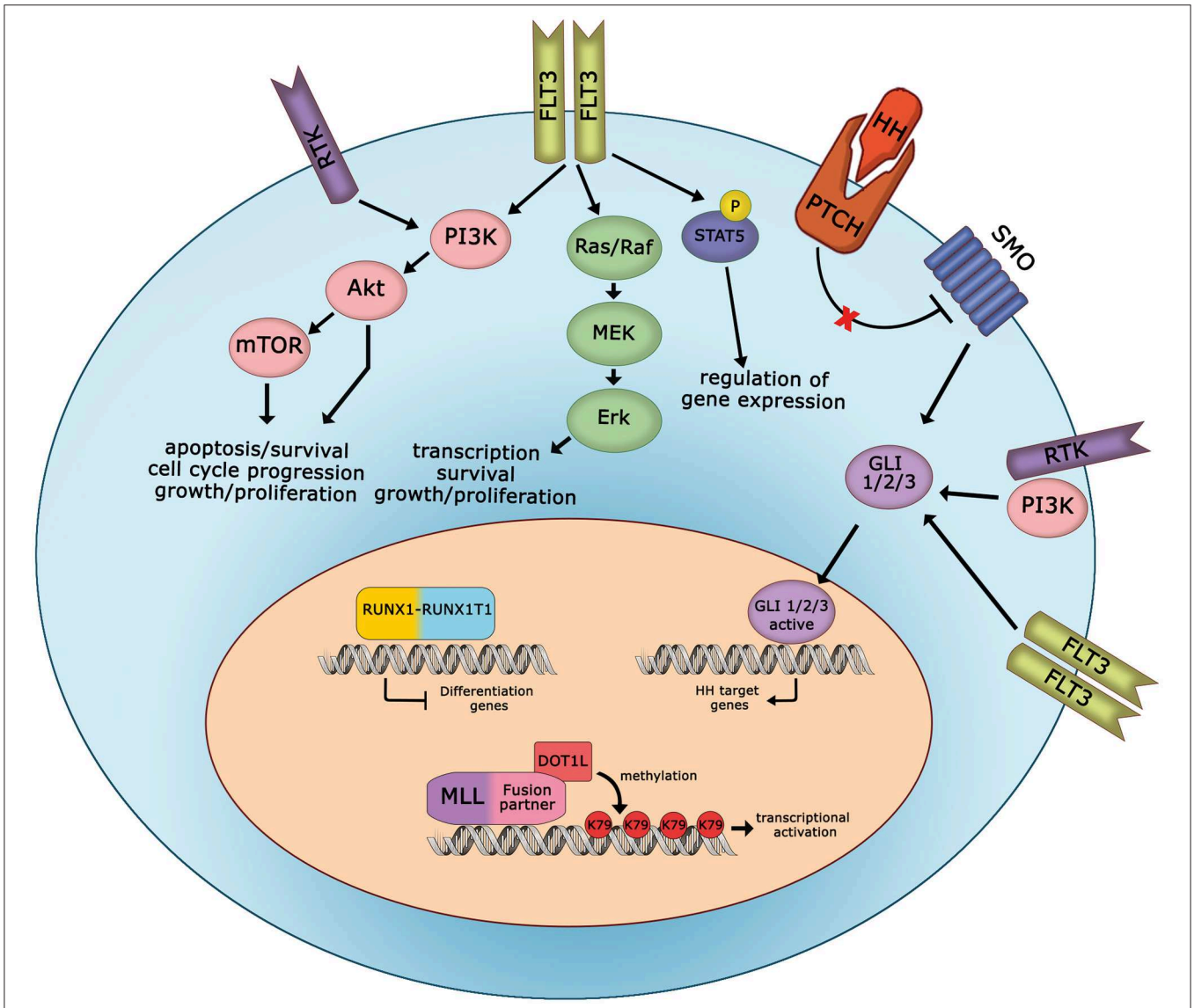

FIGURE 1 | Cooperation between oncogenic signaling pathways in pediatric AML. Schematic representation of deregulated signaling and mutated proteins, involved in AML cell proliferation and survival, that can be targeted in pediatric patients. HH, hedgehog; RTK, receptor tyrosine kinase.

progresses in drug development, together with continuous efforts in our understanding of the molecular landscape of AML, provide great hope that more effective treatments may be offered to pediatric AML patients in the near future.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and editing of this review and approved the final manuscript. AL conducted literature review and wrote the manuscript in consultation with RM. AP critically revised the work. RM provided final approval of the version to publish.

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Treatment Outcome and the Genetic Characteristics of Acute Promyelocytic Leukemia in Children in Poland From 2005 to 2018

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Background: The aim of the study was to analyze the treatment outcome and genetic characteristics of acute promyelocytic leukemia (APL) in children in Poland from 2005 to 2018.

Methods: All 41 patients diagnosed with APL in Poland during the analysis period were eligible for the study. In period I (2005–2015), 33 patients were treated with chemotherapy and all-trans retinoic acid (ATRA), and in period II (2015–2018), 3 patients (high risk) received induction chemotherapy with ATRA and arsenic trioxide (ATO), and 5 patients (standard risk) received ATRA and ATO without chemotherapy.

Results: Probability of 5-years overall survival (OS), event-free survival (EFS), and relapse-free survival (RFS) was 0.819 ± 0.069, 0.831 ± 0.063, and 0.961 ± 0.037, respectively, in the whole cohort. Four (11%) early deaths were observed. One patient died of severe infection in the course of disease progression. Relapse occurred in one patient, who died finally because of disease progression. All events occurred in the patients from period I. Variant APL was identified in one patient (successfully treated with chemotherapy with ATRA) and complex translocation in one patient (the only patient with relapse). Additional chromosomal aberrations were found in 26% of patients and

FLT3-ITD mutation was detected in 44% of patients; none of those changes influenced clinical outcome.

Conclusion: Treatment outcome in the analyzed group is similar to the results reported by other study groups. The main cause of death was coagulation disorders in the early stage of disease. Early, accurate diagnosis followed by specific treatment enables the reduction in the number of early deaths.

Keywords: acute promyelocytic leukemia, children, variant translocations, treatment results, ATO, ATRA

INTRODUCTION

Acute promyelocytic leukemia (APL) is a specific subtype of acute myeloid leukemia (AML). In most cases, it is characterized by translocation (15;17) with the PML-RARA fusion gene (1–3), but about 1–2% of APL cases are due to rare variant translocations including ZBTB16/RARA, NMP/RARA, NUMA/RARA, STAT5B/RARA, PRKAR1a/RARA, BCOR/RARA, and FIP1L1/RARA (4–6). APL comprises about 5–10% of pediatric AML (1) and about 0.4% of all malignancies in children. Methods of the treatment used in children are based on clinical studies performed on greater adult population. Until the late 1980's, APL was the most lethal subtype of AML (2, 3). The result of the treatment improved significantly since the 1980's when specific treatment with all-trans retinoic acid (ATRA) was introduced (2, 3, 7–9). Since the 1990's, another specific drug—arsenic trioxide (ATO)—has been implemented. Efficacy and safety were first proven in adult patients (10–12) followed by the studies in children (1, 13–16). The combination of ATRA, ATO, and anthracycline-based chemotherapy ensures remission achievement in almost all patients (1, 13–16). Use of the specific treatment in APL allowed reduction of the chemotherapy especially cumulative anthracycline doses (13, 15–17). The main causes of the treatment failure are still early deaths, mostly in the course of intracranial hemorrhage (2, 3, 18). In the large analysis comprising 683 patients from different international studies, initial high WBC counts and obesity were found as likely predictors of thrombohemorrhagic early deaths in childhood APL (18).

Here, we present retrospective analysis of the treatment results of pediatric APL in Poland from 2005, when genetic analysis became widely available to confirm diagnosis of APL, to 2018. Children were treated according to two consecutive protocols, first (2005–2015) based on combination of chemotherapy and ATRA and second (2015–2018) based on ATRA and ATO with or without chemotherapy depending on number of leukocytes at diagnosis.

The aims of the study were to assess clinical outcome and determine the causes of treatment failures. We also performed analysis of additional genetic changes found in APL patients.

PATIENTS AND METHODS

From January 2005 to December 2018, 41 children (age 0–18) with newly diagnosed APL were treated in 16 centers of the Polish Pediatric Leukemia and Lymphoma Study Group. They comprised 6.5% of all 627 pediatric patients diagnosed with AML in that period. They were treated according to two consecutive protocols (AML-BFM 2004 Interim and AML-BFM 2012). Patient characteristics are summarized in **Table 1**. All of them were eligible for the study. The last patient was enrolled in May 2018 and the last follow-up was done in December 2018. Median observation time was 61.7 months (range, 7.0–145.5 months). The data were collected in Polish AML registry and analyzed retrospectively.

Informed consent to participation in the studies was obtained from guardians of all patients, in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of Jagiellonian University Medical College.

All patients with characteristic bone marrow morphology and immunophenotype had diagnosis confirmed by the use of conventional cytogenetics, showing t(15;17) or variant translocations, and/or by fluorescence *in situ* hybridization tests for PML/RARa fusion or positive reverse transcription polymerase chain reaction (RT-PCR) assay.

The FLT3-ITD mutation analyses were routinely performed in all children. Patients were also screened for additional chromosomal aberrations.

In the first period (January 2005–June 2015, period I) 33 patients were treated according to AML-BFM 2004 Interim Protocol; in the second period (July 2015–December 2018, period II) 8 children were treated according to AML-BFM 2012 Protocol. In period I, treatment consisted of four intensive chemotherapy cycles (AIE: cytarabine, idarubicine, etoposide, AI: cytarabine, idarubicine, haM: high-dose cytarabine, mitoxantron, HAE: high-dose cytarabine, etoposide; additional intrathecal cytarabine in every cycle) and maintenance

TABLE 1 | Characteristics of the patients.

		Total $n = 41$	Period I AML-BFM 2004 Interim (2005–2015) $n = 33$	Period II AML-BFM 2012 (2015–2018) $n = 8$
Age, years		12.4	12.34	13.51
median (range)		(0.1–17.9)	(0.1–17.9)	(3.7–17.8)
Gender	Males	21 (51%)	18 (54.5%)	3 (37%)
	Females	20 (49%)	15 (45.5%)	5 (63%)
Observation time, months		61.7	61.7	18.4
median (range)		(7.0–145.5)	(12.7–145.5)	(7.0–30.0)

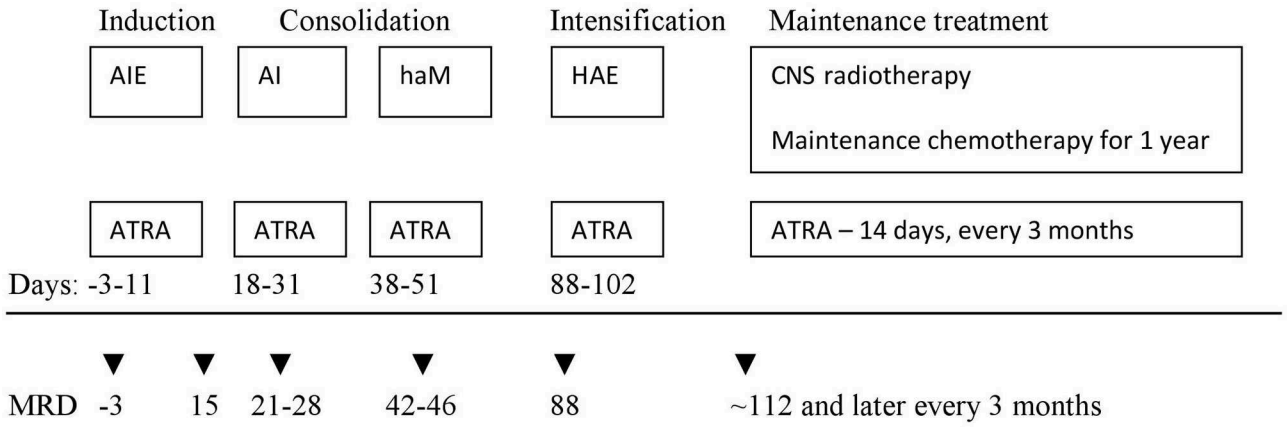

FIGURE 1 | First period (AML-BFM 2004 Interim) treatment scheme. AIE—cytarabine 100 mg/m^2/day [days 1–2] and 100 mg/m^2 every 12 h [days 3–8], idarubicin 12 mg/m^2/day [days 3, 5, and 7], and etoposide 150 mg/m^2/day [days 6–8]. AI—cytarabine 500 mg/m^2/day [days 1–4] and idarubicin 7.5 mg/m^2/day [days 3 and 5]. haM—cytarabine 1 g/m^2 every 12 h [days 1–3] and mitoxantrone 7 mg/m^2/day [days 3–4]. HAE—cytarabine 3 g/m^2/every 12 h [days 1–3], etoposide 125 mg/m^2/day [days 2–5]. Maintenance chemotherapy—lasting 1 year: thioguanine 40 mg/m^2/day orally, cytarabine 40 mg/m^2/day intravenously for 4 consecutive days, every 4 weeks. ATRA—all trans-retinoid acid 25 mg/m^2/day for 14 days. Intrathecal cytarabine in the age-depended dose (<1 year 20 mg; 1–2 years 26 mg; 2–3 years 34 mg; >3 years 40 mg) in each chemotherapy cycle and during maintenance treatment. CNS, central nervous system.

therapy (6-thioguanine, cytarabine) for 1 year. All patients received ATRA concomitant with chemotherapy in 14-days cycles (**Figure 1**). Median observation time was 61.7 months (12.7–145 months).

In period II, according to the AML-BFM 2012 Protocol, patients with APL were classified into two groups according to initial number of white blood cells (WBC). There were five patients in the standard risk (SR) group with initial WBC <10,000/μl treated with the ATRA and ATO regimen. Three other patients were in the high-risk (HR) group (WBC more than 10.000/μl), and received one chemotherapy cycle (AI: cytarabine, idarubicine) with ATRA and ATO (**Figure 2**). Median observation time was 18.4 months (range, 7.0–30 months).

Molecular response was assessed centrally according to Europe Against Cancer program (19). It was monitored in period II, while the data from period I are very limited (complete data from 1 patient and two results in 5 patients—15 and 24 months from diagnosis). In period II and in 1 patient from period I, molecular minimal residual disease (MRD) was monitored on days 21, 56, 84, and 112 from the beginning of the treatment and then every 3 months for 12 months in the SR group and 18 months in the HR group.

In our database, we also collected data about differentiation syndrome (DS), severe adverse effect of ATO, and long-lasting consequences of the treatment in patients observed for at least 18 months. DS was defined as having at least three of the following symptoms: unexplained fever, weight gain, dyspnea, and pulmonary infiltrates.

The data that support the findings of the study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Statistical Analysis

Descriptive statistical analysis was performed to assess patient baseline characteristics. We used Fisher's exact test or a chi-square test (categorical variables) and Mann–Whitney test (continuous variables) for analysis of clinical and laboratory features. Early mortality was defined as death within 42 days of the induction therapy. Overall survival (OS), event-free survival (EFS), and disease-free survival (DFS) were calculated using the Kaplan–Meier method. OS was defined as the time diagnosis to death from any cause; patients alive or lost to follow-up were censored at the date they were last known alive. EFS was defined as the time from diagnosis to disease progression, relapse, or death from any cause. Patients who were alive without disease progression or relapse were censored at the last time they were seen alive and event-free. DFS was defined as the time from complete remission (CR) to disease relapse, or death from any cause. Patients who were alive without disease relapse were censored at the time of last follow-up. For comparisons of Kaplan–Meier curves, we used the log-rank test. Because there were just eight patients in period II and observation time was much shorter than that in period I, we did not perform any statistical analysis to compare these two periods. All statistical analyses were performed using STATISTICA 12 software.

RESULTS

Genetic Aberrations

In 27 patients (65.8%), complete cytogenetic analysis was available. Variant APL with t(11;17) (q23;q12) was identified in one patient and complex translocation involving chromosomes 4, 15, 16, and 17 in another patient (**Table 2**).

Additional chromosomal aberrations were found in seven patients (26% of 27 patients with available karyotype results). We identified the trisomy of chromosome 8 in three patients, derivated chromosome 8 in one patient, complex karyotype in two patients (involving chromosomes 3 and 9 with marker chromosome in one patient and involving chromosome 6 with two marker chromosomes in one patient), and additional material from chromosome 16 in the above-described patient

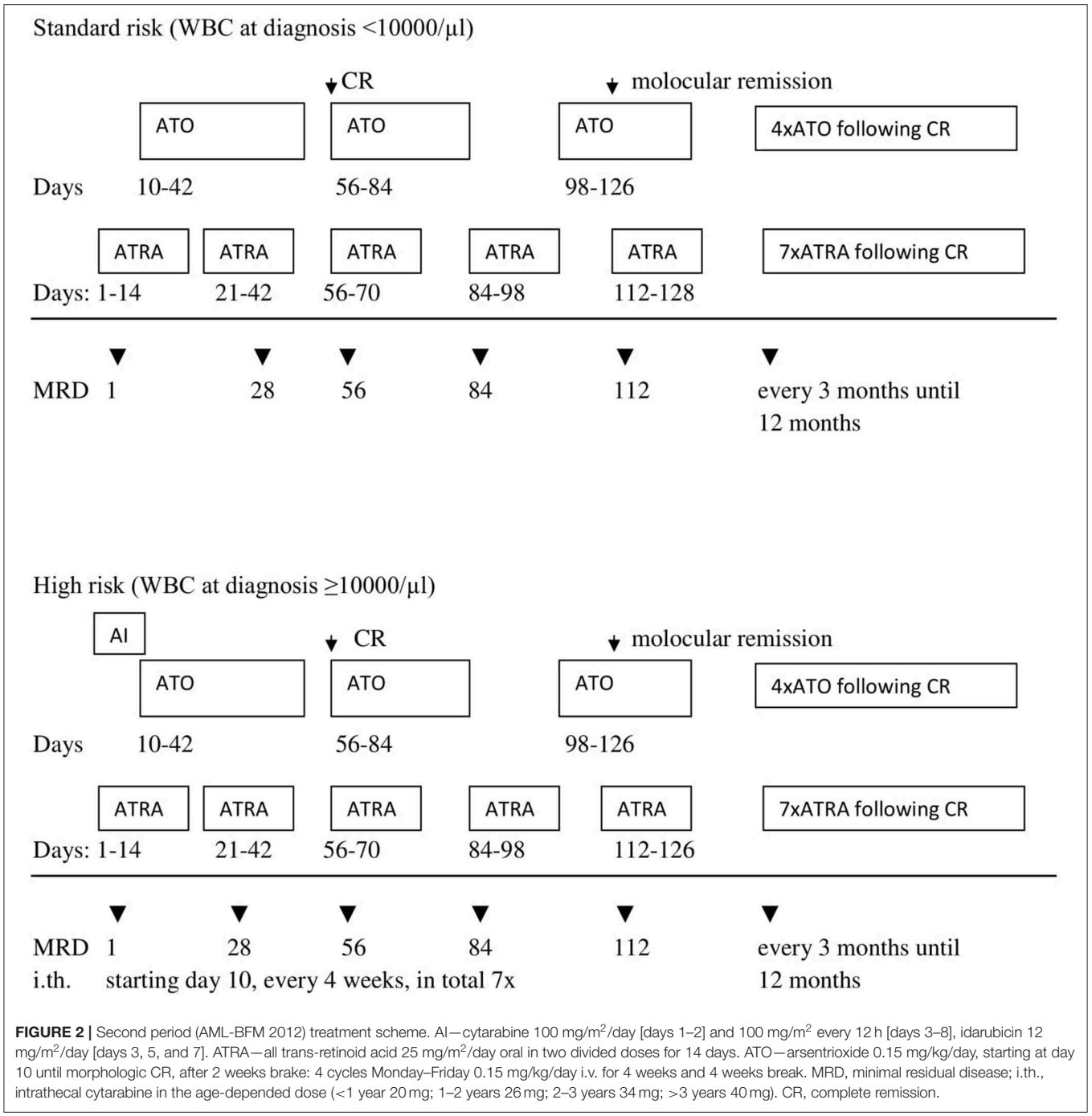

FIGURE 2 | Second period (AML-BFM 2012) treatment scheme. AI—cytarabine 100 mg/m^2/day [days 1–2] and 100 mg/m^2 every 12 h [days 3–8], idarubicin 12 mg/m^2/day [days 3, 5, and 7]. ATRA—all trans-retinoid acid 25 mg/m^2/day oral in two divided doses for 14 days. ATO—arsentrioxide 0.15 mg/kg/day, starting at day 10 until morphologic CR, after 2 weeks brake: 4 cycles Monday–Friday 0.15 mg/kg/day i.v. for 4 weeks and 4 weeks break. MRD, minimal residual disease; i.th., intrathecal cytarabine in the age-depended dose (<1 year 20 mg; 1–2 years 26 mg; 2–3 years 34 mg; >3 years 40 mg). CR, complete remission.

with complex translocation involving chromosomes 4, 15, 16, and 17 (**Table 2**).

Result of FLT3-ITD mutation analysis was available in 32 (78%) patients. This mutation was detected in 14 patients (44%).

Treatment Response and the Treatment Failures

Thirty six (88%) patients achieved complete hematological remission. Data concerning molecular response were available in nine patients (one from period I and eight from period II). On day 21, molecular MRD was negative in two of seven patients with available result, and on day 56, it was negative in seven of eight patients with available results from that time point. All nine patients with available molecular monitoring had negative MRD results from day 84. No molecular relapse occurred. Four (9.7%) early deaths (5–10 days from diagnosis) caused by severe coagulation disorders were observed. Nine patients (21.9%) suffered from severe bleedings including those four children who died. One patient (2.4%) died of severe infection in the course of disease progression 1.9 months after diagnosis (no data

TABLE 2 | Characteristics of the patients with additional genetical changes and variant APL.

	Genetics	Age (years)	WBC ($\times 10^9$/L)	PLT ($\times 10^9$/L)	Complications	Outcome	Observation time (months)
1	46,XY,t(11;17)(q23;q12)	<1	72.8	236	ND	Alive	14.7
2	46,XY,der(4)(4pter→4q26::16q22→16qter),der(15)(15pter→15q23::4q31.3→4qter),der(16)(16q22→16p11.2::17q25→17q21::16p11.2::→16pter)[20] PML/RARA(+), FLT3-ITD (–)	1–5	21.2	8	-	Death in course of progression after II relapse	37.7
3	45-47, XX,−6, add (6)(p25), t(5,17)(q22;q21),+mar, +mar (7)/46XX,PML/RARA(+),MLL(–),FLT3-ITD(–)	1–5	5.2	7	Subcutaneous and gingival bleeding	Alive	95.0
4	47,XX, +8, t(15;17)(q22;q21); PML/RARA(+) FLT3-ITD(–),	15–18	32.3	23	DIC, ATRA syndrome	Alive	35.0
5	46,XY,add(3),add(9),t(15;17),+mar; PML/RARA(+)	1–5	0.45	20	-	Alive	56.6
6	47XX,+8,t(15,17)(q22,q21)	10–15	2.0	187	ATRA syndrome	Alive	83.6
7	46,XY, t(15,17)(q22,q21)[9]/47XY+8; FLT3-ITD(–)	1–5	4.7	5	-	Alive	92.3
8	46XY, t(15;17)(q22.q21)[5]/46XY, t(15;17)(q22,q21), der(8)(pterq24…q11 qter), PML/RARA (+), FLT3-ITD(–)	13	20.0	24	-	Alive	61.7

WBC, number of white blood cells at diagnosis; PLT, number of platelets at diagnosis; ND, no data; DIC, disseminated intravascular coagulation; ATRA, all trans-retinoic acid.

TABLE 3 | Characteristic of the patients with the treatment failure.

	Age (years)	WBC ($\times 10^9$/L)	PLT ($\times 10^9$/L)	Genetics	Events	EFS (months)	OS (months)
1	10–15	40	26	46,XX,t(15;17)(q22;q21), PML/RARA(+), FLT3-ITD(+)	Early death (DIC, MOF)	0.17	0.17
2	1–5	151	0	PML/RARA (+), FLT3-ITD (+)	Early death (intracranial bleeding)	0.2	0.2
3	10–15	92	30	46,XY,t(15;17), FLT3-ITD non-available	Early death (intracranial bleeding)	0.3	0.3
4	1–5	21	8	46,XY,der(4)(4pter→4q26::16q22→16qter),der(15)(15pter→15q23::4q31.3→4qter),der(16)(16q22→16p11.2::17q25→17q21::16p11.2::→16pter) (20) PML/RARA(+), FLT3-ITD (–)	Relapse, death in progression after second relapse	17.3	37.7
5	10–15	24	6	46,XY,t(15;17), FLT3-ITD(–)	Death in progression	0	1.87
6	1–5	196	21	PML/RARA (+), FLT3-ITD analysis non-available	Early death (DIC, leukostasis)	0.33	0.33

DIC, disseminated intravascular coagulation; MOF, multiorgan failure; WBC, number of white blood cells at diagnosis; PLT, number of platelets at diagnosis; EFS, event-free survival; OS, overall survival.

concerning molecular MRD were available). Relapse occurred in one patient (2.4%), 17 months after first hematological remission. That was the patient with complex translocation. The patient did not respond to second-line chemotherapy (Idarubicine, Fludarabine) with ATRA, but achieved hematological remission after ATO treatment, followed by hematopoietic stem cell transplantation (HSCT). Fourteen months after HSCT, second relapse confirmed by molecular examination occurred, and the patient died 2 months later despite second-line therapy because of disease progression. No data concerning molecular response are available in that patient. **Table 3** displays characteristics of the patients with the treatment failures. All events occurred in the patients from period I. There was no event in patients diagnosed after 2011 (21/41 children, 51%). Treatment results in two periods are presented in **Table 4**.

Analysis of Survival

The probability of 5-years OS, EFS, and RFS was 0.832 ± 0.069, 0.848 ± 0.066, and 0.964 ± 0.035, respectively, for all analyzed patients (**Figure 3**).

White blood cell count at diagnosis was significantly higher in children who died early compared to the other patients (median

[range]: 121 × 10^3/μl [40–196 × 10^3/μl] vs. 5.2 × 10^3/μl [0.45–140 × 10^3/μl], $p = 0.003$).

Patients with more than 10,000/μl WBC at diagnosis had significantly lower probability of 5-years OS and EFS compared to patients with WBC <10,000/μl at diagnosis (1.0 vs. 0.63, $p = 0.004$ and 1.0 vs. 0.67, $p = 0.005$, respectively) (**Figure 4**).

The time from diagnosis to ATRA introduction did not differ between patients with treatment failure and other patients [median (range): 2 (1–4) days vs. 3 (0–56 days) $p = 0.67$].

There was no significant difference in OS, EFS, and DFS between patient FLT3-ITD positive and FLT3-ITD negative. Among 14 FLT3-ITD-positive patients, two early deaths occurred (14.3%), compared to 1 early death (5.5%), 1 progression (5.5%), and 1 relapse (5.5%) in 18 FLT3-ITD-negative patients. Number of WBC at diagnosis did not differ significantly in patients with and without FLT3-ITD mutation (median [range]: 18.2 × 10^9/L [0.9–150.7 × 10^9/L] vs. 6.5 × 10^9/L [0.4–140 × 10^9/L], respectively, $p = 0.4$).

Patients with additional chromosomal aberrations did not differ significantly from patients without those aberrations in terms of survival rates (OS, EFS, and RFS). The only event occurred in the patient with complex translocation and additional material from chromosome 16 who had two relapses and died of the disease progression 36.7 months after diagnosis.

The patient with variant translocation (11;17) was treated successfully with a combination of chemotherapy and ATRA and remains in remission with an observation time of 4 years.

TABLE 4 | The treatment results in two consecutive periods.

	Total $n = 37$	Period I AML-BFM 2004 Interim (2005–2015) $n = 33$	Period II AML-BFM 2012 (2015–2018) $n = 8$
Complete remission (CR) achieved (%)	36 (87.8)	28 (84.8)	8 (100)
Early deaths (%)	4 (9.7)	4 (12.2)	0
Death in progression (%)	1 (2.4)	1 (3)	0
Relapse (%)	1 (2.4)	1 (3)	0
Continuous CR (%)	35 (85.3)	27 (81.8)	8 (100)
Severe bleedings (%)	9 (21.9)	8 (24.2)	1 (12.5)

Adverse Events of ATRA and ATO

DS was observed in 29% of patients (9/31, no data from 10 patients), eight of whom were treated with steroids; in three patients, ATRA was held and then restarted.

There were no severe adverse events in patients treated with ATO. Transient rash occurred in one patient at the beginning of the therapy.

Late Consequences of the Treatment

Data concerning long-lasting consequences of the treatment were available in 23 of 34 patients with an observation time of at least 18 months. One patient was diagnosed with aseptic bone necrosis and one was diagnosed with ischemic stroke of brain. We did not find any long-lasting toxicities in 21 patients (91%).

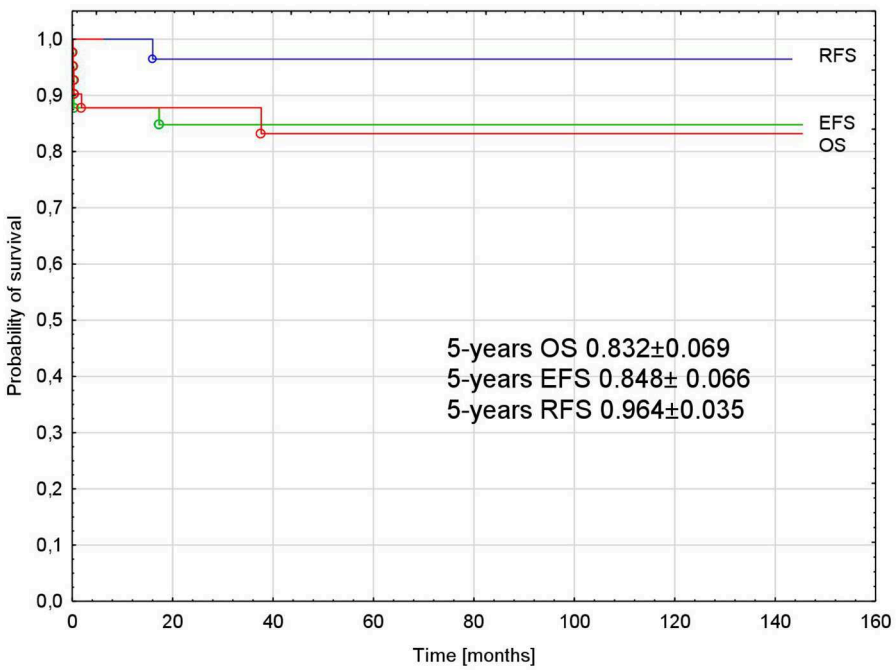

FIGURE 3 | Probability of overall, event-free, and relapse-free survival in all analyzed patients with APL. APL, acute promyelocytic leukemia; OS, overall survival; EFS, event-free survival; RFS, relapse-free survival.

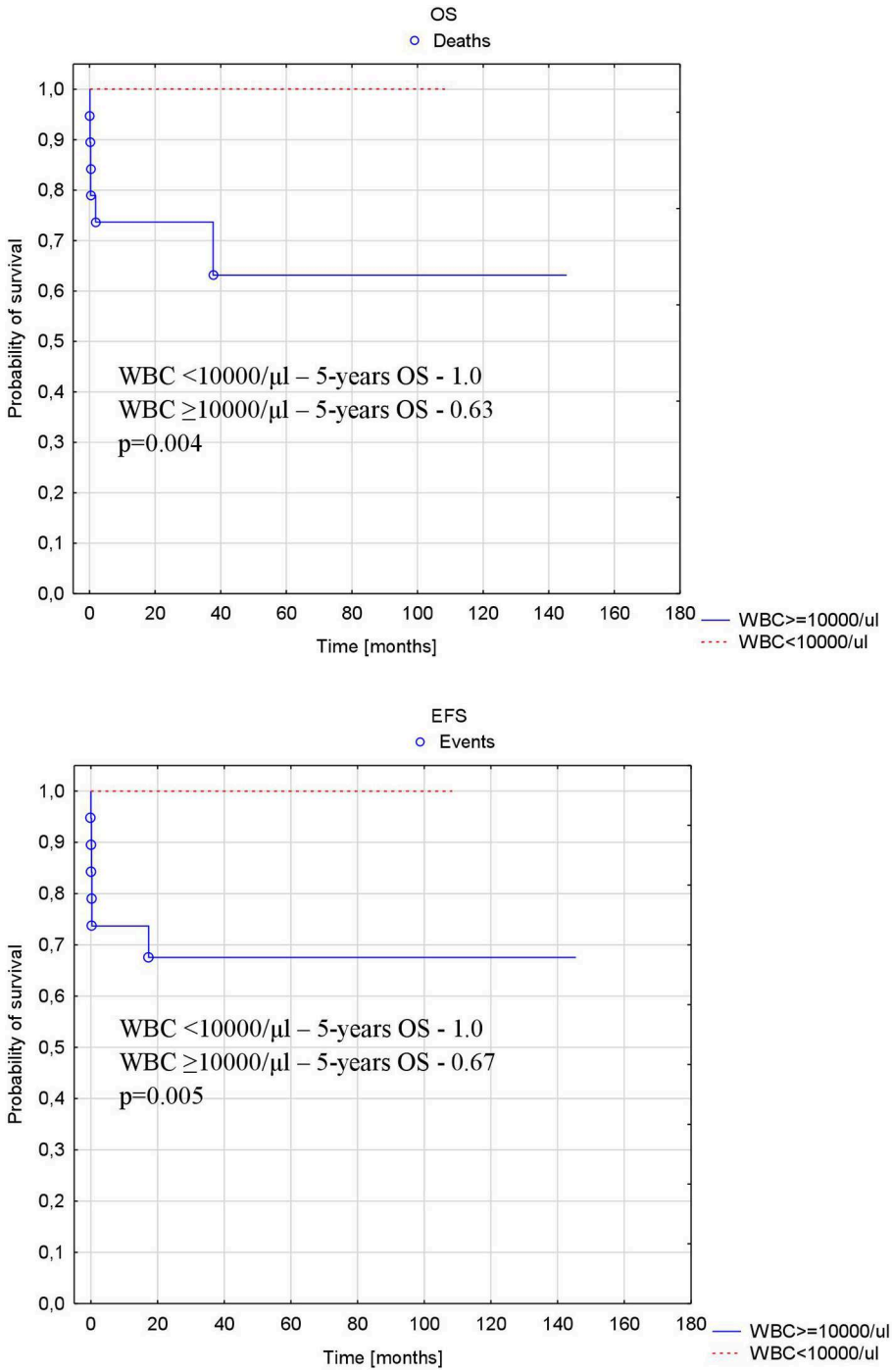

FIGURE 4 | Probability of overall and event-free survival depending on number of WBC at diagnosis. OS, overall survival; EFS, event-free survival; WBC, white blood cell number at diagnosis.

DISCUSSION

APL comprises about 5–10% of childhood acute myeloid leukemia (1). In the analyzed period, the percentage of the patients with APL among all children with AML in Poland was 6.5%. The treatment results achieved in the study group (OS, 0.832 ± 0.069; EFS, 0.848 ± 0.066; RFS, 0.964 ± 0.035) are comparable to the results described by other authors (1, 2, 7, 8, 13, 16, 17); however, the number of enrolled patients is relatively low. The data concerning molecular monitoring were limited in period I so comparison between two groups was not possible.

The bleeding complications remain the main cause of the treatment failures in APL. They were found in 4 (9.7%) patients in our cohort. It is worth noticing that there was no early death in patients with APL in Poland from 2011. It seems that early introduction of ATRA/ATO as well as oncology centers experience reduced the risk of early deaths. New treatment protocol introduced in 2015 with reduction of chemotherapy and use of ATO seems to be very effective. There were no events in that period; however, follow-up was rather short (median 18.4 months).

Variant translocation described as simple translocation involving chromosome 15 or 17 with any other chromosomes or complex translocations characterized by the involvement of additional chromosomes in addition to chromosomes 15 and 17 (4–6, 20–24) was found in two patients in the analyzed group. In one of them, t(11;17) (q23;q12) was identified, and in the second complex, translocation involving chromosomes 4, 15, 16, and 17 was found. The first patient was treated successfully with combination of chemotherapy and ATRA and remains in remission with an observation time of 4 years. The patient with complex translocation died of disease progression after second relapse.

Two different variant APL translocations involving chromosomes 11 and 17 were described before, t(11;17)(q23;q21) producing ZBTB16-RARA (formerly PLZF-RARA) fusion gene and t(11;17)(q13;q21) generating NUMA/RARA (20, 22). In cases of APL with t(11;17)(q23q21), there is evidence of resistance to ATRA and ATO both *in vivo* and *in vitro*, particularly in those patients who have the reverse rearrangement RARα-PLZF (20), while patients with t(11;17)(q13;q21) have better prognosis (22).

There are a number of studies concerning the alternate translocation in APL; however, little is still known about the complex variant translocations in APL (22, 23). Both 4, 15, 17, and 15, 16, 17 translocations have been described by other authors (4, 6, 22, 23), but the translocation found in our patient involving four chromosomes 4, 15, 16, and 17 has not been reported before.

In our cohort, additional chromosomal aberrations were found in 28% of patients with complete cytogenetic analysis, with the most common trisomy 8 (43%), similarly described by Cervera et al. (25). All but one patient from that group remain in CR. The only patient with treatment failure (death of disease progression after second relapse) was the child with complex translocation involving chromosomes 4, 15, 16, and 17 with additional material from chromosome 16. Analyzing all patients with additional chromosomal aberration in the study group, no differences in the treatment outcome were found compared to the patients with isolated t(15;17). It was reported by Cervera et al. that patients with and without additional chromosomal abnormalities had similar CR rates, and despite univariate analysis, they showed that additional chromosomal abnormalities were associated with a lower relapse-free survival in the LPA99 trial; such association was not present in the LPA96 trial. Neither additional chromosomal abnormalities overall nor any specific abnormality was identified as an independent risk factor for relapse in multivariate analysis (25).

In the current study, FLT3-ITD was present in 44% of patients. This is in accordance with the other studies where the frequency of FLT3-ITD mutation was 20–46% (26–30). No differences in WBC at diagnosis and the treatment outcome were found in patients with and without FLT3-ITD mutation but analyzed groups were rather small. The prognostic impact of FLT3-ITD mutation in APL remains controversial. Some authors describe negative influence on prognosis. Lucena-Araujo et al. screened for FLT3-ITD mutations in 171 APL patients (including nine children) and reported that FLT3-ITD mutations were associated with high WBC counts and may independently predict a shorter survival in patients with APL treated with ATRA and anthracycline-based chemotherapy (28). Association between FLT3-ITD mutation and high WBC was also confirmed by Barragan et al. in PATHEMA and HAVON groups, but the study did not demonstrate an independent prognostic value of FLT3-ITD mutation in patients with APL treated with ATRA and anthracycline-based regimens (29).

The COG AAML0631 study on childhood APL did not show an association of FLT3-ITD mutations with early death or bleeding/clotting events in induction; however, it was revealed that the relapse rate following ATO consolidation was significantly higher in FLT3-ITD mutant patients (30).

To conclude, the treatment outcome in children with APL in Poland is similar to results reported by other study groups. Reduction of early deaths in the last years was noticed. That could be the effect of introducing ATRA to a treatment protocol and the better experience of the study centers in the care for this challenging group of patients. A new treatment approach with the use of ATO and ATRA without chemotherapy in the SR group or with reduced chemotherapy in HR seems to be safe and effective. The limitation of the study is the relatively low number of enrolled patients. Further studies are needed to confirm the results with longer follow-up.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Bioethics Committee of Jagiellonian University Medical College. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MC and WBal designed the study. MC, KP-W, BS-F, MM, AR-K, AC, JS-S, JW, KM-R, MK-R, DG, JK, KZ, TU, RT, TS, IK-D, MW, JP, GK, WM, KM, WBad, SS, and WBal were involved in participant recruitment. TK was involved in the laboratory work and interpretation of its results. MC, KP-W, BS-F, AR-K, JS-S, KM-R, DG, KZ, RT, IK-D, JP, and KM collected the clinical data. MC was involved in the statistical analysis and interpretation

of its results, and wrote the first draft of the manuscript. WBal, KP-W, and SS edited the first draft of the manuscript. All authors reviewed the manuscript and approved the final version of the manuscript.

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Polymorphisms of SLC19A1 80 G>A, MTHFR 677 C>T, and Tandem TS Repeats Influence Pharmacokinetics, Acute Liver Toxicity, and Vomiting in Children With Acute Lymphoblastic Leukemia Treated With High Doses of Methotrexate

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Introduction: High dose methotrexate (HD-Mtx) is highly effective and significantly improves overall acute lymphoblastic leukemia (ALL) patients survival. The pharmacodynamics of Mtx depends on the polymorphism of genes encoding proteins engaged in the folate metabolism pathway. The aim of the current study is to determine the relationship between variants of folate metabolism-related genes and the frequency of acute toxicities of HD-Mtx.

Material and Methods: A group of 133 patients aged 1.5–18.1 years (median: 6.3) was treated in accordance with the ALL-IC-2002 and ALL-IC-2009 protocols. The following polymorphisms were determined: 80 G>A *SLC19A1* (solute carrier family 19 member 1; rs1051266) with direct DNA sequencing, as well as 677 C>T *MTHFR* (methylenetetrahydrofolate reductase; rs1801133) and the tandem repeats of the *TS* (thymidylate synthase) with PCR technique. HD-Mtx organ toxicities were evaluated based on the laboratory tests results and the National Cancer Institute criteria.

Results: In patients with genotypes AA for *SLC19A1* and CC or CT for *MTHFR* Mtx steady state concentrations (C_{ss}) and AUC_{inf} were distinctly higher. In patients with genotype 3R/3R for *TS* initial elimination rate constant was significantly higher ($P = 0.003$). Patients receiving Mtx at the dose of 5 g/m^2 had lower clearance (4.35 vs. 8.92 L/h/m^2) as compared to the ones receiving 2 g/m^2 that indicates non-linear Mtx elimination at the higher dose. Liver impairment was the most frequently observed

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toxicity. The homozygous genotype was associated with a significantly higher incidence of hepatic toxicity for both the *SLC19A1* ($P = 0.037$) and *TS* ($P = 0.002$). Logistic regression analysis indicated an increased risk of vomiting for the 2R/3R genotype of the *TS* gene (OR 3.20, 95% CI 1.33–7.68, $P = 0.009$) and for vomiting and hepatic toxicity for the 3R/3R genotype (vomiting: OR 3.39, 95% CI 1.12–10.23, $P = 0.031$; liver toxicity: OR 2.28, 95% CI 1.05–4.95, $P = 0.038$). None of the acute toxicities differed between the analyzed dosing groups.

Conclusions: Determination of polymorphisms of *SLC19A1*, *MTHFR*, and *TS* genes might allow for a better prior selection of patients with higher risk of elevated Mtx levels. Our study is the first one to report the increased risk of hepatotoxicity and vomiting in patients with *TS* polymorphisms.

Keywords: acute lymphoblastic leukemia, children, genes, polymorphism, methotrexate, pharmacokinetics, toxicity

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is diagnosed in about 30% of children with neoplastic diseases and is the most common neoplasm in pediatric population (1). Methotrexate (Mtx) is one of the key chemotherapeutic agents used in a high doses (HD) treatment regimens of childhood ALL. Due to the observed severe toxicity, HD-Mtx, defined as Mtx doses $\geq$ 1 g/m^2, requires a proper monitoring of drug elimination and an adequate leucovorin rescue administration. Nonetheless, in some of ALL patients toxic plasma concentrations of Mtx are observed, causing severe acute chemotherapy complications. The resulting modifications of treatment regimens might negatively affect overall patient survival (2, 3).

To date, well-known risk factors of toxicity after prolonged Mtx exposition include drug-drug interactions, insufficient prehydratation, older age, obesity or so called "third space fluid collections." However, they do not explain all the changes observed in pharmacokinetics (PK) of Mtx in patients with childhood ALL. Numerous centers have performed comprehensive studies to explain the molecular basis of Mtx pharmacological activity and to identify genetic risk factors of its abnormal PK (3–6). As determined, Mtx enters the cell through the cell membrane by binding to the solute carrier family 19 member 1 (*SLC19A1*) (7–9). Inside the cell Mtx and its more active derivatives—polyglutamates block function of several enzymes of folate cycle, mainly dihydrofolate reductase (DHFR) responsible for production of active form of folate—tetrahydrofolate and thymidylate synthase (TS), involved in DNA synthesis (6, 10, 11). The final effect of Mtx pharmacological activity is blocking purine *de novo* synthesis and cells division. One of the main enzymes of the complex folate metabolism is methylenetetrahydrofolate reductase (MTHFR), that catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. Although Mtx does not directly inhibit MTHFR function, the activity of this enzyme is crucial for the body resources of tetrahydrofolate, that are necessary in DNA synthesis, as well as in methylation of DNA, lipids and proteins, including transformation of homocysteine into methionine. All aspects of Mtx disposition and mechanism of action that we attempt to consider are summarized in **Figure 1**.

As has been shown previously *SLC19A1*, *TS* and *MTHFR* genes polymorphisms are common in the European population (12, 13). Different variants of these proteins can influence cytotoxic effect of Mtx and contribute to acute side effects of HD-Mtx therapy (12, 13). Although published, until now, results regarding relationship between polymorphisms of *SLC19A1*, *TS*, and *MTHFR* genes, increased Mtx plasma concentrations and intensive toxicities caused by HD-Mtx indicated importance of genetic polymorphisms, they have been sometimes conflicting (6, 8, 14–16). There are also studies showing a clear relationship between the polymorphisms of above-mentioned genes involved in folate metabolism with worse therapeutic prognosis for children with ALL (14, 17).

The aims of the current study were to assess the prevalence of *SLC19A1* 80 G>A and *MTHFR* 677 C>T genes polymorphisms as well as *TS* gene tandem repeats in the group of children treated due to ALL and its influence on Mtx pharmacokinetics and incidence of acute toxicities caused by HD-Mtx.

MATERIALS AND METHODS

Patients

The study group included 133 patients (**Table 1**), 1.5–18.1 years old (median: 6.3 years), treated in Department of Oncology and Hematology University Children's Hospital in Krakow, Poland, in accordance with ALL-IC-2002 (132 patients) and ALL-IC-2009 (1 patient) protocols. Both protocols for SR and IR risk pre-B ALL as well as T-ALL patients had the same frame. They were composed of induction (prednisone, vincristine, daunorubicin, L-asparaginase, cyclophosphamide, arabinoside cytosine, 6-mercaptopurine, and intrathecal methotrexate); consolidation (high dose methotrexate, 6-mercaptopurine); reinduction (dexamethasone, vincristine, doxorubicin, L-asparaginase, cyclophosphamide, arabinoside cytosine, 6-thioguanine and intrathecal methotrexate) and maintenance therapy (6-mercaptopurine, low dose methotrexate, and intrathecal methotrexate). The consolidation Mtx dose in

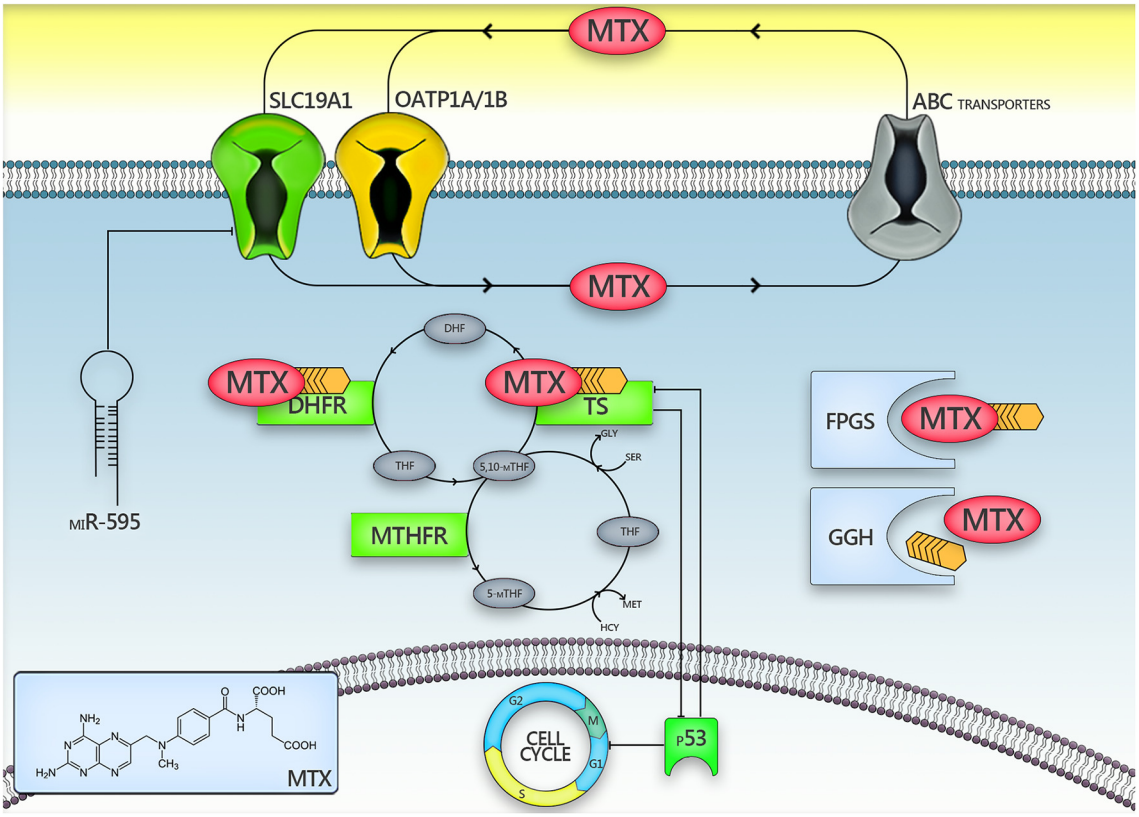

FIGURE 1 | The presentation of intracellular methotrexate metabolism pathways. Factors that may be responsible for the inconsistency among studies assessing the role of *SLC19A1*/TS/MTHFR polymorphisms in methotrexate toxicity are marked with an asterisk (*).5,10-mTHF, 5,10-methylenetetrahydrofolate; ABC, ATP-binding cassette; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthase; GGH, γ-glutamyl transferase; miR-595, micro RNA 595; MTHFR, methylenetetrahydrofolate reductase; Mtx, methotrexate; OATP1A/1B, organic anion–transporting polypeptides 1A/1B; p53, p53 protein; *SLC19A1*, solute carrier family 19 member 1; TS, thymidylate synthase.

TABLE 1 | Characteristic of patients according to Mtx dose.

Mtx dose g/m^2	Patients nb.	Chemotherapy cycles (%)	Age (median)	Gender (%)	BSA (%)	Type of ALL	Risk group	Cycles with delayed Mtx elimination (%)
2	123	478 (91%)	1.7–16.2 (4.9)	64 girls (52) 59 boys (48)	75: N (61) 29: > 75p. (23.6) 19: <3 p. (15.4)	121–pre B 2–pre T	SR 58.5% IR 41.5%	69 (14.4)
5	13	47 (9%)	1.5–18.1 (7.3)	2 girl (15.4) 11 boys (84.6)	9: N (69.2) 3:>75 p. (23.1) 1: < 3 p. (7.7)	1–pre B 12–pre T	IR 100%	28 (59.6)

p., percentyl.

ALL-IC-2002 was 2 g/m^2 for all children with precursor-B, standard and intermediate risk ALL group. For T-ALL the dose was 5 g/m^2. In ALL-IC-2009 the IR group and T-ALL were treated with Mtx at the dose of 5 g/m^2, SR patients had the same dose of 2 g/m^2. In both protocols the only additional drug given simultaneously was 6-mercaptopurine at the dose of 25 mg/m^2. Together, 525 Mtx-chemotherapy cycles given in consolidation phase (protocol M) were studied. Two patients were given 1 cycle at the Mtx dose of 2 g/m^2, and 3 cycles at the dose of 5 g/m^2. One patient was given 3 cycles at the Mtx dose of 2 g/m^2, and 1 cycle at the dose of 5 g/m^2 (**Table 1**).

Genetic Analysis

Genetic analysis was performed in the laboratory with the international QC certificates (EMQN). DNA for molecular analyses was extracted with standard methods from blood mononuclears (0.5 ml of blood was collected from every patient; QIAamp DNA Blood Mini Kit was used, manufactured by

QIAGEN). Assessment of 80 G>A *SLC19A1* polymorphism was performed with direct DNA sequencing (Sanger's method). In turn, 677C>T *MTHFR* polymorphism was analyzed with PCR-RFLP technique and *TS* tandem repeats were assessed based on the PCR with subsequent agarose gel electrophoresis (12). Based on genotyping results the patients were divided into 3 groups ("wild" genotype, heterozygotes, homozygotes). The sequences of primers that were used for genotyping were presented in the **Supplementary Material**.

Pharmacokinetic Analysis

Pharmacokinetic parameters of Mtx were calculated based on the routinely measured concentrations after the HD-Mtx administration. The blood samples were taken at the end of 24 h infusion (steady state) and in the elimination phase at 36, 42, and 48 h from the beginning of the Mtx administration (i.e., 12, 18, and 24 h after the end of infusion). In the cases where the last concentration measured was above 0.4 μM (indication of prolonged Mtx elimination) subsequent samples were taken at the selected time points until the Mtx level decreased below 0.25 μM. Because of the potential for capacity-limited intracellular transport and renal clearance the elimination of Mtx is not accurately described by linear pharmacokinetic model. However, relatively simple two-compartment model appears to represent quite well the elimination phase. Therefore, the elimination constants (k_{el}) for both initial α (up to 12 h after the end of infusion) and terminal β (from 12 to 24 h after the end of infusion) phases were calculated by log-linear regression of the drug concentration data in the appropriate phase. The area under the concentration vs. time curve extrapolated to infinity (AUC_{inf}) was estimated using the log-linear trapezoidal rule and the total clearance (normalized per m^2 of BSA) was calculated from dose/AUC_{inf}. Mtx concentrations were analyzed with immunoenzymatic method on the VIVA-Vitalab analyzer, DADE-BEHRING, USA.

Pharmacodynamic Analysis

The pharmacodynamics study was concentrated on the analysis of acute toxicity observed during the chemotherapy cycles at the Mtx doses of 2 and 5 g/m^2. Every cycle, independent of HD-Mtx dose, was administered to a patient in good clinical condition, after exclusion of acute infection and with normal renal and liver functions. The routine blood tests panel included complete blood count, blood urea nitrogen, creatinine, total protein, albumin, bilirubin, alanine transaminase, aspartate transaminase, and electrolytes (all tests were measured in SI units). Tests were performed 1 day before HD-Mtx administration and 48 h after starting the infusion (24 h after the end of infusion). HD-Mtx toxicity was evaluated based on the analysis of laboratory tests results and clinical features according to the National Cancer Institute criteria (NCI 3.0 version). Liver (SGOT/SGPT, bilirubin), blood/bone marrow (WBC, PLT, Hgb, ANC) and gastrointestinal (vomiting, stomatitis) toxicities as well as concomitant infections were studied. Grades ≥ 2 according to NCI criteria were analyzed. Liver function was considered to be impaired if the following criteria (based on our own experience) were met: increase in transaminases level at least 1 grade and/or bilirubin grade ≥ 2 and/or decrease in protein level at least 13% comparing to the values observed before the actual cycle. Data concerning toxicity were collected prospectively at each cycle of chemotherapy, and were the basis for the subsequent therapeutic decisions, than all patients charts were reviewed.

Statistical Analysis

Statistical analyses were performed with Statistica 12.0 (StatSoft, Statistica 12.0, Tulsa, Oklahoma, USA) software. Chi-square, Pearson chi-square and Fisher exact tests were used to identify relations between categorical variables. Comparison of numerical variables was performed using one-way ANOVA with *post-hoc* Tukey test or non-parametric Kruskal-Wallis test depending on the sample size. Allelic separation consistency within observed group of patients with expected allele distribution according to Hardy-Weinberg's rule was checked with use of the Chi-square test. Multiple logistic regression analysis was performed to identify risk factors of increased HD-Mtx therapy toxicities. Bonferroni correction for multiple comparisons was applied when assessing associations of toxicities and genetic variants, separately for each gene assessed. P-value of <0.05 was considered statistically significant.

RESULTS

The distribution of observed genotypes was consistent with the Hardy-Weinberg equilibrium (**Table 2**).

Pharmacokinetic Results

As expected, steady state concentrations of Mtx in patients treated with 5 g/m^2 were significantly higher than in those receiving 2 g/m^2 (137 vs. 38.5 μM). Moreover, the overall mean AUC_{inf} values were higher than proportionally expected (2,510 μM·h for 5 g/m^2 vs. 717 μM·h for 2 g/m^2) indicating lower total clearance in patients receiving Mtx at the dose of 5 g/m^2 as compared to the ones receiving 2 g/m^2 (4.35 vs. 8.92 L/h/m^2, respectively). Furthermore, the percentage of patients with prolonged elimination, defined as the concentration > 0.4 μM at 48 h after the beginning of infusion, was much higher in the group receiving Mtx at the dose of 5 g/m^2 (59.6 vs. 14.4%) (**Table 1**). These data might indicate that at the higher dose Mtx elimination process approaches saturation resulting in non-linearity of PK (**Tables 3**, **4**). The possible variations in the Mtx levels resulting from non-linear elimination could influence the statistical analysis of the relationship between the genetic polymorphism and Mtx PK parameters. Moreover, due to limited number of observations, in the group receiving Mtx at the dose of 5 g/m^2 the non-parametric statistical tests were used which are less powerful. Therefore, analysis of the influence of genetic polymorphism on PK of Mtx was mostly based on the parameters calculated after the dose of 2 g/m^2. All the obtained results are presented in **Tables 3**, **4** as well as in **Figure 2** (multiple comparison). Mean steady state concentrations of Mtx were significantly higher (42.9 vs. 36.9 or 37.3 μM) in homozygotes AA of 80 G>A gene *SLC19A1* polymorphism ($P = 0.0467$). Also homozygotes CC and heterozygotes 677 C>T of *MTHFR* gene had significantly higher (41.3 or 37.3

TABLE 2 | The distribution of observed genotypes.

Gene	Variant 1	Variant 2	Variant 3	Consistent with the Hardy-Weinberg
80 G>A gene *SLC19A1*	GG−45 patients (33.8%)	AG−50 patients (37.6%)	AA−38 patients (28.6%)	($P = 0.054$, $\chi^2 = 5.84$, df = 2)
677 C>T gene *MTHFR*	CC−66 patients (49.6%)	CT−54 patients (40.6%)	TT−13 patients (9.8%)	($P = 0.89$, $\chi^2 = 0.22$, df = 2)
TS gene tandem repeats	2R/2R−29 patients (21.8%)	2R/3R−76 patients (57.1%)	3R/3R−28 patients (21.1%)	($P = 0.37$, $\chi^2 = 1.99$, df = 2)

TABLE 3 | Methotrexate (Mtx) steady-state concentrations and basic pharmacokinetic parameters, calculated after administration of Mtx at the dose of 2 g/m^2, depending on the observed genotype.

	Total ($n = 478$)	**GG ($n = 175$)**	**GA ($n = 180$)**	**AA ($n = 123$)**	***P* value**
***SLC19A1* gene**					
Prolonged elimination	69 (14.4%)	26 (14.9%)	31 (17.2%)	12 (9.8%)	NS
C_{SS} [μM]	38.5 (36.5–40.5)	36.9 (33.6–40.1)	37.3 (34.4–40.2)	42.9 (38.3–47.6)	0.0467
k_{el} alfa [1/h]	0.30 (0.29–0.30)	0.29 (0.28–0.30)	0.29 (0.28–0.30)	0.31 (0.31–0.32)	0.0007
AUC_{inf} [μM·h]	717 (680–755)	690 (630–740)	696 (640–751)	797 (712–883)	0.0566
CL [L/h/m^2]	8.92 (7.96–9.88)	9.51 (8.06–10.97)	9.25 (7.22–11.3)	7.40 (6.55–8.25)	NS
	Total ($n = 478$)	**CC ($n = 246$)**	**CT ($n = 184$)**	**TT ($n = 48$)**	***P* value**
***MTHFR* gene**					
Prolonged elimination	69 (14.4%)	37 (15.0%)	25 (13.6%)	7 (14.6%)	NS
C_{SS} [μM]	38.5 (36.5–40.5)	41.3 (38.3–44.3)	37.3 (34.3–40.3)	28.4 (24.4–32.9)	0.0007
k_{el} alfa [1/h]	0.30 (0.29–0.30)	0.30 (0.29–0.31)	0.30 (0.29–0.31)	0.28 (0.26–0.30)	0.0465
AUC_{inf} [μM·h]	717 (680–755)	770 (714–826)	694 (639–750)	531 (452–611)	0.0073
CL [L/h/m^2]	8.92 (7.96–9.88)	8.42 (6.9–9.9)	8.66 (7.71–9.61)	12.50 (8.10–16.91)	0.0496
	Total ($n = 478$)	**2R2R ($n = 100$)**	**3R2R ($n = 283$)**	**3R3R ($n = 95$)**	***P* value**
***TS* gene**					
Prolonged elimination	69 (14.4%)	12 (12.0%)	43 (15.2%)	14 (14.7%)	NS
C_{SS} [μM]	38.5 (36.5–40.5)	35.9 (31.1–40.7)	37.9 (35.5–40.3)	42.9 (37.9–47.7)	NS
k_{el} alfa [1/h]	0.30 (0.29–0.30)	0.28 (0.27–0.30)	0.30 (0.29–0.30)	0.31 (0.30–0.32)	0.0034
AUC_{inf} [μM·h]	717 (680–755)	680 (589–771)	705 (661–751)	792 (702–881)	NS
CL [L/h/m^2]	8.92 (7.96–9.88)	10.29 (7.88–12.70)	8.96 (7.62–10.3)	7.35 (6.31–8.38)	NS

Values are given as mean and 95 CI. Comparisons were performed using one-way ANOVA. The number of patients with prolonged elimination (Mtx concentration measured at 48 h from the beginning of the infusion ≥ 0.4 μM) is given in both unrelative (N) and relative (%N) way. Pearson chi-square was used to identify relations between prolonged Mtx elimination and genotype. C_{ss}, steady state concentration; k_{el} alfa, initial elimination rate constant; AUC_{inf}, area under the concentration-time curve extrapolated to infinity; CL, clearance; NS, non significant.

vs. 28.4 μM) mean Mtx steady state plasma concentrations in comparison to TT homozygotes ($P = 0.0007$). In the case of TS gene polymorphism slightly higher (42.9 vs. 35.9 or 37.9 μM) concentrations were observed for homozygotes 3R/3R for tandem repeats of the TS gene however the difference did not reach statistical significance. In the patients receiving Mtx at the dose of 2 g/m^2 initial elimination rate constant and AUC_{inf} were significantly lower ($P = 0.0465$ and $P = 0.00073$, respectively) in homozygotes TT C>T of *MTHFR* gene, thus indicating higher clearance (12.5 vs. 8.42 L/h/m^2 in e.g., homozygotes CC). Furthermore, the significant correlation has been found between initial elimination rate constant and polymorphism of *SLC19A1* and TS genes. Elimination rate constant was significantly higher in homozygotes 3R/3R for tandem repeats of the TS gene ($P = 0.00343$) and in homozygotes AA of 80 G>A gene *SLC19A1* ($P = 0.0007$), however in the latter case the AUC_{inf} was also higher ($P = 0.05$). On the contrary, for the dose of 5 g/m^2 in homozygotes AA of 80 G>A gene *SLC19A1* initial elimination rate constant was significantly lower (GG vs. AA $P = 0.0319$) (**Figure 2C**). There was no significant influence of studied genetic polymorphism on the terminal elimination rate constant, although this is not a surprise since most of Mtx is eliminated during the α phase.

Pharmacodynamic Results

In the case of homozygotes AA (80 G>A gene *SLC19A1* polymorphism), a statistical trend for higher incidence of transaminase elevation was observed ($P = 0.037$ without

TABLE 4 | Methotrexate (Mtx) steady-state concentrations and basic pharmacokinetic parameters, calculated after administration of Mtx at the dose of 5 g/m^2, depending on the observed genotype.

	Total (*n* = 47)	**GG (*n* = 4)**	**GA (*n* = 20)**	**AA (*n* = 23)**	***P* value**
***SLC19A1* gene**					
Prolonged elimination	28 (59.6%)	1 (25.0%)	14 (70.0%)	13 (56.5%)	NS
C_{SS} [μM]	137 (24–230)	185 (139–230)	147 (42–200)	76 (24–200)	0.0015
k_{el} alfa [1/h]	0.33 (0.18–0.46)	0.43 (0.35–0.46)	0.34 (0.21–0.41)	0.32 (0.18–0.39)	0.0345
AUC_{inf} [μM·h]	2510 (446–5467)	3617 (2527–4216)	2718 (776–3645)	1392 (446–5467)	0.0019
CL [L/h/m^2]	4.35 (0.80–24.6)	3.11 (2.61–4.35)	4.05 (3.02–14.17)	6.66 (0.8–24.6)	0.0295
	Total (*n* = 47)	**CC (*n* = 13)**	**CT (*n* = 30)**	**TT (*n* = 4)**	***P* value**
***MTHFR* gene**					
Prolonged elimination	28 (59.6%)	11 (84.6%)	15 (50.0%)	2 (50.0%)	NS
C_{SS} [μM]	137 (24–230)	140 (24–197)	138 (35–230)	109 (89–146)	NS
k_{el} alfa [1/h]	0.33 (0.18–0.46)	0.34 (0.27–0.37)	0.33 (0.18–0.46)	0.34 (0.21–0.36)	NS
AUC_{inf} [μM·h]	2510 (446–5467)	2551 (446–3603)	2519 (680–5467)	1985 (1626–2727)	NS
CL [L/h/m^2]	4.35 (0.80–24.6)	4.31 (3.0–24.6)	4.28 (0.8–16)	5.55 (4.0–6.8)	NS
	Total (*n* = 47)	**2R2R (*n* = 16)**	**3R2R (*n* = 19)**	**3R3R (*n* = 12)**	***P* value**
***TS* gene**					
Prolonged elimination	28 (59.6%)	7(43.8%)	13 (68.4%)	8 (66.7%)	NS
C_{SS} [μM]	137 (24–230)	109 (24–200)	145 (44–230)	122 (35–195)	NS
k_{el} alfa [1/h]	0.33 (0.18–0.46)	0.34 (0.21–0.37)	0.33 (0.18–0.46)	0.32 (0.18–0.41)	NS
AUC_{inf} [μM·h]	2510 (446–5467)	1985 (446–3645)	2645 (861–5467)	2229 (680–3533)	NS
CL [L/h/m^2]	4.35 (0.80–24.6)	5.55 (3.0–24.6)	3.89 (0.8–12.8)	5.0 (1.29–14.2)	NS

Values are given as median and range. Comparisons were performed using Kruskal-Wallis test (small samples size). The number of patients with prolonged elimination (Mtx concentration measured at 48 h from the beginning of the infusion ≥ 0.4 μM) is given in both unrelative (N) and relative (%N) way. Pearson chi-square was used to identify relations between prolonged Mtx elimination and genotype. C_{ss}, steady state concentration; k_{el} alfa, initial elimination rate constant; AUC_{inf}, area under the concentration-time curve extrapolated to infinity; CL, clearance; NS, non significant.

correction for multiple comparisons). Furthermore, similar trend was observed in the case of 3R/3R genotype of *TS* tandem repeats ($P = 0.002$ without correction for multiple comparisons or 0.01 with Bonferroni correction). No significant influence of all analyzed polymorphisms on the incidence of hematological toxicity, vomiting, gastrointestinal mucositis, and infections was observed (**Table 5**).

Occurrences of particular acute toxicities induced by HD-Mtx were also analyzed with logistic regression models (**Table 6**). All commonly known risk factors of acute adverse reactions to HD-Mtx, such as: dose, prolonged drug exposure, age, as well as genotype were included. Heterozygous genotype 2R/3R of *TS* tandem repeats was associated with significant increase in stated intensive vomiting (OR adjusted to the wild-type genotype 3.20, 95% CI 1.33–7.68; $P = 0.009$). Similar relationship was also observed in the case of 3R/3R homozygotes (adjusted OR 3.39, 95% CI 1.12–10.23; $P = 0.031$). Additionally, as also demonstrated by logistic regression, 3R/3R polymorphism was associated with a higher risk of hepatotoxicity (adjusted OR 2.28, 95% CI 1.05–4.95; $P = 0.038$). No such relationships were observed for the other analyzed polymorphisms as well as for other acute toxicities.

Impact of Particular Mtx Dosage (2 vs. 5 g/m^2) on Toxicity

In this study, 525 chemotherapy cycles with 2 and 5 g/m^2 Mtx doses (478 and 47 cycles, respectively) were analyzed. Impaired liver function, the most common acute toxicity, was observed with the same frequency in both groups. For the dosing of 2 and 5 g/m^2 it was 68.4 and 66.7% of all patients, respectively ($P = 0.816$). Surprisingly, none of the acute toxicities differed between the analyzed groups (**Table 7**). Only an insignificant relationship was observed toward a higher incidence of hematological toxicity in the group treated with higher doses of Mtx (12.3 vs. 22.9%, $P = 0.124$) (**Table 7**). In all patients adequate antitoxic therapy according to the requirements of protocols prevented life-threatening complications.

DISCUSSION

We showed in our study that the genetic polymorphisms of the *SLC19A1*, *MTHFR*, and *TS* genes can influence pharmacokinetics of Mtx. Importantly, we have observed, for the first time in the literature, the increased risk of hepatotoxicity (significant

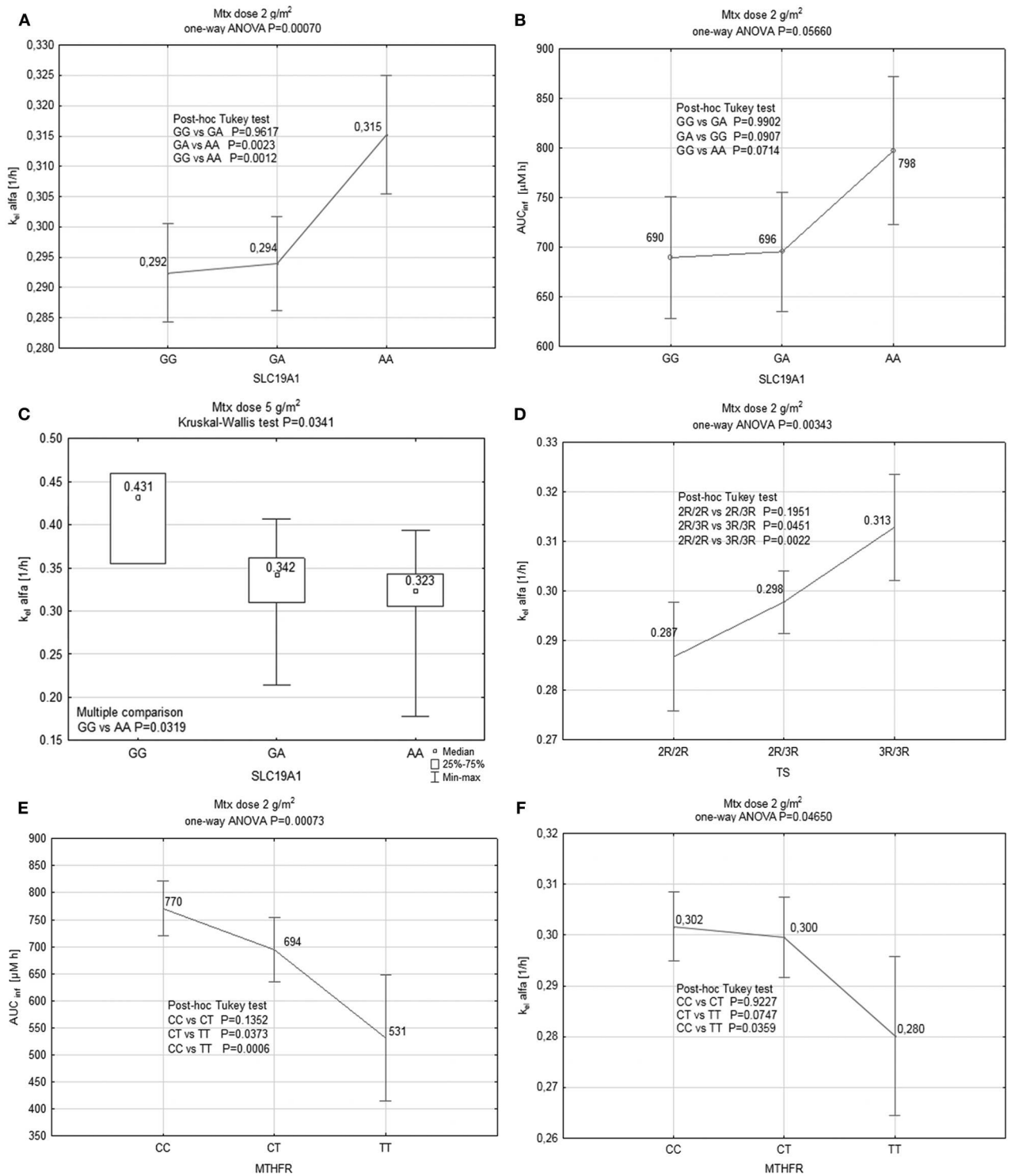

FIGURE 2 | Results of analysis of influence of genetic polymorphisms on elimination of Mtx in both dosing groups: **(A)** Relationship between the initial elimination rate constant (k_{el} alfa) and SLC19A1 gene polymorphism in the patients receiving Mtx at the dose of 2 g/m^2. Values are presented as mean and 95CI. **(B)** Relationship between the area under the concentration-time curve extrapolated to infinity (AUC_{inf}) and SLC19A1 gene polymorphism in the patients receiving Mtx at the dose of 2 g/m^2. Values are presented as mean and 95CI. **(C)** Relationship between the initial elimination rate constant (k_{el} alfa) and SLC19A1 gene polymorphism in the patients receiving Mtx at the dose of 5 g/m^2. Values are presented as median and range. **(D)** Relationship between the initial elimination rate constant (k_{el} alfa) and TS gene polymorphism in the patients receiving Mtx at the dose of 2 g/m^2. Values are presented as mean and 95CI. **(E)** Relationship between the area under the concentration-time curve extrapolated to infinity (AUC_{inf}) and MTHFR gene polymorphism in the patients receiving Mtx at the dose of 2 g/m^2 Values are presented as mean and 95CI. **(F)** Relationship between the initial elimination rate constant (k_{el} alfa) and MTHFR gene polymorphism in the patients receiving Mtx at the dose of 2 g/m^2. Values are presented as mean and 95CI.

TABLE 5 | Statistical significance (*Bonferroni correction for multiple comparisons) of particular acute toxicities depending on the three analyzed genes polymorphisms.

Type of toxicity	AA *SLC19A1*, *P* value	N (%)	TT *MTHFR*, *P* value	N (%)	3R/3R *TS*, *P* value	*N* (%)
Features of impaired liver function	0.037 (*NS)	110 (75.3)	0.609 (*NS)	38 (73.1)	0.002 (*0.01)	83 (76.9)
Hematological toxicity	0.657 (*NS)	17 (11.6)	0.248 (*NS)	5 (9.6)	0.453 (*NS)	18 (16.7)
Vomiting	0.056 (*NS)	19 (13.0)	0.682 (*NS)	7 (13.5)	0.102 (*NS)	15 (13.9)
Mucositis	0.590 (*NS)	10 (6.9)	0.341 (*NS)	2 (3.9)	0.207 (*NS)	14 (13.0)
Infections	0.056 (*NS)	12 (8.2)	0.424 (*NS)	6 (11.5)	0.560 (*NS)	7 (6.5)

NS, non significant.

TABLE 6 | Logistic regression analysis of acute toxicities adjusted to prolonged exposure to methotrexate, drug dose, age, and genotype.

Polymorphism	Genotype	Hepatotoxicity	*P* value	Vomiting	*P* value
80 G>A *SLC19A1*	hom GG	1.00 (–)	–	1.00 (–)	–
	het GA	1.44 (0.81–2.55)	NS	0.40 (0.16–1.02)	0.054
	hom AA	1.87 (0.96–3.63)	0.066	0.85 (0.35–2.03)	NS
2R>3R *TS*	hom 2R/2R	1.00 (–)	–	1.00 (–)	–
	het 2R/3R	1.46 (0.78–2.73)	NS	3.20 (1.33–7.68)	0.009
	hom 3R/3R	2.28 (1.05–4.95)	0.038	3.39 (1.12–10.23)	0.031
677 C>T *MTHFR*	hom CC	1.00 (–)	–	1.00 (–)	–
	het CT	1.07 (0.63–1.81)	NS	0.83 (0.43–1.63)	NS
	hom TT	1.22 (0.65–2.29)	NS	1.34 (0.32–5.59)	NS

Data are shown as odds ratios with 95% confidence intervals.
NS, non significant.

TABLE 7 | The incidence of chemotherapy toxicities depending on the methotrexate doses.

Toxicity	2 g/m^2 *N* (%)	5 g/m^2 *N* (%)	*P* value
Impaired liver function	327 (68.4)	32 (66.7)	NS
Vomiting	52 (10.9)	9 (18.8)	NS
Stomatitis/skin inflammation	42 (8.8)	4 (8.3)	NS
Infections	39 (8.2)	4 (8.3)	NS
Hematological toxicity	59 (12.3)	11 (22.9)	NS

NS, non significant.

even with Bonferroni correction) and vomiting in patients with particular *TS* polymorphism and for the second time the increased risk of hepatotoxicity in the *SLC19A1* homozygous genotype. Surprisingly, the Mtx dose did not affect the incidence of individual toxicities, which may indicate a congenital predisposition to their development in individual ALL patients.

SLC19A1 80 G>A Polymorphism and Its Influence on Hepatotoxicity

SLC19A1 80 G>A is a common single nucleotide polymorphism among genes responsible for Mtx transport into a cell (6, 14, 17–20). Our results indicate a relationship between the AA genotype of the *SLC19A1* 80 G>A polymorphism and significantly elevated steady state Mtx concentrations after HD-Mtx infusions (e.g., 42.9 vs. 36.9 μM) (**Table 3**). Since this particular mutation is responsible for the lower affinity of the transporter protein to Mtx, it is expected that in these patients the higher amount of drug stays in the central circulation. Although patients, with this genotype, receiving the lower dose of Mtx had higher initial elimination rate constant (e.g., 0.315 vs. 0.292 1/h for AA vs. GG; $P = 0.0012$) (**Figure 2A**), the total clearance was lower (higher $AUC_{inf;}$ e.g., 798 vs. 690 μM·h for AA vs. GG) (**Figure 2B**). On the contrary patients with AA genotype of the *SLC19A1* receiving 5 g/m^2 of Mtx had significantly lower initial elimination rate constant (e.g., 0.323 vs. 0.431 1/h for AA vs. GG; $P = 0.0319$) (**Figure 2C**). However, these changes are rather consequences of the saturation of Mtx elimination processes. Higher Mtx exposure in AA homozygotes might result in the impaired liver function, defined by us as increase in transaminases level at least 1 grade and/or bilirubin grade $\geq$ 2 and/or decrease protein level, that was observed significantly more frequently (not significant with Bonferroni correction) in these patients. Until now, only one study showed a correlation between the 80 AA variant and significant liver function impairment caused by Mtx. Moreover, it was referred only to the group of patients with an additional, specific variant of *GSTM1* gene (15). The mechanism explaining increased liver toxicity, despite the presence of a variant of reduced folate carrier (RFC) protein with lower ability to transport Mtx (also into hepatocytes), may involve the participation of other transporters, with higher expression in liver tissues. The explanation could

be the activity of OATP1A/1B which determine transport of drugs (including Mtx) into hepatocytes (21), alteration of the polyglutamylation pattern (22) or the intracellular level of miR-595 acting as a phenotypic regulator of Mtx sensitivity in cells by targeting *SLC19A1* (23) (**Figure 1**).

The association between *SLC19A1* polymorphism and Mtx levels, similar to the one observed in our study, was also shown by Laverdiére et al. (9). Since then, several researchers have observed the impact of this polymorphism on incidence of specific adverse reactions. Kotnik et al. showed an association of AA genotype with leukopenia, while Gregers et al. with significant percentage of serious myelotoxicities (6, 24). In the study conducted by Salazar et al. the wild GG genotype was associated with thrombocytopenia and mucositis of grade at least 2 according to WHO (14). However, the opposite results were presented by some other researchers who did not find the impact of *SLC19A1* polymorphism on acute toxicities of HD-Mtx therapy, although liver toxicity was not investigated (3, 25–28).

It should be emphasized that the number of patients in our study almost 3-fold exceeds the total number of HD-Mtx treated patients reported in previous studies of Mtx hepatotoxicity. Thus, our results showing possible increased risk of HD-Mtx induced hepatotoxicity in patients with the *SLC19A1* 80 AA variant seem to be better substantiated. It should also be emphasized that previous studies were based on assessment of various ethnic groups and of patient cohorts of variable size. The polymorphisms of numerous genes involved in Mtx disposition might differ among various populations thus possibly determining the toxic effects of treatment (29).

The Role of *TS* Repeats and Their Impact on Hepatotoxicity and Vomiting

The results of our study suggest that determination of the *TS* gene polymorphism in pediatric population may have significant clinical implications in predicting liver impairment associated with HD-Mtx (significant even with Bonferroni correction).

Mtx is an uncompetitive, irreversible inhibitor of TS and, from a pharmacokinetic point of view, this enzyme should have a relatively low contribution to Mtx elimination (30). However, slightly higher (without statistical significance) steady state Mtx concentrations were seen in homozygotes 3R/3R for tandem repeats of the *TS* gene (e.g., 42.9 vs. 35.9 μM for 3R/3R vs. 2R/2R) (**Table 3**). Since simultaneous increase in the initial elimination rate has been also observed (e.g., 0.313 vs. 0.287 1/h for 3R/3R vs. 2R/2R; $P = 0.0022$) (**Figure 2D**) the decrease in total clearance in the patients with 3R/3R variant for tandem repeats of the *TS* gene, although visible (7.35 vs. 10.29 L/h/m^2), did not reach statistical significance (**Table 3**). The vast majority of Mtx is eliminated by renal route and the higher amount of proteins able to irreversibly bind Mtx might impair the elimination process, but this hypothesis needs further investigation. However, it should also be stressed that TS plays additional (not directly related to folate metabolism) role in cell homeostasis control through associations with numerous cell cycle proteins, in particular with p53. The mutual regulation of TS and p53 is based on negative feedback loop. In 3R/3R cells with the higher TS expression the p53 level will be lower (31), what decreases ability of intensively dividing cells (e.g., hepatocytes and gastrointestinal epithelium cells) to block the cell cycle during exposure to the severely damaging factors acting during the S phase (such as Mtx). It may explain the increased incidence of mucosal and liver damage observed in our study (32). The published results regarding the influence of TS gene polymorphism on the incidence of different toxicities are not consistent. Ongaro et al. showed a significant increase in the risk of anemia in adult patients with 3R/3R genotype, while other researchers reported increased toxicity in the central nervous system (33–37). Opposite results were presented by Kotnik et al. and Erculj et al. showing that 3R/3R genotype was associated with a reduced risk of mucositis, leukopenia and thrombocytopenia (6, 38). Demonstrated by us relationship between 3R/3R genotype and increased hepatotoxicity, as we mentioned earlier, has not been reported previously.

The Role of *MTHFR* 677 C>T Polymorphism in Toxicity of HD-Mtx

In our study, patients homozygotes CC and heterozygous for the common 677C>T polymorphism of the *MTHFR* gene achieved significantly higher steady state Mtx plasma concentrations (41.3 or 37.3 vs. 28.4 μM; $P = 0.0007$) (**Table 3**). Elevated levels of Mtx in the carriers of CT variant of the *MTHFR* gene are consistent with several previous observations (16, 39, 40). However, we did not show significant differences in the frequency of acute side-effects of HD-Mtx in carriers of this polymorphism. Such results observed in our study could be partially explained by the fact that despite the higher steady state concentrations and higher AUC_{inf} values (694 vs. 531 μM·h for CT vs. TT; $P = 0.0373$) (**Figure 2E**) the carriers of this gene mutation had also slightly higher, although not significantly, initial elimination rate constant (0.3 vs. 0.28 1/h) (**Figure 2F**) indicating faster decrease of Mtx concentration immediately after the end of infusion. Lack of relationships between toxicity and *MTHFR* gene polymorphism have already been noticed by Seidemann et al. (41) and Shimasaki et al. (16), except for mucosal toxicity directly caused by higher drug plasma concentrations. However, other authors found higher frequency of mucosal toxicity or increased hematological toxicity associated with *MTHFR* gene polymorphism (29, 42, 43). El-Khodary et al. described increased hepatic and myeloid toxicity in *MTHFR* 677TT homozygotes (44). The presence of the 677T allele was also associated with a higher risk of thrombocytopenia (45), and an overall increase in toxicity, if in combination with the 1298AC variant (46). Considering the conflicting reports presented in the literature it is highly possible that, in the case of 677C>T *MTHFR* gene polymorphism some other concomitant factors are likely to affect toxicity of HD-Mtx treatment.

Limitations of the Study

Our study has some limitations. First, the tested polymorphisms seem to be important for the Mtx disposition, but the influence of other genetic and biochemical factors should be also considered. The influence of combinations of specific alleles of several genes, including possible synergistic or antagonistic effects are possible.

Second, more advanced genetic methods, such as whole exome sequencing, could provide more information on other potential polymorphisms associated with Mtx toxicity (47, 48). Third, our study group was homogenous as far as ethnicity is concerned, so this makes the discussion focused solely on the central European population.

CONCLUSIONS

The genetic polymorphisms has an unquestionable effect on pharmacokinetics and toxicity of Mtx. Determination of polymorphisms of *SLC19A1*, *MTHFR*, and *TS* genes allows for a better selection of patients with higher risk of elevated Mtx levels. According to our knowledge, our study is the first one to report the increased risk of hepatotoxicity and vomiting in patients with particular *TS* polymorphisms. In addition, we were able to confirm the previous data showing that the increased risk of hepatotoxicity has been associated with the *SLC19A1* homozygous genotype. Surprisingly, the administered Mtx dose did not affect the incidence of individual toxicities. Further research, considering also polymorphisms of other folate metabolism pathways and some mutual gene associations, including meta-analyzes of the previous studies, is necessary for the final determination of the role of individual polymorphisms in the pharmacokinetics, pharmacological activity, and toxicity of Mtx. Such studies could lead to the pharmacogenetically improved, individualized dosing of Mtx, that in turn could compensate for its interindividual PK variations.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by local Permanent Ethical Committee for Clinical Studies (KBET/96/B/2008). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MCw, MCz, KK, AM-T, MB-M, WB, and SS contributed to the study concept and design. MCw, MCz, KK, AM-T, KP, AW, TK, and MR performed diagnostic tests and collected relevant clinical data. MCw, MCz, AM-T, MS, KS, PH, AL, and KM conducted statistical analysis. MCw, MCz, MS, KS, PH, AL, and KM wrote sections of the manuscript. MB-M, WB, and SS critically revised the article. All authors were responsible for the integrity and accuracy of the data and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.00307/full#supplementary-material

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Retrospective Analysis of the Treatment Outcome in Myeloid Leukemia of Down Syndrome in Polish Pediatric Leukemia and Lymphoma Study Group From 2005 to 2019

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Background: Children with Down syndrome (DS) have increased risk of myeloid leukemia (ML), but specific treatment protocols ensure excellent outcome. This study was a retrospective analysis of the treatment results and genetic characteristics of ML of DS (ML-DS) in Poland from 2005 to 2019.

Methods: All 54 patients with ML-DS registered in the Polish Pediatric Leukemia and Lymphoma Study Group in analyzed period were enrolled to the study. There were 34 children treated with Acute Myeloid Leukemia–Berlin-Frankfurt-Munster 2004 Interim Protocol (group I) and 20 patients treated with ML-DS 2006 Protocol (group II). In the first protocol, there was reduction of the antracyclines doses and intrathecal treatment for ML-DS compared to non-DS patients. In the second protocol, further

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reduction of the treatment was introduced (omission of etoposide in the last cycle, no maintenance therapy).

Results: Probabilities of 5-year overall survival (OS), event-free survival (EFS), and relapse-free survival in the whole analyzed group were 0.85 ± 0.05, 0.83 ± 0.05, and 0.97 ± 0.03, respectively. No significant differences were found between two protocols in the terms of OS and EFS (0.79 ± 0.07 vs. 0.95 ± 0.05, $p = 0.14$, and 0.76 ± 0.07 vs. 0.95 ± 0.05, $p = 0.12$, respectively). All deaths were caused by the treatment-related toxicities. Reduction of the treatment-related mortality was noticed (20% in group I and 5% in group II). The only one relapse in the whole cohort occurred in the patient from group I, older than 4 years, without *GATA1* gene mutation. He was treated successfully with IdaFLA cycle followed by hematopoietic stem cell transplantation from matched sibling donor. No significant prognostic factor was found in the study group probably due to low number of patients in the subgroups.

Conclusions: The study confirms that the reduced intensity protocols are very effective in ML-DS patients. The only cause of deaths was toxicities; however, systematic decrease of the treatment-related mortality was noticed.

Keywords: myeloid leukemia, down syndrome, children, treatment results, genetic characteristics

INTRODUCTION

Children with Down syndrome (DS) have increased risk of myeloid leukemia (ML) compared to children without DS (150-fold before the age of 5 years) (1). Myeloid leukemia in DS children (ML-DS) is characterized by several unique features. Approximately 50% of patients are diagnosed within the first year of life, and only 1–2% at 4 years or older (1). It shows a high prevalence of the acute megakaryocytic leukemia phenotype (AMKL), which is rare in non-DS acute myeloid leukemia (AML) (2, 3). Myeloid leukemia of DS can be preceded by transient abnormal myelopoiesis (TAM) observed in approximately 10% of neonates with DS (4, 5). Approximately 20 to 30% of TAM can progress to ML-DS (5, 6). Both TAM and ML-DS, especially AMKL, are associated with mutations of the hematopoietic transcription factor GATA1 (7–9). Development of ML-DS is frequently preceded by myelodysplastic phase characterized by thrombocytopenia and anemia. Myeloid neoplasms of DS are not subclassified into myelodysplastic syndrome (MDS) or AML because they have a similar behavior independently of blast cell count (10). In case of evidence for leukemic blasts in the bone marrow, it is recommended that children with DS should be diagnosed with AML, even if the blast threshold of 20% is not reached (11). According to the World Health Organization 2008 and 2016 classifications, both MDS and AML in children with DS are classified as a separate entity—ML of DS (ML-DS) (10, 12).

Myeloid blasts in children with DS have high drug sensitivity especially to cytarabine and anthracyclines (13). That determines excellent response to the treatment. The risk of the therapy-associated toxicities is higher in children with DS compared to other patients with AML (14). Introduction of the DS-specific therapeutic protocols with reduced intensity of the treatment resulted in excellent outcome (14–17).

The aim of the study was retrospective analysis of the treatment results and genetic characteristics of pediatric ML-DS patients treated in Poland from 2005 to 2019.

PATIENTS AND METHODS

From January 2005 to December 2019, there were 54 newly diagnosed ML-DS patients registered in the Polish Pediatric Leukemia and Lymphoma Study Group (PPLLSG) registry. They were treated in 16 pediatric oncology centers in Poland. All were eligible to the study. Inclusion criteria included age up to 18 years, diagnosis of DS and ML, and written informed consent. Exclusion criteria comprised accompanying diseases that do not allow ML-DS therapy. Thirty-four children were treated with DS-specific arm of the Acute Myeloid Leukemia–Berlin-Frankfurt-Munster (AML-BFM) 2004 Registry Protocol and 20 patients with ML-DS 2006 Protocol (**Figure 1**).

The last patient was enrolled in August 2019, and the last follow-up was done in November 2019. Median observation time was 62.7 months (range, 2.6–174.8 months). Characteristics of the patients are presented in **Table 1**.

The data were collected in PPLLSG AML registry and analyzed retrospectively.

Informed consent to participation in the studies was obtained from guardians of all patients, in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of Jagiellonian University Medical College.

From January 2005 to June 2015, 34 patients were treated according to the DS-specific arm of the AML-BFM 2004 Interim Protocol (group I). The treatment consisted of four chemotherapy cycles AIE (cytarabine, idarubicin, etoposide), AI (cytarabine, idarubicin), haM (high-dose cytarabine, mitoxantrone), HAE (high-dose cytarabine, etoposide;

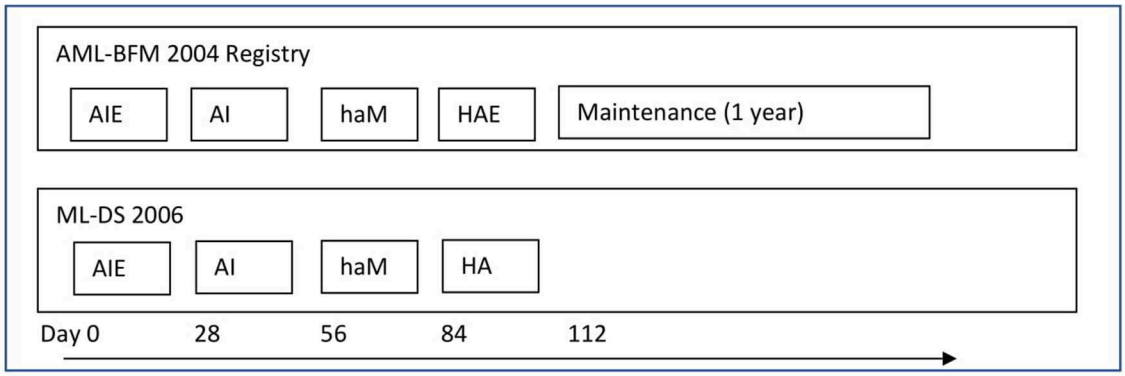

FIGURE 1 | The treatment protocols. AML-BFM 2004 Registry—group I (reduced intensity arm for children with ML-DS)—group I: AIE (cytarabine 100 mg/m^2/day [days 1–2] and 100 mg/m^2 every 12 h [days 3–8], idarubicin 8 mg/m^2/day [days 3, 5, and 7], and etoposide 150 mg/m^2/day [days 6–8]); AI (cytarabine 500 mg/m^2/day [days 1–4] and idarubicin 5 mg/m^2/day [days 3 and 5]); haM (cytarabine 1 g/m^2 every 12 h [days 1–3] and mitoxantrone 7 mg/m^2/day [days 3–4]); HAE (cytarabine 3 g/m^2/every 12 h [days 1–3], etoposide 125 mg/m^2/day [days 2–5]). Maintenance therapy lasting 1 year: thioguanine 40 mg/m^2/day orally, cytarabine 40 mg/m^2/day intravenously for 4 consecutive days, every 4 weeks. Intrathecal cytarabine (CNS prophylaxis)—in the each intensive treatment block, not during maintenance therapy (in total, six aged adapted doses—20 to 40 mg per dose). The cumulative doses: 29,400 mg/m^2 cytarabine, 950 mg/m^2 etoposide, 34 mg/m^2 idarubicin, and 14 mg/m^2 mitoxantrone. ML-DS 2006—group II: AIE (cytarabine 100 mg/m^2/day [days 1–2] and 100 mg/m^2 every 12 h [days 3–8], idarubicin 8 mg/m^2/day [days 3, 5, and 7], and etoposide 150 mg/m^2/day [days 6–8]); AI (cytarabine 500 mg/m^2/day [days 1–4] and idarubicin 5 mg/m^2/day [days 3 and 5]); haM (cytarabine 1 g/m^2 every 12 h [days 1–3] and mitoxantrone 7 mg/m^2/day [days 3–4]); HA (cytarabine 3 g/m^2/every 12 h [days 1–3]). Intrathecal cytarabine (CNS prophylaxis)—at the start of each treatment block (in total, four aged adapted doses—20–40 mg per dose). The cumulative doses: 27,400 mg/m^2 cytarabine, 450 mg/m^2 etoposide, 34 mg/m^2 idarubicin, and 14 mg/m^2 mitoxantrone.

additionally intrathecal cytarabine in every cycle, total six doses), and maintenance therapy (6-thioguanine, cytarabine) for 1 year (**Figure 1**). Compared to the therapy for non-DS AML, there was reduction of doses of antracyclines (idarubicin 8 mg/m^2 in AIE and 5 mg/m^2 in AI instead of 12 mg/m^2 and 7 mg/m^2, respectively, and mitoxantrone 7 mg/m^2 in haM instead of 10 mg/m^2). Median observation time in group I was 91.5 months (range, 38.3–174.8 months).

From June 2015, ML-DS Protocol was introduced. There were additionally three children treated with ML-DS 2006 before 2015 according to individual decision of the treating center. In total, 20 patients were treated according to the ML-DS 2006 Protocol (group II). It consisted of four chemotherapy cycles: AIE (cytarabine, idarubicin, etoposide), AI (cytarabine, idarubicin), haM (high-dose cytarabine, mitoxantrone), and HA (high-dose cytarabine) (**Figure 1**). Intrathecal treatment with cytarabine was given in every cycle (totally four doses). There was no maintenance treatment. Median observation time in group II was 21.1 months (range, 2.6–89.1 months).

Cytogenetic analyses were performed in local laboratories. Karyotype results were available in 49 patients (91%). From 2015, the status of mutation in *GATA1* gene was done centrally in the Department of Medical Genetics, Institute of Pediatrics, Jagiellonian University Medical College, Krakow, Poland. From each patient, DNA was isolated from 300 μL bone marrow sample collected at diagnosis by the nucleic acid isolation system QuickGene-Mini80 with DNA Blood kit (KURABO Industries Ltd, Osaka, Japan). The variation in *GATA1* gene coding sequence fragments was detected by Sanger sequencing method (3500 Genetic Analyzer; Applied Biosystems, Foster City, California, USA). Separate analyses included 2, 3, and 4 exons of the gene. The *GATA1* gene coding sequence was checked against the reference sequence no. ENST00000376670.7. Results of *GATA1* mutation status was available in 18 patients (33%).

The data that support the findings of the study are available on request from the corresponding author. The data are not publicly available because of privacy or ethical restrictions.

Statistical Analysis

Descriptive statistical analysis was performed to assess patient baseline characteristics. We used Fisher exact test or a χ^2 test (categorical variables) and Mann–Whitney *U*-test (continuous variables) for analysis of clinical and laboratory data. Overall survival (OS), event-free survival (EFS), and relapse-free survival (RFS) were calculated using the Kaplan–Meier method. Overall survival was defined as the time from diagnosis to death from any cause; patients alive or lost to follow-up were censored at the date they were last known alive. Event-free survival was defined as the time from diagnosis to disease progression, relapse, or death from any cause. Patients who were alive without disease progression or relapse were censored at the time they were last seen alive and event-free. Relapse-free survival was defined as the time from complete remission (CR) to disease relapse or death from any cause. Patients who were alive without disease relapse were censored at the time of last follow-up. For comparisons of Kaplan–Meier curves, we used the log-rank test. Cumulative incidence of relapse (CIR) was also counted and compared between groups using Gray test. Statistical analysis was performed using STATISTICA 13 software (StatSoft Polska, Krakow, Poland).

TABLE 1 | Characteristics of the patients.

Characteristic	All patients ($n = 54$)		AML-BFM 2004 interim ($n = 34$)		ML-DS 2006 ($n = 20$)		*p*
	n	%	*n*	%	*n*	%	
Sex							
Male	27	50	19	56	8	60	0.29
Female	27	50	15	44	12	40	
Age at diagnosis (median, range), years	1.9	0.7–17.4	1.9	0.7–17.4	2.2	0.9–4.9	0.26
<4	50	92.6	31	91.2	19	95	0.60
>4	4	7.4	3	8.8	1	5	
History of TAM							
Yes	15	44.1	8	38.1	7	53.8	0.29
No	19	55.9	13	61.9	6	46.2	
No data	20		13		7		
FAB							
M0	5	9.8	5	15.6	0	0	M7 vs. others **0.03**
M1	9	17.6	8	25	1	5.3	
M2	4	7.8	4	12.5	0	0	
M4	1	2	1	3.1	0	0	
M5	1	2	1	3.1	0	0	
M6	2	3.9	1	3.1	1	5.3	
M7	29	56.9	12	37.5	17	89.5	
No data	3		2		1		
Cytogenetics							
Trisomy 21 only	21	42.8	14	45.2	7	38.9	Isolated trisomy 21 vs. others 0.65
Trisomy 8	8	16.3	5	16.1	3	16.7	
Del(7)	5	10.2	4	12.9	1	5.5	
Del(16)	3	6.1	3	9.7	0	0	
Del(6)	3	6.1	3	9.7	0	0	
Dup(7)	2	4.1	0	0	2	11.1	
i(7)	2	4.1	1	3.2	1	5.5	
Complex karyotype	6	12.2	4	12.9	2	11.1	
No data	5		3		2		
***GATA1* gene mutation**							
Yes	7	38.9	1	20	6	46.2	0.63
No	11	61.1	4	80	7	53.2	
No data	36		30		7		
Congenital heart defect							
Yes	24	47.1	16	51.6	8	40	0.25
No	27	52.9	15	48.4	12	60	
No data	3		3		0		
History before diagnosis, months							
≤3	23	52.3	15	51.7	8	53.3	0.83
>3	21	47.7	14	48.3	7	46.7	
No data	10		5		5		
WBC at diagnosis (median, range), $\times 10^9$/L	6.5	1.9–282	8.83	2.2–282	3.9	1.9–30.9	**0.010**
Platelets at diagnosis (median, range), $\times 10^9$/L	26	2–247	24	2–247	31	9–87	0.52
Peripheral blasts (median, range), %	11	0–92	16	0–92	6.5	0–79	0.24
Bone marrow blasts (median, range), %	30	5–91	39	10–91	25	8–59	**0.015**

TAM, transient abnormal myelopoiesis. P-values are bolded where there are significant differences ($p < 0.05$) between groups.

RESULTS

Patient Characteristics

There were 54 patients enrolled to the study, including 27 girls and 27 boys. The median age at diagnosis was 1.9 years (range, 0.7–17.4 years). Fifty children were younger than 4 years, and four patients were older (4.01, 4.9, 10.5, and 17.4 years). There were no significant differences between groups I and II concerning sex and age. Median number of white blood cells at diagnosis was 6.5×10^9/L (range, 1.9–282 $\times$ 10^9/L). It was significantly higher in group I (median, 8.83; range, 2.2–282 $\times$ 10^9/L) than in group II (median, 3.9; range, 1.9–30.9 $\times$ 10^9/L; $p = 0.01$). Median percentage of peripheral blasts in the whole cohort was 11% (range, 0%−92%), with no significant differences between the groups. The percentage of bone marrow blasts was significantly higher in group I (median, 39%; range, 10–92%) compared to group II (median, 25%; range, 8–59%). Detailed characteristics are presented in **Table 1**.

Treatment Outcome

Forty-nine patients (90.7%) achieved CR (30/34 patients in group I and 19/20 patients in group II). Five patients (9.3%) died of toxicities before CR (0.5–2.2 months from diagnosis). The deaths were caused by infections in course of aplasia (two due to sepsis, two due to pneumonia, one due to typhlitis). Forty-five patients (83%) remain in continuous CR (26/34 in period I and 19/20 in period II). There were three deaths in CR (all in group I), two patients died of pneumonia (1 and 7.4 months after diagnosis), and one patient because of cardiac tamponade (1.4 months from diagnosis). In total, eight deaths from toxicities occurred (14.8%): seven in group I (20.6%) and one in group II (5%) ($p = 0.078$). The only one relapse in the whole cohort occurred in the patient from group I, diagnosed of ML-DS at age of 10.5 years. His karyotype revealed isolated chromosome 21 trisomy; *GATA1* mutation was excluded. No additional genetic changes were found in molecular analysis. Leukocyte count at diagnosis was 74.8×10^9/L, with 90% of blasts, much higher than median in the analyzed cohort. The proportion of blast in the bone marrow (91%) was also much higher than median value in the whole group. The patient responded well to the first-line chemotherapy. Relapse occurred 21.7 months from CR. The patient was successfully treated with IdaFLA cycle followed by allogenic hematopoietic stem cell transplantation (HSCT) from the matched sibling donor. Conditioning with fludarabine, melphalan, and total body irradiation was used. The patient received cyclosporine and methotrexate as graft-vs.-host disease (GvHD) prophylaxis. Chronic GvHD with skin involvement occurred in the patient. He remains in the second CR for 33 months.

Probabilities of 5-year OS, EFS, and RFS in the whole analyzed group were 0.85 ± 0.05, 0.83 ± 0.05, and 0.97 ± 0.03, respectively (**Figure 2**). There was a trend toward an improved OS and EFS in group II compared to group I (OS and EFS 0.95 ± 0.05 vs. 0.79 ± 0.07, $p = 0.14$, and 0.95 ± 0.05 vs. 0.76 ± 0.07, $p = 0.12$, respectively; **Figures 3**, **4**). There was one relapse in group I and no relapses in group II. In the whole cohort, 5-year CIR was 0.0286: in group I, 0.037; and in group II, 0. The difference was not statistically significant ($p = 0.58$).

Genetic Analyses

The *GATA1* gene analysis was available in 18 patients, mainly from group II ($n = 13$). Analysis of *GATA1* gene was not done in the patients who died. Mutation was found in 7 of 18 patients (38.9%). The group of patients with *GATA1* gene mutation did not differ from the group without mutation regarding percentages of blasts at diagnosis and age. There were only two patients older than 4 years with known *GATA1* status, both without mutation. There were no events in the patients with *GATA1* gene mutation and one relapse in the group without *GATA1* gene mutation.

Results of the cytogenetic analysis were available in 49 patients. There were no significant differences in OS nor EFS depending on karyotype (isolated trisomy 21 vs. additional genetic changes). Presence of trisomy 8; chromosome 7, 6, or 16 deletion; or complex karyotype did not influence significant probabilities of OS and EFS (**Table 2**). Detailed genetic characteristics are presented in **Table 3**.

Analysis of Medical History Before Diagnosis

Data concerning medical history from the first symptoms to the diagnosis of ML-DS were available in 34 patients. No significant differences in OS or EFS were found depending on the length of the history (<3 vs. >3 months; **Table 2**).

In 15 patients (44% from 34 with available data concerning TAM), ML-DS was preceded by TAM. Treatment results were similar in the patients with and without TAM (**Table 2**).

Congenital heart defect was detected in 24 patients (58.5% of 41 children with available data). In one of those patients with history of atrioventricular canal correction before AML diagnosis, severe cardiac insufficiency occurred during induction therapy. The patient needed second cardiac surgery (closure of secundum atrial septal defect, mitral, and tricuspid valvuloplasty). After stabilization of the general condition, the patient continued chemotherapy and stays in remission. One patient with congenital heart defect died during the treatment because of cardiac tamponade. There were no differences in survival between patients with and without congenital hearts defect (**Table 2**).

DISCUSSION

Introduction of the ML-DS–specific treatment protocols with reduced intensity of chemotherapy improved the outcome in that special group of patients (14–17). It resulted in reduction of the risk of toxicities and allowed to maintain excellent treatment response at the same time (14–17). In this study, we analyzed retrospectively the treatment outcome in the two consecutive treatment protocols specific for ML-DS. In AML-BFM 2004 Interim Protocol, the DS-specific arm had reduced antracyclines doses compared to standard treatment,

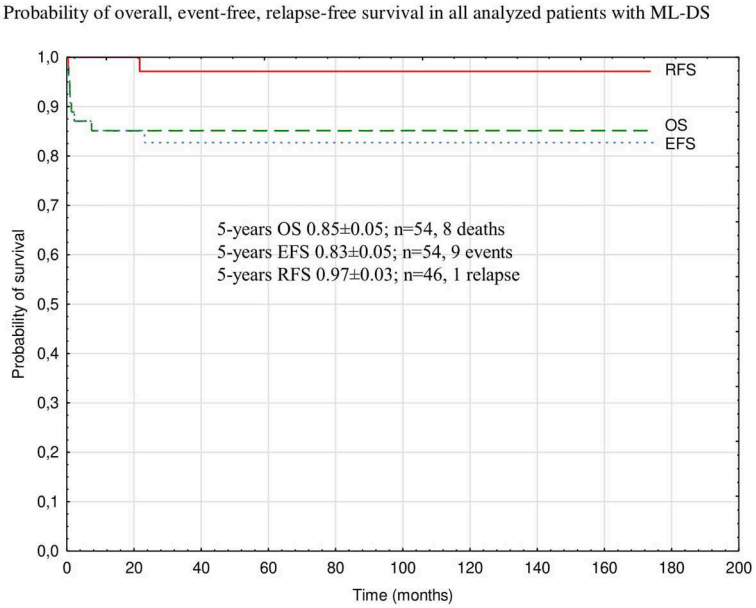

FIGURE 2 | Probability of overall, event-free, relapse-free survival in all analyzed patients with ML-DS. ML-DS, myeloid leukemia of Down syndrome; OS, overall survival; EFS, event-free survival; RFS, relapse-free survival.

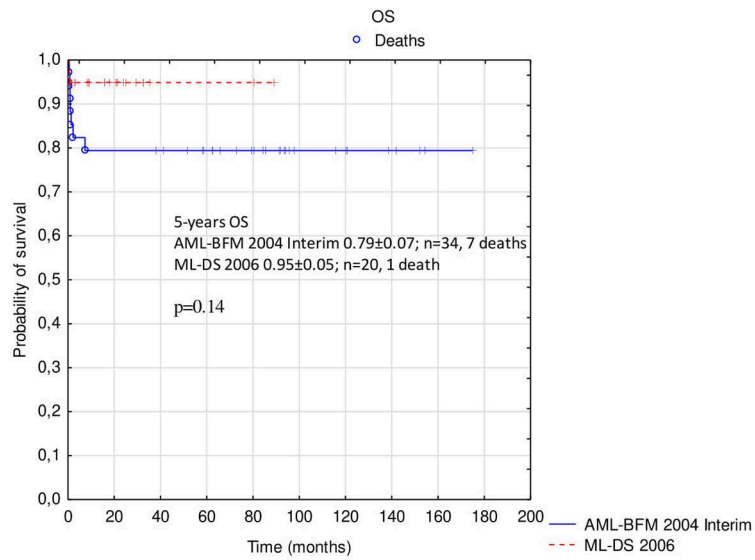

FIGURE 3 | Comparison of overall survival in patients with ML-DS treated with AML-BFM 2004 Interim Protocol and ML-DS 2006 Protocol. ML-DS, myeloid leukemia of Down syndrome; OS, overall survival.

and intrathecal therapy and central nervous system (CNS) irradiation were omitted in the maintenance treatment. In the ML-DS 2006 Protocol, further reduction of chemotherapy was introduced. There was no maintenance treatment; all doses of etoposide were omitted in the last cycle of chemotherapy, and there were four doses of intrathecal cytarabine instead of six in the previous protocol. The treatment outcome in the whole analyzed group of patients (5-year OS 0.85 ± 0.05 and EFS 0.83 ± 0.05) is comparable to the results recently described by large pediatric oncology groups: Children Oncology Group (COG) with 5-year OS 93.0% and EFS 89.9% (15) joined the Nordic Society for Pediatric Hematology and Oncology, Dutch

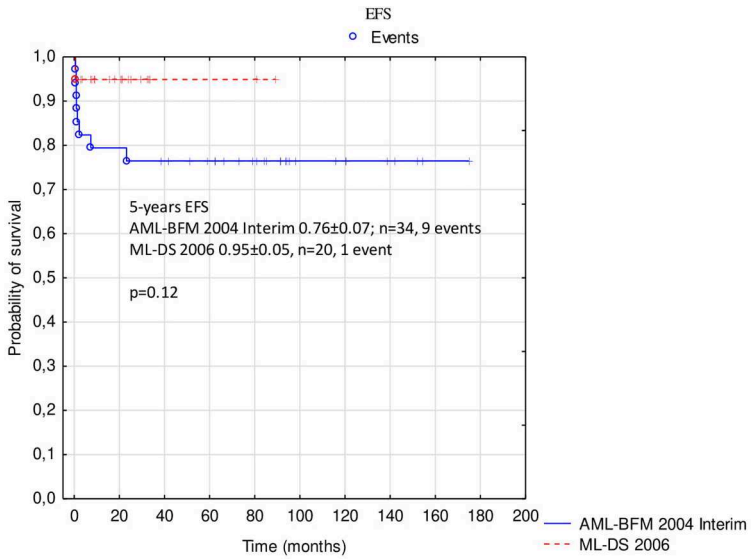

FIGURE 4 | Comparison of event-free survival in patients with ML-DS treated with AML-BFM 2004 Interim Protocol and ML-DS 2006 Protocol. ML-DS, myeloid leukemia of Down syndrome; EFS, event-free survival.

Childhood Oncology Group, and the AML-BFM study group with OS 89 ± 3% and EFS 87 ± 3% (16), as well as Japanese Pediatric Leukemia/Lymphoma Study Group with OS 87.5 ± 3.9% and EFS 83.3 ± 4.4% (17).

In the analyzed group of patients, reduction of the chemotherapy intensity in the ML-DS 2006 Protocol compared to AML-BFM 2004 Interim DS-specific arm resulted in improvement of survival rates (OS 0.95 ± 0.05 vs. 0.79 ± 0.07, $p = 0.14$, and EFS 0.95 ± 0.05 vs. 0.76 ± 0.07, $p = 0.12$), but the differences were not statistically significant. In group I, the treatment-related mortality (TRM) of 20.6% was much higher than described by other authors (1.5–4.9%) (15, 16, 18). Proportion of deaths from toxicities decreased to 5.0% in group II; however, the differences were not statistically significant. Decrease of the TRM probably resulted mainly from improvement of supportive treatment and experience of the treating centers, as almost all deaths from toxicities in group I occurred after the first chemotherapy cycle (reduction of chemotherapy concerned the last chemotherapy cycle and maintenance therapy). Reduction of the intensity of chemotherapy did not affect the treatment efficacy. There were no relapses in group II, compared to one relapse in group I. The 5-year CIR in the whole cohort was 3%. The result is excellent compared to other studies with the 5-year CIR of 6–10.0% (15, 16). Despite generally poor prognosis in relapsed ML-DS (OS, 25.9–34.3%) (15, 19), our patient was treated successfully with IdaFLA chemotherapy followed by HSCT.

In order to optimize the treatment of ML-DS, many efforts are made to find prognostic factors. Uffmann et al. (16) performed multivariate analysis and revealed that poor early response and the gain of chromosome 8 were independent prognostic factors. In the recent study of COG, the only one significant predictor of outcome was MRD on day 28 of induction (15). According to the Japanese Pediatric Leukemia/Lymphoma Study Group AML-D05 study, age at diagnosis of <2 years was a significant favorable prognostic factor for risk of relapse (17). Retrospective international study including 451 ML-DS patients from 13 collaborative study groups participating in the International-BFM AML Study Group revealed that patients with normal karyotype had a higher CIR (21 ± 4%) than cases with an aberrant karyotype ($n = 255$) with a CIR of 9% (±2%) (20).

In our study, no significant prognostic factor was found probably because of low number of patients in the subgroups.

Efforts are made to define mechanisms of leukemic transformation from TAM to ML-DS. In the recent study, Labuhn et al. (21) showed that trisomy 21 and *GATA1* mutation are sufficient for the development of TAM. They identified transforming hotspot mutation in myeloid cytokine receptor *CSF2RB*. Using a multiplex CRISPR/Cas9 screen in an *in vivo* murine TAM model, they found that loss of 18 from 22 tested recurrently mutated ML-DS genes led to leukemia phenotypically, genetically, and transcriptionally similar to ML-DS (21).

The most frequent chromosomal alterations associated with ML-DS are as follows: dup(1q), del(6q), del(7p), dup(7q), +8, +11, del(16q), and +21 (22). In our study among 49 patients with cytogenetic analysis, 43% had isolated trisomy 21. The most frequent numerical abnormality involved trisomy 8 (16.3%); structural abnormalities comprised del(7) (10.2%), del(6) (6.1%), del(16q) (6.1%), dup(7) (4%), isochromosome 7 (4%) (**Table 3**). Other abnormalities were found in the single patients (**Table 3**). Complex karyotype was revealed in 12.2% of patients.

The *GATA1* gene analysis became available for most ML-DS patients from 2015. Status of the gene was analyzed in 18 patients. Surprisingly, mutation was found in only 38% of those children, whereas the prevalence of *GATA1* mutation in

TABLE 2 | Treatment outcome in the defined groups of the patients.

	n	Deaths	OS	*p*	Events	EFS	*p*	Relapse
Total	54	8	85.5		9	85.5		1
Sex				0.46			0.30	
Male	27	3	0.89		3	0.76		1
Female	27	5	0.81		6	0.89		0
Age at diagnosis, years				0.39			0.72	
<4	50	8	0.84		8	0.84		0
>4	4	0	1.0		1	0.67		1
History of TAM				0.41			0.41	
Yes	15	1	0.93		1	0.93		0
No	19	3	0.84		3	0.84		0
No data	20							
Cytogenetics								
Trisomy 21 only				0.72			0.54	
Yes	21	4	0.84		5	0.80		1
No	28	3	0.87		3	0.87		0
Trisomy 8				0.82			0.74	
Yes	8	1	0.87		1	0.87		0
No	41	6	0.85		7	0.82		1
del(7)				0.66			0.77	
Yes	5	1	0.80		1	0.80		0
No	44	6	0.86		7	0.83		1
del(6)				0.47			0.41	
Yes	3	0	1.0		0	1.0		0
No	46	7	0.85		8	0.82		1
del(16)				0.33			0.42	
Yes	3	1	0.67		1	0.67		0
No	46	6	0.87		7	0.84		1
Complex karyotype				0.29			0.26	
Yes	6	0	1.0		0	1.0		0
No	43	7	0.84		8	0.81		1
No data	5							
Congenital heart defect				0.30			0.51	
Yes	24	2	0.92		3	0.87		1
No	27	5	0.81		5	0.81		0
No data	3							
History before diagnosis, months				0.70			0.45	
≤3	23	3	0.87		4	0.81		1
>3	21	2	0.90		2	0.90		0
No data	10							

EFS, probability of event-free survival; OS, probability of overall survival; TAM, transient abnormal myelopoiesis.

other studies was 85–89% (15, 16). It could not be explained by low percentage of blasts at diagnosis because it was similar in the group with and without mutation. There could be some false-negative results as the Sanger sequencing used to detect *GATA1* mutation has limited sensitivity. Finally, the group of patients who had *GATA1* analysis was small and could be not representative.

No significant influence of the genetic features on the treatment outcome was revealed in the analyzed cohort.

In conclusion, the study confirms that reduced intensity protocols are very effective in ML-DS patients. The main cause of deaths remained toxicities (there were no deaths from the disease); however, systematic decrease of the TRM was noticed in the analyzed group.

TABLE 3 | Patients with additional genetic abnormalities.

Cytogenetics	Age, years	FAB	Outcome	Follow-up, months
47,XX,+21,del(16)	1–2	7	Death in CR	0.90
47,XY,der(1)add(1)(p?36.3)add(1)(q44),+21c[5] /48,XY,+9,+21c[1]/47,XY,+21c[24]	3–4	0	CCR	85.4
47,XX, +21, add(5),del(1)(q32),del(9)(q13-22),del (12)(q15-24.1)	2–3	7	CCR	32.7
47,XX,dic(5;7)(p12;p12),+21c[4]/47,XX,+21c[10]	1–2	7	CCR	20.7
48-50,XY,+8[10],+8[10],t(18;21)(q10;q10)c, +der(18;21) (q10;q10),+21,+21[2][cp20]	1–2	7	CCR	15.6
48,XY,+8,+21	1–2	1	Early death in aplasia	2.2
47,XY, +21 [3]/48, XY, +8, +21 [2]	2–3	2	CCR	154.4
47,XX,der(5)t(5;15)(q34;q22)+21[7]/47,XX+21[13]	4–5	7	CCR	21.3
48–50,XX,dup(7)(p13p22),+r(21)(pll.2q22.1) × 2–4	2–3	7	CCR	8.1
48,XX, inv(5)(p15q33)del(5)(p15.3),r(7)(p?)del(7) (q22), +21c,+21[10]/47,XX,+21c[15]	2–3	7	CCR	62.7
48,XY,del(6)(q13q22),+11,der(17)t(1;17)(p?;p13), +21c[2]/47,XY,+21c[28]	2–3	7	CCR	38.3
47,XY,r(7)dup(7)(q331q?32),+21c[17]/47,XY+21c[6]	1–2	7	CCR	9.2
50,XY,+19,+20,+21c,+22 [2]/47,XY,+21c[18]	1–2	nd	CCR	66.0
47,XX,+21c,del(6)(q23) (20)	1–2	0	CCR	79.3
48,XX,+8[11],+21[16][cp18]/46,XX[2]	1–2	7	CCR	62.7
47,XY,del(11)(q23),+21c[2]/49,XY,+8,del(11)(q23),+15,+21c[6]/47,XY,+21c[15]	2–3	7	CCR	3.5
47,XX,+21; del(6)(q21qter),del(12)(p12pter),del(5)(q4pter),	4–5	1	CCR	152.1
trisomy 18, trisomy 19, trisomy 22, hyperdiploidy in 25% of metaphases				
47,XX, del(7)(q32),+21c; 47XX, +21	2–3	0	Early death in aplasia	0.6
47,XX, del(16)(q12q22), +21c[11]	<1	7	CCR	120.6
48,XX,+8+21c[5]/47,XX,+21c[12]/46,XX[3]′	2–3	1	CCR	115.8
47,XY,+21c[3]	2–3	7	CCR	18.1
FISH—trisomy 8 in 9% of 200 interphase nuclei				
47,XY,+ 21, i(7q)	1–2	nd	CCR	94.3
47,XX, i(7)(q10), +21c[18]/47,XX, +21c[2]	1–2	nd	CCR	8.8
47,XY,+21c[18]/46, XY [2] FISH del(7q22)(7q35)	2–3	7	CCR	95.4
47,XX, del(16)(q13),t21c [2]/47,XX,+21c	2–3	7	CCR	93.5
47,XY,+21, FISH trisomy 8	1–2	5	CCR	80.5
47,XX,+21,t(7;21)	2–3	7	CCR	89.7
47,XY,+21,der(7)t(7;11)	2–3	7	CCR	80.6

CCR, continuous complete remission; CR, complete remission; nd, no data.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Jagiellonian University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MCz and WBal designed the study. MCz, KP-W, BS-F, MM, AR-K, AC, JS-S, JW, KM-R, MK-R, DG, JK, LM-K, EA-D, KZ, TU, RT, TS, MN, MW, JP, AC-G, GK, AM-M, KB, WM, KM, WBad, MCi, RC, SS, and WBal were involved in the participates recruitment. TK involved in the laboratory work, and interpretation of its results. MCz, KP-W, BS-F, AR-K, JS-S, KM-R, DG, LM-K, KB, KZ, RT, MN, JP, AC-G, AM-M, MCi, and KM collected the clinical data. MCz involved in the statistical analysis, interpretation of its results, and wrote the first draft of the manuscript. WBal and SS edited the first draft of the manuscript. All authors reviewed the manuscript and approved the final version of the manuscript.

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High Frequency of Fusion Gene Transcript Resulting From t(10;11)(p12;q23) Translocation in Pediatric Acute Myeloid Leukemia in Poland

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11q23/*MLL* rearrangements are frequently detected in pediatric acute myeloid leukemia. The analysis of their clinical significance is difficult because of the multitude of translocation fusion partners and their low frequency. The presence of t(10;11)(p12;q23) translocation was previously identified in pediatric acute myelogenous leukemia (AML). It is considered as the second most common translocation detected in pediatric 11q23/*MLL*-rearranged (present *KMT2A*) AML, after t(9;11)(p22;q23). The presence of the above translocation was previously identified as an unfavorable prognostic factor. Since June 2015, the Polish Pediatric Leukemia/Lymphoma Study Group has applied the therapeutic protocol requiring extensive diagnostics of genetic changes in

pediatric AML. Until November 2019, molecular genetic studies were performed in 195 children with diagnosed AML to identify carriers of fusion gene transcripts for 28 most common chromosomal translocations in acute leukemia. The fusion gene transcript for translocation t(10;11)(p12;q23) involving *MLL* gene was detected with unexpectedly high frequency (8.9%) in our research. It was the highest frequency of all detected *MLL* rearrangements, as well as other detected fusion gene transcripts from chromosomal aberrations characteristic for AML. It seems that chromosomal aberration between chromosomes 10 and 11 can be relatively frequent in some populations. Paying attention to this fact and ensuring proper genetic diagnosis seem to be important for appropriate allocation of patients to risk groups of pediatric AML treatment protocols.

Keywords: acute myeloid leukemia, 11q23/*KMT2A* rearrangements, *MLL* rearrangements, children, risk stratification, treatment results

INTRODUCTION

Acute myelogenous leukemia (AML) is a heterogeneous group of hematologic malignancies, characterized by unregulated, clonal proliferation of abnormal myeloid progenitor cells. Despite major improvements in outcome over the past decades, it remains a life-threatening malignancy in children. Several constitutive genetic variants and acquired chromosomal abnormalities have been identified as prognostic markers in leukemia (1, 2). In AML in children, genetic diagnostics play an extremely important role in stratifying the risk of treatment failure. Identified genetic markers together with the response to induction treatment are the most important factors that allow selection of optimal therapy for the patient. Among the most relevant factors are chromosomal translocations and gene mutations, which recently have become crucial for risk stratification in pediatric AML (3, 4).

Characteristics for AML genetic changes include structural chromosomal aberrations (translocations, inversions), leading to the formation of gene fusions. The fusion genes present in leukemia cells undergo expression that cause malfunctioning of their protein products (4–6). Common genetic abnormalities in AML with high frequency and well-established favorable prognostic significance include t(8;21)(q22;q22) (*RUNX1-RUNX1T1*), inv(16) (p13;q22) (*CBFB-MYH11*), and t(15;17)(q24;q21) (*PML-RARA*). A basic diagnostic tool for detecting these changes is cytogenetic analysis using the karyotyping technique, supported by fluorescence *in situ* hybridization (FISH). However, nowadays, molecular biology methods are increasingly used to detect transcripts of specific fusion genes in patient's leukemia cells as an important part of the diagnostic process. Molecular techniques have found wide diagnostic application in the detection of not only the best known AML-related fusion genes but also the detection of fusion partners, for example, after the *MLL* (present *KMT2A*) gene rearrangement (7).

A variety of recurrent chromosomal rearrangements involving 11q23/*MLL* gene have been reported in adult and pediatric acute leukemias. So far, more than 135 different fusion partners of *MLL* gene have been identified in acute leukemias (8). Most common for pediatric AML are translocations t(9;11)(p21.3;q23.3) (*MLL-MLLT3*), t(10;11)(p12;q23.3) (*MLL-MLLT10*), t(11;19)(q23.3;p13.1) (*MLL-ELL*), and t(6;11)(q27;q23.3) (*MLL-AFDN*) (7–9). The prognostic impact of *MLL* rearrangement depends on detected fusion partner but is not always clearly defined. However, the presence of translocation t(10;11)(p12;q23) is usually associated with a poor prognosis (3, 7, 9).

MATERIALS AND METHODS

Since June 2015, the Polish Pediatric Leukemia/Lymphoma Study Group has applied the therapeutic protocol for pediatric AML requiring extensive diagnostics of genetic changes in leukemic cells. For all patients, conventional and molecular cytogenetics analyses were recommended—classical karyotype and FISH analyses for the most common translocation in AML: t(8; 21)(q22; q22)/*RUNX1-RUNX1T1*, inv(16)(p13; q22)/*CBFB-MYH11*, t(15;17)(q24; q21)/*PML-RARA*, and *MLL* rearrangements by break-apart FISH probes. Molecular analyses were also performed to screen for further, less common transcripts of fusion genes founded in leukemia, as well as mutation in *WT1*, *FLT3*, *NPM1*, *CEBPA*, and *GATA1* genes. Until November 2019, molecular genetic studies were performed in 195 children with *de novo* diagnosed AML. This represents nearly all pediatric patients with AML in Poland during this period.

One of the performed molecular analyses was genotyping for carriers of fusion gene transcripts for 28 most common chromosomal translocations with prognostic significance in leukemia by a CE-marked (approved for use in Europe) *in vitro* diagnostic test based on multiplex reverse transcription–polymerase chain reaction (RT-PCR) screening assay, HemaVision-28N RT-PCR (DNA Diagnostic A/S, Risskov, Denmark). The detailed information about 28 leukemia-causing translocations detected by HemaVision-28N is presented in **Supplement Table 1**. Diagnostic tests were conducted on mRNA samples isolated from mononuclear cells from bone marrow collected before the treatment. For this purpose, bone marrow samples were centrifuged in a density gradient (Histopaque-1077; Sigma-Aldrich, St. Louis, MO, USA), and the obtained bone marrow mononuclear cells were lysed

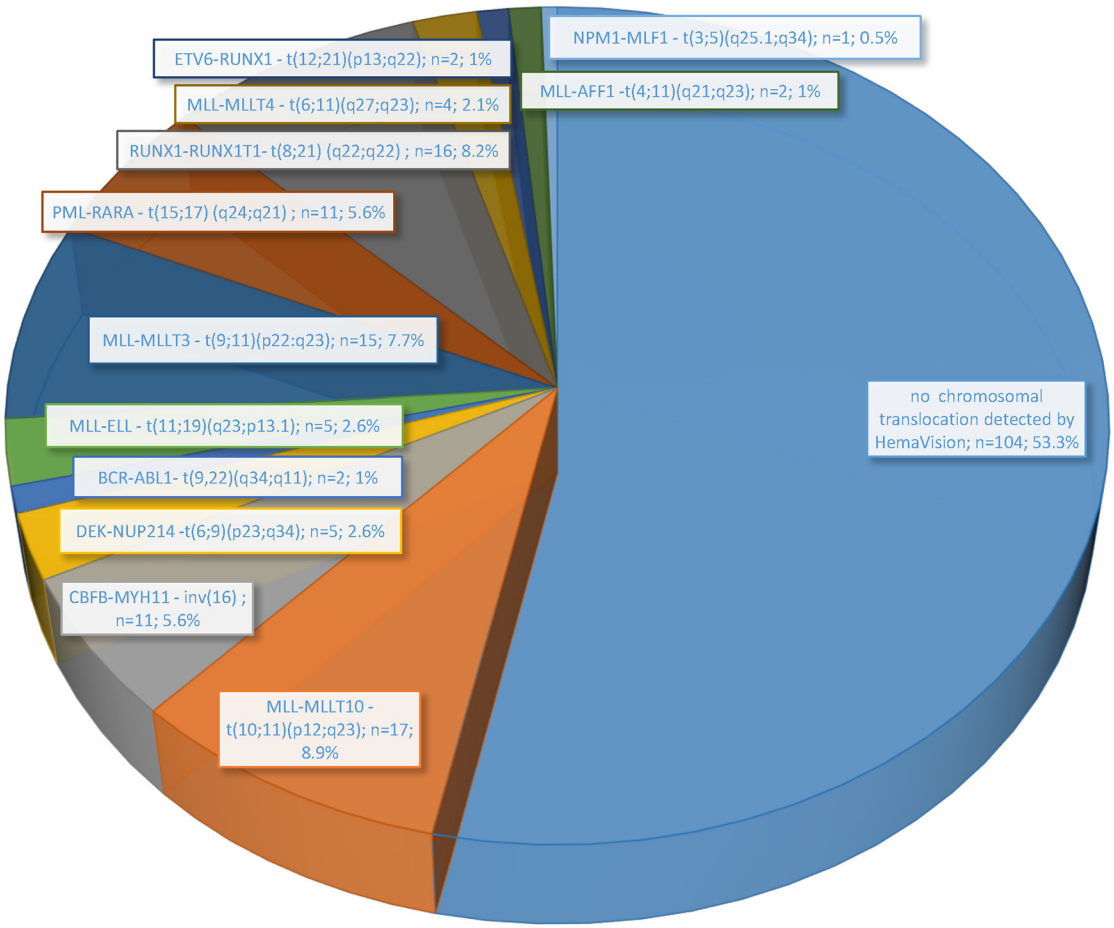

FIGURE 1 | Frequency of chromosomal translocations associated with leukemia detected by molecular methods (HemaVision RT-PCR; DNA Diagnostic A/S) in 195 pediatric AML patients treated in Poland from June 2015 to November 2019.

in TRI Reagent Solution (Thermo Fisher Scientific Baltics, Vilnius, Lithuania). The isolation of total RNA was performed according to the manufacturer's instructions. Next, 2 μg of non-degraded RNA was used as a template for synthesis of cDNA in RT reaction (SuperScript™ II Reverse Transcriptase; Thermo Fisher Scientific). HemaVision RT-PCR assay used cDNA as a template for multiplex PCR amplification reactions, followed by nested PCR reactions. All PCR reactions were performed with primer mixes from the HemaVision HV01-28N kit and Multiplex PCR Master Mix (Eur_x, Gdansk, Poland). The final PCR products were analyzed by agarose gel electrophoresis.

All children were treated according to the same therapeutic protocol—AML BFM 2012/2019. The genetic tests and individual persons' data used in publication were obtained and processed according to written informed consent obtained from guardians of all patients in accordance with the Declaration of Helsinki. The study was approved by the Ethical Committee of the Jagiellonian University in Krakow (KBET 122.6120.17.2015, dated January 29, 2015). The data that support the findings of the study are available on request from the corresponding author.

RESULTS

In the performed molecular genetic studies, the presence of fusion genes transcripts was revealed in 46.7% (91/195 patients) (**Figure 1**). The fusion gene transcript for translocation t(10;11)(p12;q23) (*MLL-MLLT10*) was detected in 8.9% (17/195) of cases with surprisingly the highest frequency among all other marked chromosomal abnormalities determined in HemaVision-28N. Slightly lower frequency was observed for fusion transcripts generated by chromosomal translocation t(8;21) (q22;q22) (*RUNX1-RUNX1T1*) (8.2%; 16 patients) and t(9;11)(p22:q23) (*MLL-MLLT3*) (7.7%; 15 patients). Furthermore, other AML-characteristic transcripts related to 11q23/*MLL* rearrangements, such as t(11;19)(q23;p13.1), t(6;11)(q27;q23), and t(4;11)(q21;q23), were also found. Together, five different fusion gene transcripts for 11q23/*MLL* rearrangements were detected in 43 patients (22.1%).

For 14 of 17 children with molecularly confirmed of *MLL-MLLT10* fusion transcript, the results of karyotype and FISH analysis were obtained, and a summary is shown in **Table 1**. In some cases, the karyotype result indicated other position

TABLE 1 | Clinical and genetic characteristics of 17 children with diagnosed AML and confirmed t(10;11)(p12;q23) fusion gene transcript in the molecular genetic study.

Case no.	Sex	Age at diagnosis	FAB	% Leukemic blasts in BM	Treatment results	Karyotype*	FISH analysis of *MLL* rearrangements
1	F	<6 months old	M5	77	CR, HSCT	47,XX,der(1)t(1;10;11)(q32;p13;q23)del(1)(p22.1)?add(1)(p22.1) dup(1)(q21q32,der(10)t(1;10;11)(q32;p13;q23) ins(10;11)(p13;q23q23),der(11)t(1;10;11)(q32;p13;q23),+19	Positive
2	F	6 months old	M5	66.6	Death due to pulmonary embolism in early relapse treatment	48,XX,+6,der(10)(p?),+19	No data
3	M	16 years old	M5	78.2	CR, HSCT	46,XY,der(3)t(3;19),t(10;11)(p13;q23),r1(19)/47,idem,+r2	No data
4	M	17 years old	M5	85	Death due to pulmonary leukostasis, acute liver failure and cardiopulmonary failure before achieving remission	46,XY	Positive ish der(9)t(9;10)(p21;p12)(wcp10+),der(10) t(10;11)(p11;q23)(wcp10+,wcp11+),der(11)t(11;10;9)(p11pter-q23::10p11 -10p12::9p21-9pter)(wcp11+,wcp10+)[20]
5	M	1 year old	M5	77	Death in CR in course of cytomegalovirus infection after HSCT	46,XY,t(10;11)(p11.2q23)	Positive
6	F	1 year old	M5	76.5	CR, HSCT	50,XX,+8, +8, t(10;11)(p12;q23?),+20, +21/54-57,XX,+4,+5,+6,+8+8,t(10;11)(p12;q23"), +12,+16,+18,+19,+20,+21;+21	Positive
7	F	10 years old	M5	41	CR, HSCT	54-56, X, t(X;10;11)(q22;p13;q23),+5,+6,+8,+8,+10, del(10)(p13),+14,+18,+19,+21,+21 [cp19]	Positive
8	M	<1 year old	Myeloid Sarcoma	6.4	CR, HSCT	No cell division	Positive
9	F	3½ years old	M5	97	CR, HSCT	46,XX,t(10;11)(p12;q23)	Positive
10	F	5 years old	M0	No data	CR, HSCT	No data	No data
11	F	7 years old	M5	98.2	CR, HSCT	No data	No data
12	M	6 years old	M5	69	Early relapse	No data	No data
13	M	12 years old	M5	88	CR, HSCT	46,XY,t(10;11)(p12;q13),inv(11)(q13q23)	Positive
14	F	7 years old	No data	No data	No data	46,XX,t(10;11)(p12;q23),del(12)(p12),del(16)(q12)/46,XX	Positive
15	M	1½ years old	No data	No data	No data	46,XY,t(10;11)(p12;q23),t(12;20)(p11.2;p13) /46,XY,t(12;20)(p11.2;p13)	Positive

(Continued)

TABLE 1 | Continued

Case no.	Sex	Age at diagnosis	FAB	% Leukemic blasts in BM	Treatment results	Karyotype*	FISH analysis of *MLL* rearrangements
16	F	<6 months old	M5	81.6	CR, HSCT	46,XX	Positive ish der(10)ins(10;11)(p12;q13q23)(5'KMT2A+)(2) nuc ish(5'KMT2Ax3,3'KMT2Ax2)(5'KMT2A con 3'KMT2Ax2)[184/230]
17	F	<1 year old	M5	76.4	Death due to sepsis and ischemic stroke before achieving remission	46,XX,t(7;10)(q11.2;p11.2)/46,XX	No data

M, male; F, female; BM, bone marrow; CR, complete remission; HSCT, allogenic hematopoietic stem cell transplantation.

**In some cases, the karyotype result indicated other position of chromosome breaks than expected due to molecular result. Obtained cytogenetic results (karyotype, FISH) were performed in local laboratories, and molecular verification was carried out in a reference laboratory. No correction of chromosome breakpoints assessed in the classical karyotype after molecular analysis results was performed.*

of chromosome breaks than expected due to molecular result, especially in the case of chromosome 10 (p11, p13, p?). Pediatric karyotypes always pose a great diagnostic challenge due to achieved resolution and G-band quality. Obtained cytogenetic results (karyotype, FISH) were performed in local laboratories, and molecular verification was carried out in a reference laboratory. No correction of chromosome breakpoints assessed in the classical karyotype after molecular analysis results was performed.

In four of those 14 patients, only rearrangements in chromosomes 10 and 11 were observed (cases 5, 9, 8, and 16, for whom rearrangements of *MLL* gene were confirmed only by FISH). For the next seven cases, additional chromosomal abnormalities or complex karyotype was detected (cases 1, 3, 6, 7, 13, 14, and 15; the results in detail in **Table 1**). For patient 4, the karyotyping result was normal, but in a metaphase FISH, the variant translocation with chromosomes 11, 10, and 9 was confirmed (cryptic rearrangement). Moreover, variant translocations were also observed in two other cases—patient 1: t(1;10;11) and patient 7: p t(X;10:11). It should be emphasized that in all these cases the fusion gene transcript for *MLL-MLLT10* was detected in molecular studies. For the last two children (cases 2 and 17), the presence of translocation t(10;11)(p12;q23) was not simply confirmed; however, abnormalities in chromosome 10 were indicated in both cases. Unfortunately, no data from FISH analysis were available for them. In the case of low G-band quality and resolution in the karyotype analysis for children with leukemia, such results do not exclude rearrangement of chromosomes 10 and 11 for patient 2 or cryptic variant translocation of chromosomes 7, 10, and 11 for patient 17.

The clinical characteristics of 17 presented children with diagnosed AML and molecular confirmation of *MLL-MLLT10* fusion gene transcript are presented in **Table 1**. All of the children were treated according to the AML-BFM 2012/2019—10 girls and 7 boys with median age of 3.6 years (range, 4 months to 16.2 years). In four of them, hyperleukocytosis (91–245 $\times$ 10^9/L) was observed at diagnosis. Ten patients achieved complete remission (CR) and underwent allogenic hematopoietic stem cell transplantation (HSCT). Two patients died early before achieving remission—the first because of sepsis and ischemic stroke 1.4 months after diagnosis and the second one due to pulmonary leukostasis, acute liver failure, and cardiopulmonary failure 13 days after final diagnosis of AML. Two further patients had an early relapse; one of them died because of pulmonary embolism. One patient died in CR in course of cytomegalovirus infection 2.6 months after HSCT. Median observation time was 11.5 months (range, 0.4–49.7 months). For two patients, no follow–up data were available.

DISCUSSION

The presence of t(10;11)(p12;q23) translocation was previously identified in pediatric AML and considered as an unfavorable prognostic factor, but it was observed rarely (1, 3, 8–11). This rearrangement is described in the latest large cohort studies as the second most common translocation detected in

pediatric 11q23/*MLL*-rearranged AML, after t(9;11)(p22;q23) (8). Its occurrence is being estimated at 3% (3). Surprisingly, in our research, the fusion gene transcript resulting from t(10;11)(p12;q23) frequency (8.9%) was not only the highest in the case of all detected *MLL* rearrangements but also in relation to other common chromosomal translocation in AML, among others t(8;21)(q22;q22) (*RUNX1-RUNX1T1*)-−8.2% (16/195 patients). Similar results were presented by Stasevich et al. (12), who confirmed the presence of the t(10;11)(p12;q23) at a very high level in children treated for AML in 1 year at Minsk Oncology Center. In the mentioned article, five children with of t(10;11)(p12;q23) translocation were being described among 18 pediatric patients. Worth noting, all of five children do not have the simple reciprocal translocation; in all cases, MLL-MLLT10 fusion was created by several rearrangements. Also, in our research, in 3 of 13 children with karyotype result, variant translocation of 11q23/*MLL* was revealed [t(1;10;11), t(11;10;9), t(X;10;11)]. Therefore, it seems that in some cases the ambiguity and complexity of changes detected by cytogenetic analysis do not always allow for a correct and rapid assessment of the presence of rearrangement of chromosomes 10 and 11 in leukemic blasts. Hence, the molecular genetic studies seem to be a more appropriate technique to unequivocal assessment of the presence of *MLL-MLLT10* fusion transcript, especially in a case of possible missing of this chromosomal aberration during karyotyping (two children with normal karyotype reported) or when no karyotyping result is obtained.

In the presented group of 17 patients with positive molecular results for *MLL-MLLT10*, in three patients (patients 10–12 in **Table 1**), there is no cytogenetic confirmation of the results because of the lack of access to archival data. For the next two patients (patients 2 and 17), only the karyotype analysis was performed, with the results not indicating the presence of *MLL* rearrangement. For this reason, no FISH analysis was performed in these cases. However, in both these cases, chromosome breakage in 10p was observed [48,XX,+6,der(10)(p?),+19 and 46,XX,t(7;10)(q11.2;p11.2)/46,XX]. In the context of a positive molecular result for the *MLL-MLLT10* fusion gene transcript, the presence of more complex chromosomal rearrangements, including chromosome 11 rearrangement, may be possible in these patients (cryptic or variant translocation). This also indicates the need for obligatory FISH for *MLL* rearrangements in all AML patients.

Therefore, because of the large amount of molecular diagnostic tests performed with the HemaVision-28N RT-PCR IVD assay (for 195 children diagnosed with AML) and cytogenetic confirmation of the obtained result in the 12 presented cases, also the molecular results for this group of five patients without direct cytogenetic confirmation were considered reliable and included in the analysis.

According to the recommendations of an international expert panel, the patients with translocation t(10;11)(p12;q23) should be allocated to the high-risk group of pediatric AML treatment protocols (3). The MLL gene encodes a histone methyltransferase that positively regulates homeobox (HOX) gene expression (13). The presence of MLL-MLLT10 fusion protein observed in patients with t(10;11)(p12;q23) in leukemic blasts affects the histone methylation ability and dysregulation of HOXA and MEIS1 expression, finally leading to MLL rearrangement-dependent leukemia (14). It cannot be excluded that the presence of the *MLL-MLLT10* fusion transcript detected by molecular methods in the case of variant chromosomal rearrangements or complex karyotype does not have an equally significant negative effect on cell function. However, further research is required in this matter. To sum up, it seems that fusion gene transcript resulting from t(10;11)(p12;q23) can be relatively frequent in some populations, or its frequency may be underestimated because of the difficulty of its cytogenetic assessment. Paying attention to this fact and ensuring proper genetic diagnosis seem to be important for appropriate allocation of patients to risk groups of pediatric AML treatment protocols.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of the Jagiellonian University in Krakow. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

TK, MCz, and WBal: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, and critical revision of the manuscript for important intellectual content. PK, BS, and ML: analysis and interpretation of data and critical revision of the manuscript for important intellectual content. KP-W: analysis and interpretation of data assistance, acquisition and accumulation of data, and critical revision of the manuscript for important intellectual content. MB-M and SS: critical revision of the manuscript for important intellectual content. BS-F, MM, JS-S, JW, AR-K, AC, KM-R, MK-R, DG, JK, LM-K, EA-D, WM, RT, TS, JP, GK, AM-M, KM, WBad, KZ, TU, IK-D, MW, and MCi: acquisition of data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.00278/full#supplementary-material

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