Journal of Oncology

Successes and Challenges for Diagnosis and Therapy of Acute Leukemia

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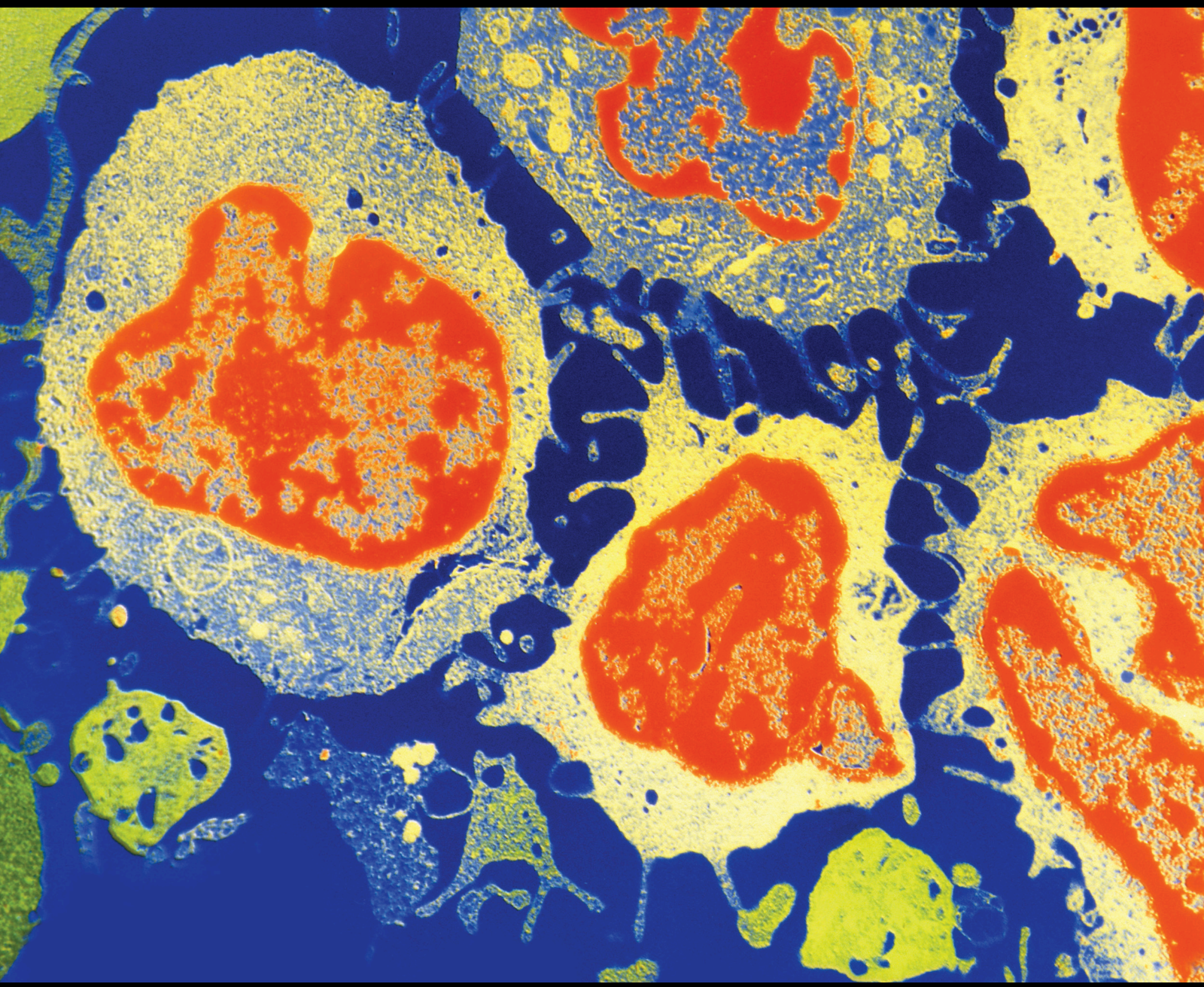

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Editorial

Successes and Challenges for Diagnosis and Therapy of Acute Leukemia

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Acute leukemia (AL) is a family of blood cancers that arises from the malignant transformation of hematopoietic cells of lymphoid (acute lymphoblastic leukemia (ALL)) or myeloid (acute myeloid leukemia (AML)) origins. While ALL occurs mostly in children, AML is mostly a disease of older adults, although it also peaks in infants (children aged 0-1 years). Treatment of AL remains challenging across age, with standard therapeutic treatments allowing 5-year overall survival rates of about 70% and 90% in children and 30–40% in adults, for AML and ALL, respectively. The major cause of treatment failure is relapse, and the prognosis for relapsed AL is extremely poor. In recent years, many efforts to improve the prognosis for these patients resulted in profound changes in supportive care and minimal residual disease monitoring. In addition, significant achievements have been made in our understanding of the genetic basis of both lymphoid and myeloid leukemias, which in turn allowed a better risk-adapted patients' stratification and the identification of novel biomarkers suitable for innovative targeted therapy-based approaches. We are pleased to introduce this special issue that contains original research as well as review articles covering several aspects of AL biology and clinical management.

Two interesting manuscripts in this special issue focus on the complexity of AML biology, which has implications for therapeutic intervention. L. Handschuh provided a comprehensive review of the molecular alterations of AML and their use for AML subtype classification, risk stratification, outcome prediction, and novel therapeutic approaches. In addition, novel findings identified by applying next-generation sequencing approaches were detailed. They include novel chimeric or alternative spliced transcripts, as well as small RNAs, piwi-interacting RNAs, small nucleolar RNAs, long noncoding RNAs, and circular RNAs. All these functional transcripts dramatically impair expression and increase the genetic heterogeneity of AML. The second review by P. Bernasconi and O. Borsani focused on AML stem cell-niche and the possible interventions that might affect the cross talk between leukemic cells and bone marrow microenvironment, aimed to restore the normal niche ecology and to sensitize AML cells to chemotherapy, thus overcoming niche-mediated drug resistance.

Three further manuscripts investigating genetic alterations or innovative therapeutic approaches in AML are included in this special issue. In the research article by N. Niktoreh et al., the prognostic relevance of co-occurrence of *WT1* mutations, *FLT3-ITD,* and *NUP98-NSD1*, was re-evaluated in a contemporary pediatric AML trial. Analysis of a large cohort of pediatric AML patients treated in Germany with AML-BFM protocols between 2004 and 2017 confirmed that co-occurrence of these three alterations defines a subgroup of AML patients with dismal outcome. The original research article by S. Bruno et al. reported the identification of two novel alterations in *DNMT3A* gene in two adult AML patients, a single nucleotide variant (p.Trp795Ter) and a 35 nucleotides insertion (p. Thr862_Glu863fsins), both resulting in a premature STOP codon. The authors demonstrated that both these mutations

affect DNMT3A protein expression and DNMT3A-mediated DNA methylation. Finally, the research article by A. Vitkevičienė et al. suggested the addition of 3-deazaneplanocin A (HMT inhibitor) and belinostat (HDAC inhibitor) to the conventional therapy (idarubicin and retinoic acid) of acute promyelocytic leukemia (APL) patients, based on enhanced efficacy observed in their *in vitro* study.

The manuscript of C. Grobbelaar and A. M. Ford described the roles of different miRNAs in pediatric ALL, particularly focusing on their role for ALL classification, risk stratification, prediction of therapy response, and potential therapeutic targets. The authors also reported an interesting section about the recently identified gut miRNAs that likely participate in shaping the gut microbiota and might be useful as potential noninvasive diagnostic and prognostic biomarkers, or even as tools for prevention of certain subtypes of childhood ALL.

Involvement of central nervous system (CNS) by leukemia is a relatively common event and it is still a major clinical issue. To target malignant cells in the CNS, intrathecal (IT) chemotherapy is commonly used. Intrathecal therapy associates with acute and long-term neurotoxicity, as it is well documented in pediatric patients but data about long-term toxicity of IT in adults are lacking. D. M. Byrnes et al. investigated the effects of IT in adult patients, including ALL patients, who were treated over a two-year period. The results of this retrospective study demonstrated a high frequency of neurological complications secondary to IT therapy, suggesting that less toxic forms of therapy may be warranted.

Finally, E. Ledesma-Martínez et al. reviewed the biological effects of caseins, the main milk proteins, and peptides released during their digestion, focusing in particular on their interaction with the immune system, normal hematopoietic and leukemic cells. Although few researches have explored the role of caseins as antileukemic agents, what emerges is the influence of dietary intake of foods on cancer development or predisposition, sustaining the importance to pay attention on patients' nutrition during leukemia therapy.

We hope that the articles included in this special issue will provide the opportunity to deepen some aspects of acute leukemias and will stimulate future researches in the field.

Conflicts of Interest

The editors declare no conflicts of interest.

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Riccardo Masetti

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Research Article

Identification of Two *DNMT3A* Mutations Compromising Protein Stability and Methylation Capacity in Acute Myeloid Leukemia

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Somatic mutations of *DNMT3A* occur in about 20% of acute myeloid leukemia (AML) patients. They mostly consist in heterozygous missense mutations targeting a hotspot site at R882 codon, which exhibit a dominant negative effect and are associated with high myeloblast count, advanced age, and poor prognosis. Other types of mutations such as truncations, insertions, or single-nucleotide deletion also affect the *DNMT3A* gene, though with lower frequency. The present study aimed to characterize two *DNMT3A* gene mutations identified by next-generation sequencing (NGS), through analysis of protein stability and DNA methylation status at CpG islands. The first mutation was a single-nucleotide variant of *DNMT3A* at exon 20 causing a premature STOP codon (c.2385G > A; p.Trp795*; NM_022552.4). The *DNMT3A* mutation load increased from 4.5% to 38.2% during guadecitabine treatment, with a dominant negative effect on CpG methylation and on protein expression. The second mutation was a novel insertion of 35 nucleotides in exon 22 of *DNMT3A* (NM_022552.4) that introduced a STOP codon too, after the amino acid Glu863 caused by a frameshift insertion (c.2586_2587insTCATGAATGAGAAAGAGGACATCTTATGGTGCACT; p.Thr862_Glu863fsins). The mutation, which was associated with reduced DNMT3A expression and CpG methylation, persisted at relapse with minor changes in the methylation profile and at protein level. Our data highlight the need to better understand the consequences of *DNMT3A* mutations other than R882 substitutions in the leukemogenic process in order to tailor patient treatments, thus avoiding therapeutic resistance and disease relapse.

1. Introduction

Genetic alterations of chromatin regulators and epigenetic modifications cooperate to the pathogenesis of acute myeloid leukemia (AML) [1]. Among epigenetic modifications, DNA methylation represents a mechanism adopted by cells to regulate DNA accessibility through the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC). It switches on/off the promoter of several target genes, thus regulating their expression levels and contributing to disease phenotype. DNA methyltransferases (DNMT3A, DNMT3B, DNMT1, and DNMT3L) are the key

enzymes involved in DNA methylation. DNMT3A and DNMT3B produce *de novo* hemimethylated DNA and *de novo* symmetric DNA methylation, respectively, while DNMT1 is involved in maintenance of DNA methylated status [2, 3].

DNA methylation has been identified as a specific functional category of mutated genes in AML that includes mutation in *DNMT3A*, *DNMT3B*, *DNMT1*, *Tet methylcytosine dioxygenase 1* and *2* (*TET1*, *TET2*), and *isocitrate dehydrogenase 1* and *2* (*IDH1*, *IDH2*) genes [4]. Moreover, *Wilms tumor 1* (*WT1*) mutations were shown to reduce DNA methylation levels significantly in AML patients, conferring the same hypomethylation signature observed in patients harbouring *TET2* mutations [5]. Haploinsufficiency of *WT1* in preclinical models was also shown to decrease 5-hmC levels and impair TET2 function, especially in elderly animals [6].

DNMT3A mutations are among the most frequent driver mutations in AML, third for frequencies to *Fms-like tyrosine kinase 3* (*FLT3*) and *nucleophosmin 1* (*NPM1*) [1]. About 20% of patients with *de novo* AML show recurrent somatic mutations in the *DNMT3A* gene [7], which represents an early lesion in leukemia development. Indeed, *DNMT3A* genomic alterations are considered a preleukemic event in hematopoietic stem cells (HSCs) and confer a proliferation advantage [8]. They have been identified in preleukemic stem cells of myelodysplastic syndrome (MDS) and AML patients [9]. Moreover, mutations in genes encoding epigenetic modifiers, including *TET2*, *ASXL transcriptional regulator 1* (*ASXL1*), and *DNMT3A* have been reported in aging healthy people and are considered the first "hit" for the "clonal hematopoiesis of indeterminate potential (CHIP)" [10, 11].

Genomic lesions of the *DNMT3A* gene mostly consist in missense mutations targeting a hotspot site at R882 codon, which exhibits a dominant negative effect [12]. Furthermore, *DNMT3A* R882 mutations are associated with a hypomethylated status of CpG islands, higher relapse rate, and poor prognosis [13–18], and they persist during remission [19, 20]. It has been recently shown that the methylation levels are dependent on the amount of active DNMT3A and higher methylation confers a better prognosis to AML patients [21]. Among patients carrying *DNMT3A* mutations, 15–20% do not show the substitution at R882 codon, but they harbour truncations, insertion, or single-copy deletions [1, 7].

The present study aimed to characterize two undescribed *DNMT3A* mutations identified by next-generation sequencing (NGS) in two different AML patients, through analysis of protein stability and DNA methylation status at CpG islands.

2. Materials and Methods

2.1. Patients. Samples were obtained from AML patients after informed consent was approved by the Institutional Ethical Committee (protocol 112/2014/U/Tess of Policlinico Sant'Orsola-Malpighi) in accordance with the Declaration of Helsinki.

2.2. Isolation of Mononuclear Cells. Bone marrow (BM) mononuclear cells were isolated by Ficoll density-gradient (Amersham Biosciences) and lysed in guanidine-thiocyanate-containing lysis buffer (RLT, Qiagen, Ltd).

2.3. Genomic DNA, RNA, and Protein Extraction. The All-Prep DNA/RNA/Protein Mini Kit (Qiagen, Ltd) was used to extract DNA, RNA, and protein from primary mononuclear cells according to the manufacturer's instructions.

2.4. Ion Torrent Next-Generation Sequencing and Variant Calling. The patients' mutational profile was determined using Oncomine Myeloid Research Assay. The libraries were quantified using the Ion Library TaqMan TM Quantitation Kit (Thermo Fisher Scientific) following manufacturer's instructions and run in the Ion 530™ chip on the Ion Torrent S5 instrument (Thermo Fisher Scientific). Sequence alignment and analysis were performed using the Ion Torrent Suite Software v.5.8.0 and the Ion Reporter software v.5.10.3.0 and v.5.10.5.0 (Thermo Fisher Scientific). Human genome build 19 (Hg 19) was used as the reference for sequence alignment. A minimum coverage depth per amplicon of 500 was required; nonsynonymous mutations with a variant allele frequency (VAF) ≥2.5% were reported; 5′ and 3′ untranslated regions (UTRs), intronic donor splice-site variants, and polymorphisms were filtered out.

2.5. Amplification and Sanger Sequencing. *DNMT3A* mutations were confirmed by Sanger sequencing. Briefly, reverse transcription was performed starting from 1 μg of RNA using reverse transcriptase M-MuLV enzyme (Sigma-Aldrich®). The obtained cDNA was used to perform polymerase chain reaction (PCR) using Applied Biosystem AmpliTaq Gold® (Thermo Fisher Scientific) and the following primers: FW: 5′-TCGAGTCCAACCCTGTGATG-3′ and REV: 5′-TAACTTTGTGTCGCTACCT CAGTT-3′. Cycling conditions were as follows: 10 minutes at 95°C; 40 cycles: 30 seconds at 94°C; 45 seconds at 54°C; 30 seconds at 72°C; and 10 minutes at 72°C. PCR amplicons were purified using Applied Biosystems ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific) according to manufacturer's instructions. Samples were sequenced according to dideoxy procedure BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) on an Applied Biosystems 3730xL genetic analyzer system (Thermo Fisher Scientific).

2.6. Capillary Electrophoresis. The allelic ratio of *FLT3*-ITD was measured by capillary electrophoresis. The reaction was performed starting from 20 μg of DNA, by using AmpliTaq™ Gold DNA polymerase enzyme, Buffer II, magnesium chloride (Thermo Fisher Scientific) and the following primers: forward, 5′-GCAATTTADGTATGAAAGC-CAGC-3′, and reverse, 5′-CTTTCAGCATTTTGACGGCA-ACC-3′. Cycling conditions were 10 minutes at 95°C; 30 seconds at 95°C; 60 seconds at 60°C; 60 seconds at 72°C for 35

cycles; 7 minutes at 72°C. The amplification products were sequenced on Applied Biosystems 3130 Genetic Analyzer (Thermo Fisher Scientific) and were analyzed with GeneMapper™ Software 5 (Thermo Fisher Scientific). The ratio of the area of mutated and wildtype (wt)-*FLT3* defined the allelic ratio.

2.7. Western Blot Analysis. Protein extracts were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad) and transferred onto nitrocellulose membranes. The following antibodies were used: rabbit anti-DNMT3A (D23G1; Cell Signaling Technologies) and goat anti-β-actin (Santa Cruz Biotechnology) as control; horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin (Ig)G (GE Healthcare) and anti-goat IgG (Santa Cruz) as secondary antibodies. ECL Prime (GE Healthcare) reagent was used for detection using ChemiDoc XRS+ System (Bio-Rad). Signal quantification was performed using Image J software.

2.8. DNA Methylation Assay. Methylation was quantified on total DNA using the MethylFlash Methylated DNA 5-mC Quantification Kit (Epigentek) using triplicates of 100 ng of DNA from each sample. The absorbance (OD) was read at 450 nm using the Multiskan EX automatic microplate reader (Thermo Fisher Scientific). To quantify the absolute amount of methylated DNA, we generated a standard curve using a negative control (ME3) and 5 dilutions of positive control (10.0; 5.0; 2.0; 1.0; 0.5 ng/μl); next we determined the slope (OD/ng) using linear regression. According to manufacturer's instructions, we used the following formulas to calculate the amount and percentage of 5-mC:

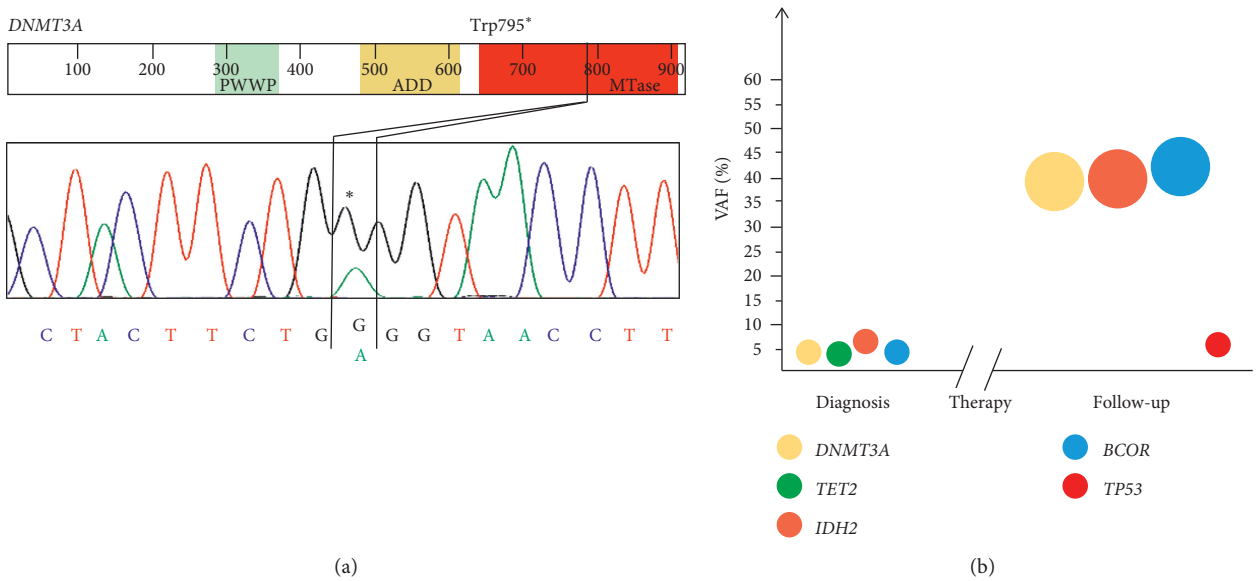
$$5\,\mathrm{mC\,(ng)} = \frac{(\text{sample OD} - \text{ME3 OD})}{\text{slope} \times 2^{*}},$$
$$5\,\mathrm{mC\,(\%)} = \frac{5\mathrm{mC\,(ng)}}{\text{sample DNA (ng)} \times 100}, \quad (1)$$

Where *2 is a factor to normalize the positive control because it contains only 50% of 5 mC.

3. Results and Discussion

3.1. Identification of a Premature Stop Codon in Exon 20 of the DNMT3A Gene in AML. NGS on a primary AML sample identified a single-nucleotide variant of *DNMT3A* at exon 20 causing a premature STOP codon (c.2385G > A; p.Trp795Ter) that was confirmed by Sanger sequencing (Figure 1(a)). Mutations at codon 795 were previously reported in angioimmunoblastic T-cell lymphoma (p.Trp795_Gly796ins3) [22] and in refractory anemia evolving to secondary AML (Trp795Cys) [23]. A STOP codon at position 795 has been reported in the Leiden Open Variation Database (c.2384G > A, https://www.lovd.nl/) and the c.2385G > A mutation has been found annotated in the database of single-nucleotide polymorphisms (dbSNP, rs1395575712). Moreover, missense variants affecting the codon 795 (chr2:25462024:A > C and chr2:25462024: A > G) were also annotated in the Genome Aggregation Database (gnomAD) with an allelic frequency of 0.000003979 and 0.000007957, respectively (https://gnomad.broadinstitute.org/). We detected the mutation (VAF = 4.5%) in the BM sample of a 74-year-old woman (AML#1), with 70% of AML blasts at diagnosis, normal karyotype, wt-*FLT3*, *NPM1*, and *tumor protein p53* (*TP53*) and intermediate cytogenetic risk according to ELN 2017 [24]. Mutations of *IDH2* (c.515G > A; p.Arg172Lys), *BCL6 corepressor* (*BCOR*) (c.2915_2916insA; p.Tyr972Ter), and *TET2* (c.3641G > A; p.Arg1214Gln) were also detected in the patient, with a VAF of 6.1%, 4.8%, and 3.2%, respectively (Figure 1(b) and Table 1). Previous evidence reported the co-occurrence of *DNMT3A* lesions with mutations of *NPM1*, *IDH2*, and *TP53* and with those affecting genes involved in chromatin and splicing in AML cases [25]. The patient received an induction therapy with the DNMT inhibitor guadecitabine [26], with persistence of disease at the bone marrow evaluation after 4 courses of therapy (40% of blasts and stable cytogenetic risk). The *DNMT3A* mutation load increased to 38.2% in the sample evaluated 4 months after treatment. NGS analysis revealed an increasing VAF during treatment of the *IDH2* (6.1% to 39.8%) and *BCOR* mutations (4.8% to 42.1%) detected at diagnosis, along with the emergence of a *TP53* mutation (c.607G > A; p.Val203Met, VAF 5.3%, Figure 1(b) and Table 1), which was not detectable at diagnosis. Nowadays, no predictive marker of response to guadecitabine has been defined for newly diagnosed AML. However, Chung et al. reported no significant association between gene mutations and complete remission in a cohort of 128 relapsed/refractory AML [27]. Trends were observed for *TET2*-mutated cases (higher CR rate) and *IDH1/2*-mutated and *TP53*-mutated AML (resistance). Moreover, *TET2* mutations have been associated with increased response to hypomethylating agents in MDS [28, 29] and AML with low blast count [28]. We believe that our results suggest the persistence during treatment of a minor clone harbouring *DNMT3A*, *IDH2*, and *BCOR* mutations, which was positively selected and progressively expanded, along with the acquisition of a novel *TP53*-mutated subclone. In parallel, *TET2*-mutated blasts were killed by the treatment.

3.2. Detection of a Novel 35 Nucleotides Insertion in Exon 22 of the DNMT3A Gene in AML. Targeted deep sequencing leads to the identification of an additional *DNMT3A* genetic alteration consisting in a new insertion of 35 nucleotides in exon 22 of the *DNMT3A* gene causing the amino acid change Glu863Ser followed by a premature STOP codon (c.2586_2587ins35:TC ATGAATGAGAAAGAGGACATCTTATGGTGCAC; p. Thr862_Glu863fsins, Figure 2(a)). The variant, which has never been reported before, was detected in primary leukemic cells isolated from a 63-year-old woman (AML#2), with 80% of blasts in the BM at diagnosis and 90% at relapse. The patient had normal karyotype, low *FLT3*-ITD allelic burden (c.1747_1748ins57: GCTCCTCAGATAATGAGTACTTCTACGTTGATTTCAGAGAATATGAATATGATCCAA; VAF: 11.7% at diagnosis), and



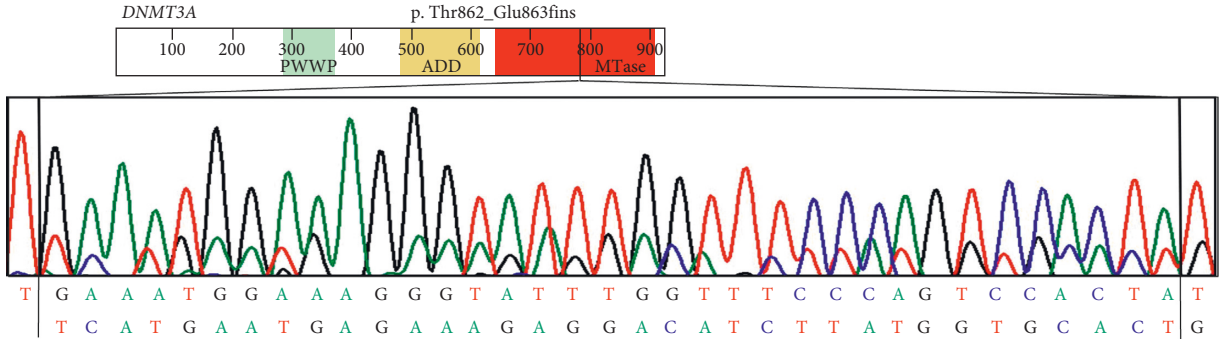
(a) (b)

Figure 1: Sanger sequencing of the *DNMT3A* single-nucleotide variant identified in AML#1 and analysis of clonal evolution based on NGS data. (a) AML#1 showed a single-nucleotide variant of *DNMT3A* at exon 20 causing a premature STOP codon (c.2385G > A; p.Trp795*; NM_022552.4). (b) Variant allele frequency (VAF) changes of the detected mutations at diagnosis and follow-up (after 4 courses of guadecitabine therapy), showing expansion of the *DNMT3A*-mutated clone.

Table 1: Mutational profile of AML#1 at diagnosis and follow-up.

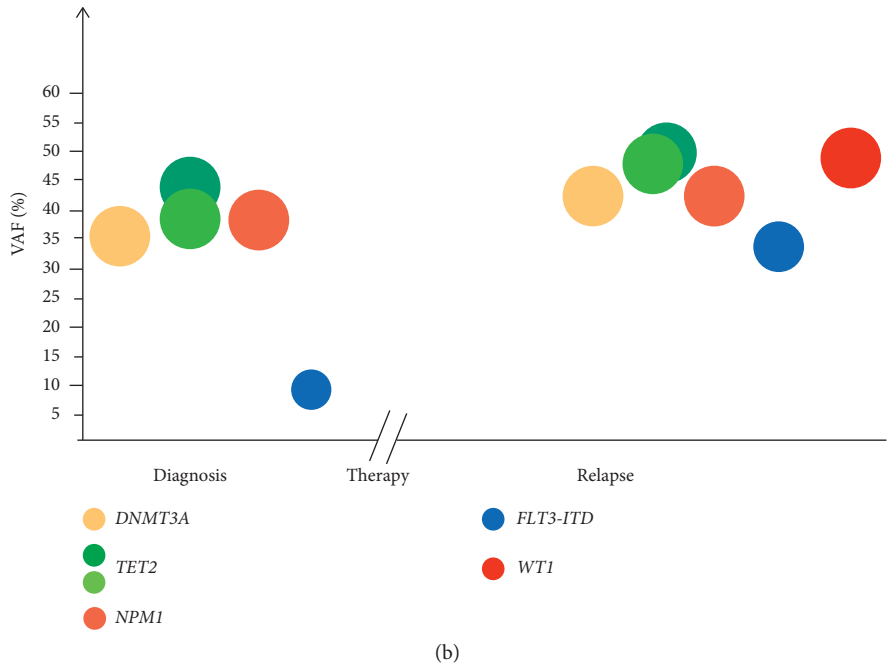
Pt	Gene	Locus	NM_ID	Exon	Type	Coding	Amino acid change	VAF (%)	Variant effect
AML#1Dx	*DNMT3A*	chr2:25462022	NM_022552.4	20	SNV	c.2385G > A	p.Trp795Ter	4.45	Nonsense
	TET2	chr4:106164773	NM_001127208.2	6	SNV	c.3641G > A	p.Arg1214Gln	3.20	Missense
	IDH2	chr15:90631837	NM_002168.3	4	SNV	c.515G > A	p.Arg172Lys	6.06	Missense
	BCOR	ChrX:39931683	NM_001123385.1	4	INDEL	c.2915_2916insA	p.Tyr972Ter	4.76	Nonsense
AML#1F-UP	*DNMT3A*	chr2:25462022	NM_022552.4	20	SNV	c.2385G > A	p.Trp795Ter	38.20	Nonsense
	IDH2	chr15:90631837	NM_002168.3	4	SNV	c.515G > A	p.Arg172Lys	39.80	Missense
	TP53	chr17:7578242	NM_000546.5	6	SNV	c.607G > A	p.Val203Met	5.25	Missense
	BCOR	chrX:39931683	NM_001123385.1	4	INDEL	c.2915_2916insA	p.Tyr972Ter	42.08	Nonsense

Pt: patient; Dx: diagnosis; F-UP: follow-up; SNV: single-nucleotide variant; INDEL: insertion/deletion; ins: insertion.



(a)

Figure 2: Continued.



(b)

Figure 2: Sanger sequencing of the insertion in the *DNMT3A* gene identified in AML#2 and analysis of clonal evolution based on NGS data. (a) AML#2 showed a novel insertion of 35 nucleotides in the exon 22 of the *DNMT3A* gene (c.2586_2587ins35: TCATGAATGAG AAAGAGGACATCTTATGGTGCAC; p Thr862_Glu863fsins). (b) Representation of clonal evolution of AML#2 from diagnosis to relapse (that occurred after 7 months of complete remission achieved with FLAI-5 chemotherapy regimen).

intermediate cytogenetic risk according to the ELN 2017 classification [24]. Molecular analysis also detected mutations of *NPM1* (c.863_864insCTTG; p.Trp288fs; VAF 37.4%) and *TET2* (c.395delA; p.Asn132fs; VAF 38.6% and c.5504delG; p.Gly1835fs; VAF 43.6%, Figure 2(b) and Table 2). The patient started fludarabine, arabinosyl cytosine, and idarubicin (FLAI-5) induction regimen [30, 31] and obtained complete hematological remission one month later, with undetectable *FLT3* mutations. After 7 months of complete remission, the patient relapsed. Mutational analysis performed on the diagnosis and relapse samples showed that the *DNMT3A* mutation loads were, respectively, 35.8% and 42.5%. The relapse sample presented an increase of 5.5% for VAF of *NPM1* mutation, along with an expanded *FLT3*-ITD clone (11.7% to 40.1%), the persistence of *TET2* mutations with a slight VAF increase (c.395delA; p.Asn132fs; VAF 48.4% and c.5504delG; p.Gly1835fs; VAF 49.7%), acquisition of a *WT1* mutation (c.1109G > C; p.Arg370Pro; VAF: 48.3%; Figure 2(b) and Table 2). In this patient, the treatment was not able to eradicate the leukemic clone harbouring *TET2*, *DNMT3A*, and *NPM1* mutations, which seems to have acquired an additional *WT1* mutation at relapse. Moreover, AML relapse was characterized by the expansion of the *FLT3*-ITD clone that was reduced but not eradicated by the treatment, with an increase of allelic ratio from 0.09 (diagnosis) to 0.21 (relapse). A recent study showed that the persistence of *DNMT3A* mutation at a VAF ≥2% at first remission is a common event in AML, occurring in 65% of cases with mutation at diagnosis and is associated with older age and inferior relapse-free survival [32].

3.3. The Identified DNMT3A Mutations Alter DNA CpG Islands Methylation and Protein Stability. To investigate the functional consequences of the identified mutations, we performed methylation analysis of CpG islands on DNA extracted from primary leukemic samples of both patients. Our cases showed DNA hypomethylation in comparison with primary AML samples with wt-*DNMT3A* gene or R882H mutation (VAF 37.0%) (Figure 3(a)). In the sample carrying Trp795* mutation, CpG islands methylation shifted from 47.1% at diagnosis to 24.4% at follow-up ($p = 0.020$), in accordance with the increase in the mutations allelic burden (from 4.5% at diagnosis to 38.2% at follow-up). At follow-up, CpG methylathion was significantly reduced compared with the wt-*DNMT3A* sample ($p = 0.017$). However, the observed hypomethylation status may be associated both with hypomethylating agent treatment and with the expansion of the *DNMT3A*-mutated clone during treatment.

In the sample carrying the insertion at Thr862_Glu863, CpG island methylation at diagnosis was significantly reduced compared with the wt-*DNMT3A* sample ($p = 0.047$). Moreover, its levels were similar to those observed in the R882H sample. No major changes occurred between disease diagnosis and relapse in terms of CpG methylation level and *DNMT3A* (c.2586_2587ins35; p. Thr862_Glu863fsins) VAF. The results suggest that the identified mutations induce DNMT3A loss of function, similarly to the R882H alteration. It has been recently shown that hypomethylation is an initiating feature of AML with $DNMT3A^{R882}$ [33] and that demethylator phenotypes, which are partially related to *DNMT3A* mutational status, have a prognostic role, independent of age and cytogenetic abnormalities [34]. Future

Table 2: Mutational profile of AML#2 at diagnosis and relapse.

Pt	Gene	Locus	NM_ID	Exon	Type	Coding	Amino acid change	VAF (%)	Variant effect
AML#2Dx	DNMT3A	chr2: 25458586	NM_022552.4	22	INDEL	c.2586_2587ins*	p.Glu863Ser	35.79	Nonsense
	TET2	chr4: 106155491	NM_001127208.2	3	INDEL	c.395delA	p.Asn132fs	38.55	fs del
	TET2	chr4: 106197168	NM_001127208.2	11	INDEL	c.5504delG	p.Gly1835fs	43.60	fs del
	NPM1	chr5: 170837545	NM_002520.6	11	INDEL	c.863_864insCTTG	p.Trp288fs	37.41	fs ins
	FLT3	chr13: 28608308	NM_004119.2	14	INDEL	c.1747_1748ins**	p.Gly583_Ser584ins***	11.70	Nonfs ins
AML#2R	DNMT3A	chr2: 25458586	NM_022552.4	22	INDEL	c.2586_2587ins*	p.Glu863Ser	42.45	Nonsense
	TET2	chr4: 106155491	NM_001127208.2	3	INDEL	c.395delA	p.Asn132fs	48.34	fs del
	TET2	chr4: 106197168	NM_001127208.2	11	INDEL	c.5504delG	p.Gly1835fs	49.65	fs del
	NPM1	chr5: 170837545	NM_002520.6	11	INDEL	c.863_864insCTTG	p.Trp288fs	42.87	fs ins
	WT1	chr11: 32417943	NM_024426.4	7	SNV	c.1109G > C	p.Arg370Pro	48.25	Missense
	FLT3	chr13: 28608308	NM_004119.2	14	INDEL	c.1747_1748ins**	p.Gly583_Ser584ins***	40.10	Nonfs ins

Pt: patient; Dx: diagnosis; R: relapse; SNV: single-nucleotide variant; INDEL: insertion/deletion; ins: insertion; fs: frameshift; del: deletion; *insertion of 35 nucleotides: TCATGAATGAGAAAGAGGACATCTTATGGTGCAC; **insertion of 57 nucelotides: GCTCCTCAGATAATGAGTACTTCTACGTTGA-TTTCAGAGAATATGAATATGATCCA; ***SerSerAspAsnGluTyrPheTyrValAspPheArgGluTyrGluTyrAspProSer.

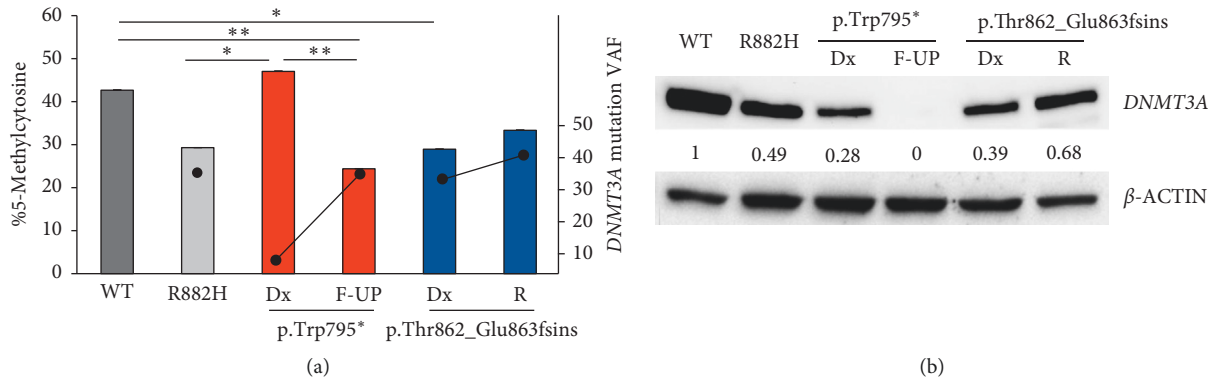


Figure 3: Effect of *DNMT3A* mutations on protein function and expression. (a) CpG islands methylation analysis. The bars represent mean ± standard deviation (SD) of the absolute 5-mC level (percentage of 5-mC left axis; SD values are barely detectable due to low values). Dots represent the *DNMT3A* VAF in the analyzed samples (right axis). A BM sample from an AML patient harbouring the *DNMT3A* R882H mutation (VAF 37.0%) was used for comparison (wt: wildtype; R882H: canonical mutation; Dx: diagnosis; F-UP: follow-up; R: relapse). (b) Western blot analyses of DNMT3A expression in representative AML cases with wt-*DNMT3A* and R882H mutation, followed by diagnosis and follow-up of AML#1, diagnosis and relapse of AML#2 (wt: wildtype; Dx: diagnosis; F-UP: follow-up; R: relapse). β-actin was used for loading normalization and quantification. Numbers represent DNMT3A levels normalized on the wt sample.

analyses of the aberrant DNA methylation pattern may help define specificities compared with $DNMT3A^{R882}$ AML and novel potential silenced or activated enhancers involved in leukemogenesis [35].

To understand whether alterations in DNMT3A protein expression were responsible for the observed changes in CpG methylation, we performed western blot on BM samples at different time points. Protein analysis revealed that despite the low *DNMT3A* mutation burden in the diagnosis sample of the case AML#1 (Trp795*), DNMT3A protein levels were reduced to 72% and 21% compared with wt-*DNMT3A* and R882H samples, respectively (Figure 3(b)). Furthermore, the increased VAF of truncated *DNMT3A* was associated with no detectable protein in the follow-up sample (Figure 3(b)). Despite the heterozygous mutational status, the wt protein isoform became undetectable. We speculated that the wt protein was unable to form a heterodimer with the truncated DNMT3A form,

which may also be unstable, thus causing a premature degradation. This hypothesis is supported by a recent study, which demonstrates that *DNMT3A* truncation mutations have a dominant negative effect with loss of function and haploinsufficiency in AML [12]. On the contrary, DNMT3A protein was detectable in the analyzed samples from AML#2 (p.Thr862_Glu863fsins). In this patient, we observed that DNMT3A levels were reduced in the diagnosis sample compared with the wt one and were similar to those obtained in the R882H-mutated case (Figure 3(b)). DNMT3A expression slightly increased in the relapse sample, showing a 30% reduction in protein level compared with the wt sample. The data suggest that the mutation, which is predicted to introduce a premature STOP codon, interferes with protein expression but does not alter the stability of the wt protein. Future investigation will be useful to demonstrate if the presence of a portion of the catalytic domain in this truncated protein allows it to bind to wt-DNMT3A. Taken together, the protein and methylation analyses indicate that this mutation results in decreased DNMT3A expression and function.

4. Conclusions

DNMT3A mutations are becoming highly relevant in hematological malignancies, thanks to NGS technologies which allow us to better characterize myeloid disorders. In this study, we presented two mutations of the *DNMT3A* gene never described in AML and investigated their consequences on protein expression and function. Both mutations localized in the catalytic domain of the DNMT3A protein and were predicted to cause loss of function. The first one is responsible for a truncated, nondetectable protein associated with hypomethylation of CpG islands, which expanded under the pressure of hypomethylating agent treatment. The second mutation was an insertion of 35 nucleotides, with a hypomethylation pattern, suggesting a negative effect on CpG methylation mediated by mutant DNMT3A. The *DNMT3A*-mutated clone escaped therapy selection and likely acquired a *WT1* mutation. Both patients showed evidence of clonal evolution, resulting from the selective pressure induced by treatment. Our strategy, based on bulk sequencing, allows us to draw a picture of AML-related mutations, along with their frequency and to speculate on mutation co-occurrence at disease diagnosis and progression. However, a single-cell sequencing approach is needed to precisely evaluate the clonal complexity of AML across the different disease stages, drive conclusions on the clonal and subclonal architecture, and uncover genomic trajectories of leukemia evolution [36–39]. The presence of cooperating mutations and their contribution to the leukemic phenotype along with the identified *DNMT3A* mutations deserve further investigation. Our data highlight the need to characterize and monitor patient-specific genomic alterations in AML, in order to tailor treatments and allow early detection of expanding subclonal population. Moreover, future studies are needed to define the hypomethylation pattern resulting from the described genomic lesions and its cooperation with differentiation stage-specific histone modification in the regulation of the leukemogenic transcriptional program.

Data Availability

The clinical and molecular data used to support the findings of this study are included within the article.

Conflicts of Interest

Giovanni Martinelli receives compensation as a consultant for ARIAD/INCYTE, Pfizer, Celgene, Amgen, J&J, and Roche. The other authors declare no conflicts of interest.

Authors' Contributions

Samantha Bruno and Maria Teresa Bochicchio equally contributed.

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Review Article

The Role of MicroRNA in Paediatric Acute Lymphoblastic Leukaemia: Challenges for Diagnosis and Therapy

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Acute lymphoblastic leukaemia (ALL) is the most common cancer of childhood. Although the overall survival of children with ALL is now more than 90%, leukaemia remains one of the leading causes of death from disease. In developed countries, the overall survival of patients with ALL has increased to more than 80%; however, those children cured from ALL still show a significant risk of short- and long-term complications as a consequence of their treatment. Accordingly, there is a need not only to develop new methods of diagnosis and prognosis but also to provide patients with less toxic therapies. MicroRNAs (miRNAs) are small ribonucleic acids (RNA), usually without coding potential, that regulate gene expression by directing their target messenger RNAs (mRNAs) for degradation or translational suppression. In paediatric ALL, several miRNAs have been observed to be overexpressed or underexpressed in patient cohorts compared to healthy individuals, while numerous studies have identified specific miRNAs that can be used as biomarkers to diagnose ALL, classify it into subgroups, and predict prognosis. Likewise, a variety of miRNAs identify as candidate targets for treatment, although there are numerous obstacles to overcome before their clinical use in patients. Here, we summarise the roles played by different miRNAs in childhood leukaemia, focussing primarily on their use as diagnostic tools and potential therapeutic targets, as well as a role in predicting treatment outcome. Finally, we discuss the potential roles of miRNA in immunotherapy and the novel contributions made by gut miRNAs to regulation of the host microbiome.

1. Introduction

Leukaemia, the most common paediatric cancer, accounts for approximately one-third of malignancies diagnosed in children below 16 years of age [1]. With a peak incidence between 2 and 5 years of age, acute lymphoblastic leukaemia (ALL) accounts for 85% of leukaemia in childhood [2]. Through treatment stratification by immunophenotype and genotype, the incorporation of more effective drug combinations into treatment protocols, and improved supportive care, overall survival of patients with ALL has increased to more than 80% in developed countries [3–5].

Despite the dramatically improved survival rates achieved with most treatment protocols, leukaemia in children remains one of the leading causes of death from disease. While the diagnostic classifications allowing for personalized treatment and prognostic evaluation have greatly improved, novel biomarkers for the diagnosis and classification of leukaemia are still required [6]. In addition, short-term and long-term complications arising from treatment toxicity remain a significant risk for individuals "cured" from their disease [7]. Hence, there is also a need for the development of new therapies to effectively treat children with high-risk disease, as well as a better optimization of current treatment strategies for low-risk disease.

2. MicroRNA Biology and the Role in Cancer

MicroRNAs (miRNAs) in animals are single-stranded noncoding RNAs with a length of 19 to 25 nucleotides (nt), usually formed from hairpin-shaped precursors. The canonical biogenesis of miRNAs (extensively reviewed in

[8, 9]) involves a complex process that converts the primary miRNA transcript (pri-miRNA, often up to 1000 nt in length) into an active mature miRNA. Briefly, the pri-miRNA transcript is processed by the Class 2 ribonuclease III enzyme, DROSHA, into a precursor miRNA (pre-miRNA, 60–120 nt) that bears a hairpin structure with a 2-nt overhang at the 3′ end. The overhang is recognised by EXPORTIN 5 (XPO5) and the pre-miRNAs are exported to the cytoplasm where they are processed by the endoribonuclease DICER into the mature miRNA duplex (19–25 nt). The 3′ or 5′ of the miRNA duplex is then loaded into the Argonaute family of proteins to form the miRNA-induced silencing complex (RISC) (Figure 1). Multiple non-canonical pathways also exist that make use of different combinations of DROSHA, EXPORTIN 5, and DICER [8]. For example, short-hairpin RNAs can be processed by DROSHA into miRNA through a DICER-independent pathway and "mitrons" can be produced from the spliced-out introns of RNA coding genes through a DROSHA-independent process. However, originally described as not having coding potential, more recent evidence has identified a subset of pri-miRNA that can indeed encode small peptides [10]. Such pri-miRNAs contain small open reading frames of around 300 nt that may be transported, unprocessed, into the cytosol where they become translated into micropeptides that can influence a variety of biological processes. Functional studies of micropeptides in humans are now beginning to show a significant association with disease and may possibly also present novel therapeutic opportunities [10]. miRNAs function by regulating the expression of genes usually through direct seed binding to the 3′ untranslated regions of their target messenger RNAs (mRNAs) and downregulate gene expression by acceleration of mRNA degradation (Figure 1). An alternative binding mechanism used by miRNAs involves particularly weak base pairing in the centre of the target pairing as a consequence of mismatched bulges in the miRNA sequence; this in turn leads to inhibition of translation [8]. miRNA binding sites have also occasionally been identified in the 5′ untranslated regions and open reading frames of mRNA, although these models are still under investigation [11]. Hence, miRNA are an important class of gene regulator estimated to be involved in the regulation of ~30% of all genes and can regulate almost every genetic pathway. Consequently, dysregulation of miRNA expression leads to the dysregulation of downstream mRNA targets and may be accompanied by severe implications to the homeostasis of cells and tissues [12].

Similar to traditional protein-coding genes, miRNA genes can be altered by a variety of aberrations including chromosomal amplifications, transcriptional activation, deletion, methylation, and sequence variation. Alterations of miRNAs have been identified in numerous diseases such as cardiac disorders, autoimmune diseases, and cancers; in the latter, they are highly dysregulated and can act as either oncogenes (oncomiRs) and/or tumour suppressors. For example, acting as an oncogene, overexpression of miR-21 in mice accelerates development of a precursor B (pre-B) cell malignant lymphoid-like phenotype; however, tumours regress completely within a few days when miR-21 is inactivated *in vivo* [13]. The role of miRNA as a tumour suppressor is illustrated by the miR-15a/16-1 cluster, which is frequently deleted in chronic lymphocytic leukaemia (CLL) [14]. In a knockout mouse model, induced deletion of the cluster results in the development of indolent B-cell-autonomous, clonal lymphoproliferative disorders that essentially reiterate the spectrum of CLL-associated phenotypes observed in humans [14]. Furthermore, the deletion of miR-15a/16-1 accelerates the proliferation of mouse and human B-cells by modulating the genes that control cell-cycle progression [14]. Examples of miRNAs acting as an oncogene in one cell type and as a tumour suppressor in another have also been reported; miR-221 is overexpressed in liver cancers where it targets the tumour suppressor *PTEN*, thereby promoting liver tumourigenicity [15]. However, in other tumour types, for example, gastrointestinal stromal tumours, miR-221 is downregulated and the consequent downregulation of *c-KIT* and *ETV1* (its target oncogenes) results in the promotion of this malignancy [16].

2.1. miRNAs as General Biomarkers of Cancer. A feature of an ideal biomarker is that it must present a unique expression profile in the patient compared to healthy individuals. Biomarkers must be reliable not only for early diagnosis, preferably before the development of full clinical symptoms, but also for the detection of persistence of minimal residual disease (MRD) and the recurrence of disease after treatment. They must additionally have a long half-life in clinical samples, be accessible through non-invasive methods, and be detected through simple, accurate, and inexpensive methodology. Therefore, miRNAs demonstrate tremendous potential as biomarkers for the early diagnosis of malignancy (and its prognosis), since they can be reliably detected in and extracted from blood (total, plasma, or serum) [17]. Circulating miRNAs are packaged inside microvesicles such as exosomes [6] and their profile in patients usually reflects the pattern observed in their respective tumour tissues, making them an attractive possibility as minimally invasive and robust biomarkers [18–20]. The first reported blood-based circulating miRNA was miR-21, which showed aberrant expression levels in the serum of diffuse large B-cell lymphoma patients [6].

2.2. miRNA in the Diagnosis and Classification of Paediatric ALL. ALL and acute myeloid leukaemia (AML) can be distinguished by a variety of morphological, immunophenotypic, and immunohistochemical methods; nonetheless, few single tests are currently sufficient for the establishment of diagnosis. The current classification of ALL is based on cell morphology, immunophenotypic characteristics, and cytochemical, cytogenetic, and molecular features, and can be divided into two distinct subgroups: B-cell precursor ALL (BCP-ALL) and T-cell ALL (T-ALL). BCP-ALL can be further subclassified according to recurrent genetic abnormalities [21, 22] and Figure 2.

To address this issue, Mi and colleagues [23] performed large-scale genome-wide miRNA expression profiling and identified 27 miRNAs that are differentially expressed

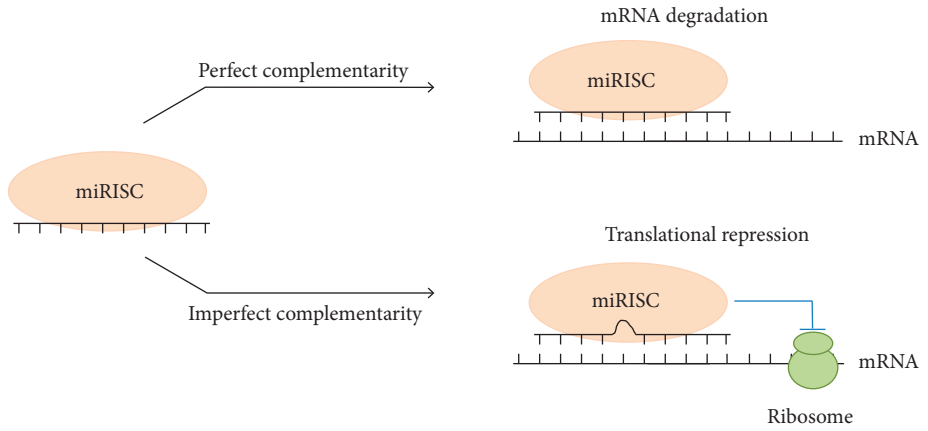

Figure 1: The functional mechanism of miRNA in posttranscriptional regulation of gene expression. miRNAs function to regulate the expression of genes by binding to the 3′ untranslated regions of their target messenger RNAs (mRNAs). miRNAs downregulate expression through acceleration of the degradation of mRNA (perfect complementarity) or by inhibition of its translation (imperfect complementarity).

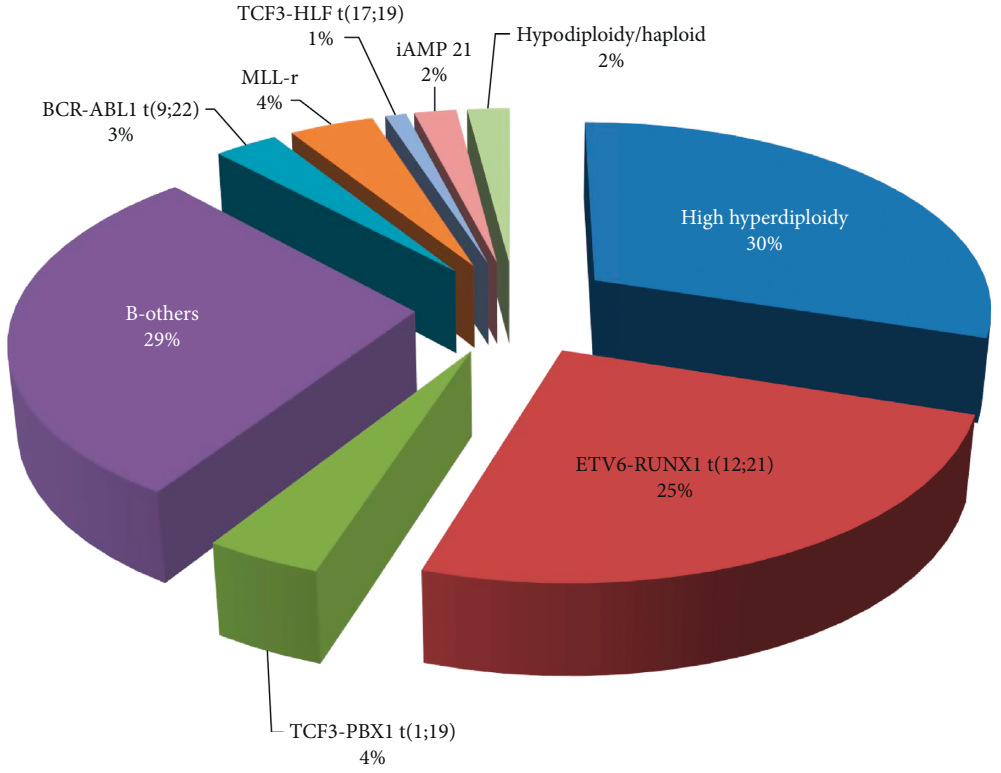

Figure 2: Major recurrent chromosome aberrations of childhood BCP-ALL. Hyperdiploidy and the *ETV6-RUNX1* translocation constitute the major subgroups of BCP-ALL and are associated with favourable clinical outcome. The t(1;19) and "B-Others" subgroups are associated with intermediate risk and the remaining subgroups are deemed poor risk.

between ALL and AML. Of these, miR-128a and miR-128b were significantly overexpressed in ALL compared to AML, whereas lethal (let)-7b and miR-223 were significantly downregulated. Their results show that ALL can be discriminated from AML with an accuracy of greater than 95% based on the differential expression pattern of these four miRNAs [23].

Certain miRNA profiles have also been shown to associate specifically with paediatric ALL [24] and reveal distinct expression profiles based on the cell of origin and cytogenetic subtype. BCP-ALL was distinguishable from normal bone marrow (BM) and CD34+ cells by presenting a significantly lower expression of miR-127 and miR-143. Similarly, T-ALL cells differed extensively from normal thymocytes with differential expression of 28 miRNAs. In high hyperdiploid ALL, high expression of a variety of miRNA was also observed [24]. Interestingly, except for *BCR-ABL1*-positive and "B-other" ALL, all the subtypes

display a unique miRNA signature that distinguishes each group from all the other subtypes. A top-ten discriminative miRNA set was proposed for each subgroup as shown in Figure 3 [25].

Significantly different levels of expression of miR-100 and miR99a have been observed in childhood ALL, with very low levels of expression seen in patients with ALL compared to those with AML or in healthy BM donors [26]. Conversely, an association between increased miR-100 levels and the presence of t(12;21)-positive ALL suggested the possibility of a t(12;21)-specific regulation of miR-100 [27]. Expression levels of two additional tumour suppressor miRNAs, miR-326 and miR-200c, appear to be significantly downregulated in BM mononuclear cells of paediatric ALL patients at diagnosis. Moreover, area under the curve (AUC) in receiver operating characteristic curve analysis (ROC) showed a high sensitivity and specificity for both miRNAs in discriminating between paediatric ALL patients and healthy individuals. These findings suggest that miR-326 and miR-200c may be involved specifically in leukaemogenesis and serve as potential reliable noninvasive diagnostic biomarkers of paediatric ALL [28].

RT-qPCR analysis of expression levels of miR-203 and miR-125b in peripheral blood (PB) isolated from 43 newly diagnosed children with ALL showed the median level of miR-125b expression to be 33-fold higher in the ALL cases than in the healthy control group. On the contrary, the median expression level of miR-203 was 31-fold higher in the control group when compared to the ALL cases. While the sensitivity of miRNA-203 was higher than that of miRNA-125b, their combination revealed absolute sensitivity, suggesting that preclinical studies targeting miRNAs for diagnosis of ALL should be strongly encouraged [29].

Disruption of the function of miR-181a, a regulator of normal haematopoiesis, has been associated with many cancer types. Depending on the cellular context and on the consequent expression of its targets, miR-181a can act as either a tumour suppressor or an oncogene. Nabhan and colleagues [30] investigated the expression levels of miR-181a in 30 newly diagnosed paediatric ALL patients, comparing these results with 30 sex-matched normal healthy children as the control group [30]. Serum samples of the ALL group showed a highly significant decrease in expression levels of miR-181a compared to those found in the healthy children. *ETV6-RUNX1*, the fusion product of t(12; 21), is the most common genetic abnormality observed in paediatric ALL and especially BCP-ALL [31, 32]. Consistent with a study that confirmed that miR-181a is able to effectively target the fusion, decrease its protein level, and induce a significant antileukaemic effect, it was noted that miR-181a plays a tumour suppressor role in ALL [30, 33]. Furthermore, miR-181a was identified as the most differentially downregulated miRNA in the PB of paediatric ALL patients that express ETV6-RUNX1 [34].

Additional investigations were performed to evaluate the expression levels of the nuclear protein "mothers against decapentaplegic homolog 7" (SMAD7), a miR-181a target pair, and transforming growth factor-b1 protein (TGF-β1), the response of which is negatively regulated by SMAD6 and SMAD7 [30]. Results showed that SMAD7 protein levels were significantly higher in ALL patients compared to the healthy control group, whereas TGF-β1 protein was significantly lower. Of note, deregulation of TGF-β1 signalling through SMAD7 overexpression has been previously associated with the pathogenesis of ALL [35]. With the combined use of miR-181a and SMAD7, the sensitivity of diagnosis was increased to 90%, whereas the combined use of miR-181a and TGF-β1 increased sensitivity to 100%. These data suggest that the diagnostic accuracy of paediatric ALL can be improved by using a small combination of biomarkers [30].

2.3. miRNA in the Prognosis of Paediatric ALL. Paediatric ALL is currently stratified according to different biological and clinical parameters with patients subsequently receiving risk-adapted therapy. Albeit current strategies yield a very high cure rate, there are a number of patients with ALL who will ultimately relapse. The classical use of both gene expression profiling and mRNA signatures in the clinical setting has disadvantages and only intermediate success has been reported. Since miRNA profiling is usually limited to a small number of genes, it can potentially offer fewer and more robust signatures that have an equally strong prognostic capacity [25].

Expression levels of miR-16, a gene lost in many cases of CLL, were assessed in 93 children with ALL and results associated to the main biological and clinical variables that define prognosis: white blood cell (WBC) count, age at diagnosis, cyto- and molecular-genetic profile, and risk groups. While the highest expression values of miR-16 associated with a poor prognosis, low levels associated with a good outcome [36]. The disease-free survival (DFS) and overall survival (OS) were evaluated according to miR-16 expression profiles in the ALL group as a whole and in the B-cell and T-cell ALL subgroups. Group analysis showed that DFS was longest for patients with miR-16 expression less than quartile 25 and the shortest for those patients with miR-16 expression above quartile 75. Relapse was rarely observed in the patients with low miR-16 levels, while the shortest DFS was in the quartile above 75. Similarly, in the T-ALL group, patients in the quartile above 75 presented the shortest survival, whilst the longest survival was observed in the quartile below 25. Statistically significant relationships were also obtained between low miR-16 levels and low WBC and good cyto- and molecular-genetic markers [36].

In addition, a multivariate analysis of miR-24 expression, including age at diagnosis, gender, and WBC, defined miR-24 as an independent prognostic marker. Upregulation of miR-24 was associated with a significant shorter OS, compared to those cases with low expression [37]. These results strongly suggest that high miR-24 expression levels could be used as a reliable and effective biomarker of poor prognosis ALL (Figure 4).

The changes in miRNA expression in relapsed ALL are the focus of several research groups and suggest that miR-223 and miR-128b levels could be possible predictors of ALL relapse. Nemes and colleagues [38] collected PB and BM samples from 24 ALL patients from all phases of treatment.

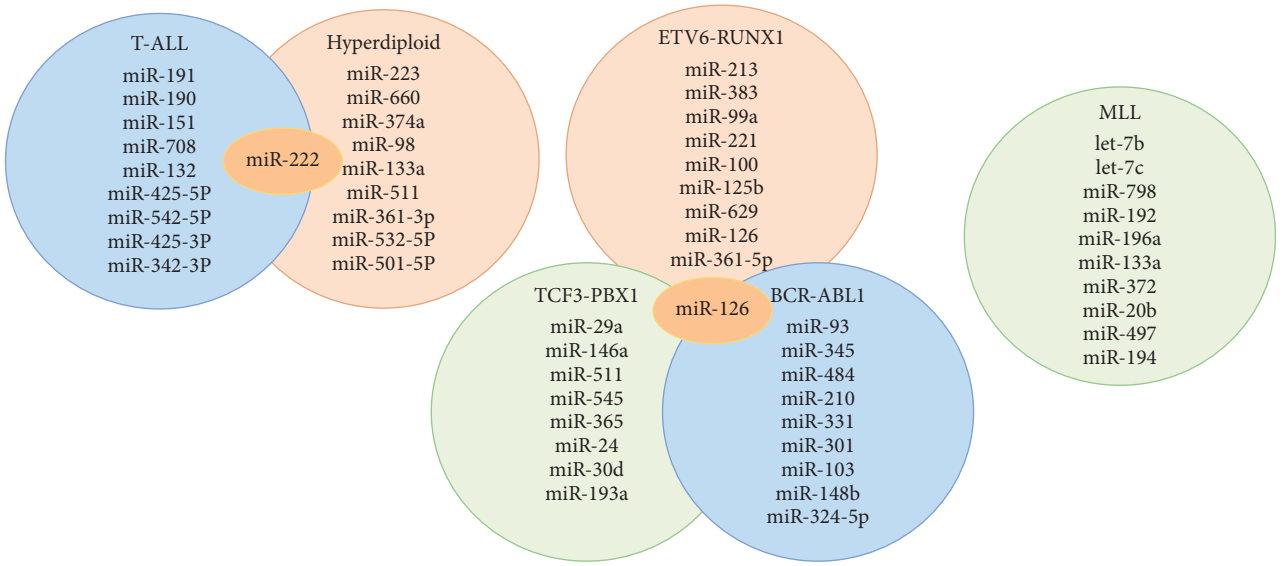

Figure 3: A top-ten discriminative miRNA set for the main leukaemia subgroups. The subtypes display a unique discriminating miRNA (except where overlap is shown) that distinguishes each subgroup from each other [25].

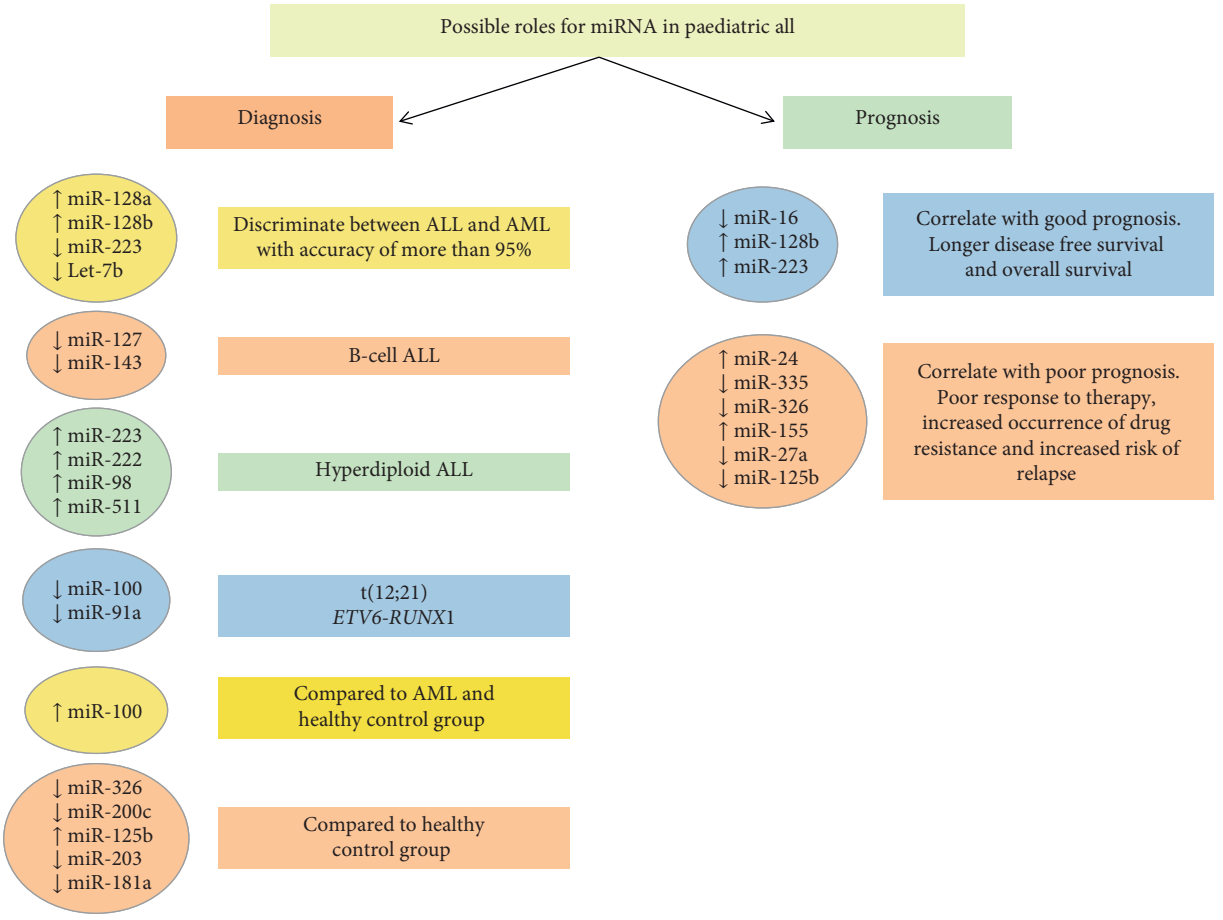

Figure 4: Potential applications of miRNA as a diagnostic and prognostic tool in paediatric ALL. The figure summarises those miRNAs that are either upregulated or downregulated in the various subsets of ALL and their potential use as a tool for its diagnosis and prognosis.

They determined that whilst miR-223 expression was almost undetectable at diagnosis, the levels of expression recovered to normal during treatment and in remission, but then decreased again at relapse. Conversely, an extreme over-expression of miR-128b was observed at diagnosis, which significantly decreased as patients entered remission where

normal levels of miR-128b expression for mononuclear cells were detected. Higher miR-128b expression levels at diagnosis also correlated with a better prognosis, suggesting that monitoring of miR-128b expression levels in PB could assist in the early detection of disease relapse (Figure 4) [38].

2.4. miRNA in Treatment, Treatment Response, and Toxicity of Paediatric ALL. Most children diagnosed with ALL follow a standard treatment protocol as part of a risk-adapted strategy that is typically divided into 3 main phases: induction, consolidation, and maintenance, and a fourth phase of CNS-directed therapy. Although in recent times the main cytotoxic agents used in the induction phase of treatment have not changed, they usually include combinations of drugs including prednisolone (PRED), vincristine (VCR), L-asparaginase (L-ASP), and daunorubicin (DNR). Multidrug regimens are used in the attempt to prevent drug resistance but all agents have undesirable late effects. Maintenance therapy, the final and most prolonged treatment in childhood ALL, involves a much less intensive regimen than the prior chemotherapy. Lasting up to two years, the maintenance phase has been demonstrated to lower the risk of relapse but may itself be a cause for the emergence of new (drug resistant) mutations.

2.5. miRNA and Multidrug Resistance. Multidrug resistance (MDR) is a ubiquitous and severe clinical problem in the treatment of paediatric ALL and is often mediated by adenosine triphosphate (ATP)-binding cassette (ABC) transporters. Various substrates, such as drug molecules, are actively transported by ABC transporters across extracellular and intracellular membranes, resulting in the restriction of drug deposition and chemotherapy failure [39]. A significant amount of evidence now shows that miRNAs tend to impact upon therapy response through modulation of expression levels of a variety of proteins, including ABC transporters [40]. Transfection of resistant HepG2 cells with miR-326 leads to enhanced sensitivity to chemotherapeutic drugs due to the downregulation of ABC transporter ABCC144. Furthermore, overexpression of ABCA2 and ABCA3 genes significantly increases the risk of MDR and relapse in children with ALL [41]. Bioinformatics analysis shows miR-326 to be a negative regulator of drug resistance-related genes, particularly ABCA2 and ABCA3 [27]. Evaluation of the expression levels of miR-326 in paediatric ALL patients resistant to chemotherapy revealed a significant decrease in miR-326 expression levels in MRD+ patients compared to the MRD- group, supporting the notion that downregulation of miR-326 has an adverse impact on chemotherapy treatment response [28]. The downregulation of miR-326 in children with relapsed ALL compared to the expression levels observed at first diagnosis, indicate that miR-326 can be considered another prognostic marker for relapse in paediatric ALL (Figure 4).

To identify novel prognostic and therapeutic predictors in ALL, a genome-wide miRNA expression study was performed on 63 newly diagnosed childhood ALL patients [42]. The miRNA profiles of the relapse/deceased cases were compared to those in complete remission (CR), identifying miR-335 as the most significantly downregulated miRNA. miR-335 gene promoter regions reveal significant methylation patterns in relapsed patients, suggesting that miR-335 is epigenetically silenced through DNA methylation. Epigenetic silencing of miRNA genes is associated with clinical outcome in infant ALL [43, 44]. Significantly poorer 5-year event-free survival was observed in patients with low expression levels of miR-335, and a multivariate analysis confirmed miR-335 levels at diagnosis to be an effective and independent prognostic marker in predicting treatment outcome [42].

miR-155 is believed to play a role in inhibition of lineage differentiation [45], holding haematopoietic stem and progenitor cells (HSPCs) at the early stem cell stage. El-Khazragy and colleagues [46] determined whether miR-155 expression levels correlate with clinic-pathological features of the disease; the effect of therapy on the expression levels of miR-155 was also evaluated by RT-qPCR. BM samples of 45 children with ALL were collected at diagnosis to determine miR-155 expression levels, and percentage of blasts post-induction was assessed on day 15 as a measure of MRD. Additionally, expression levels were determined after treatment in samples taken on day 28 and results compared with a control group of ten healthy controls. A significant high level of miR-155 expression was detected at diagnosis in the ALL group, and a significant correlation was observed between high miR-155 levels and high blast numbers (>25%), unfavourable cytogenetic abnormality, total WBC, higher relapse rate, and a higher MRD after 15 days. The overexpression of miR-155 therefore correlates with poor prognosis in paediatric ALL, due to a decrease in the response to therapy and an increase in relapse. Furthermore, miR-155 expression levels were significantly decreased 10-fold after therapy, again suggesting potential use as a biomarker of therapeutic response in childhood ALL (Figure 4) [46].

To further determine the miRNA expression profiles and miRNA patterns associated with childhood ALL relapse, a genome-wide miRNA microarray analysis was performed on paired diagnostic-relapse and diagnostic-CR series of paediatric ALL cases [47]. A differential miRNA pattern was identified between relapse and CR patients that included miR-223 and miR-27a: both miRNAs were highly expressed in patients during CR. Furthermore, the expression levels of both miR-223 and miR-27a were low in diagnostic samples of patients who subsequently relapsed during the study. miR-223 expression at initial diagnosis is another independent and reliable predictor for OS in paediatric ALL patients [47].

Piatopoulou and colleagues [48] examined miRNA profiles at diagnosis and after induction, comparing those levels with known prognostic features of ALL. Lower miR-125b expression levels at diagnosis and higher levels at the end of the induction protocol were associated with adverse disease features. The stronger risk for short-term relapse and a worse OS were clearly demonstrated by survival analysis of patients who presented with underexpressed miR-125b at diagnosis and overexpression after induction [48]. Albeit

miR-125b presented with a wide variability of expression at diagnosis, lower expression levels correlated with unfavourable prognostic features including high-risk age group, high BM blast count at diagnosis and day 15 after induction, as well as patients with a low haemoglobin concentration. Downregulation of miR-125b is also significantly associated with higher risk of relapse and a poor OS, providing convincing evidence that miR-125b could also be used as a potential prognostic biomarker in paediatric ALL [49].

With the development of miRNA inhibitors and mimics, along with antagomiRs and agomiRs, and the possible reexpression of miRNAs by delivery of their precursors, it is now becoming feasible to restore miRNA levels to normal, at least *in vitro*. A very attractive and possible future treatment strategy will therefore be to combine miRNA-based therapy with conventional chemotherapy. Gefen and colleagues [50] established that the expression of the miR-125b-2 cluster, consisting of miR-125b, miR-99a, and let-7c, is increased in *ETV6-RUNX1*+ leukaemia. Through manipulation of *ETV6-RUNX1* gene expression and chromatin immunoprecipitation, their results show that the miRNA-125b-2 cluster is not regulated by the ETV6-RUNX1 fusion protein itself, suggesting that expression of this cluster could be an independent leukaemia event. Knockdown studies of endogenous miR-125b in the *ETV6-RUNX1*+ cell line Reh increased cell sensitivity to doxorubicin and staurosporine treatment. Furthermore, overexpression of miR-125b-2 conferred a survival advantage through the inhibition of apoptosis and activation of caspase-3 [50]. These results suggest that miR-125b-2 cluster is another potential therapeutic target in paediatric ALL (Figure 5).

The *in vitro* sensitivity of cells isolated from patients with BCP-ALL following standard treatment with prednisolone (PRED), vincristine (VCR), L-asparaginase (L-ASP), or daunorubicin (DNR) has been studied for respective changes in miRNA profiles. While only one miRNA, miR-454, was linked to L-ASP resistance and none to prednisolone, resistance to VCR and DNR was characterized by a 20-fold upregulation of miR-125b, miR-99a, and miR-100. Moreover, 39% of the patients resistant to VCR were of *ETV6-RUNX1* subtype; the *ETV6-RUNX1*+ patients and the VCR-resistant cases both presented higher miR-125b expression levels. The overexpression of miR-125b reduced the amount of drug-induced apoptosis in pre-B-cells and induced the proliferation of CD34+ cells, suggesting that the interference of miR-125b function might provide a way to sensitize patients to these chemotherapeutic drugs [24]. The potential role of miR-125b in paediatric ALL treatment has now been studied *in vivo* by Bousquet and colleagues [51]. Twenty mice were transplanted with miR125b-overexpressing foetal liver cells and of these, half succumbed to a haematological malignancy within 12 to 29 weeks post transplantation. The phenotypes included both BCP-ALL and T-ALL, suggesting that miR-125b may play a role in the differentiation process of both lymphoid lineages. The authors conclude that miR-125b, as a driver mutation or secondary event, could also provide a promising therapeutic target [51]. Furthermore, miR-125b overexpression was shown to accelerate the oncogenicity of the BCR-ABL1 fusion protein, a hallmark of chronic myeloid leukaemia (CML) but one also observed in a subset of paediatric ALL [52]. To evaluate the clinical utility of miR-125b for patients treated on the Berlin-Frankfurt-Münster (BFM) protocol, BM samples of childhood ALL patients were obtained at diagnosis and day 33 and compared to healthy controls. Whilst miR-125b expression levels were downregulated at diagnosis, a significant overexpression (between 2- and 5-fold) was observed on day 33 [48]. To explore a role for miR-125b, miR-99a, and miR-100 in therapy resistance, Reh cells were treated with VCR for 3 days [53]. The baseline expression levels of these 3 miRNAs in Reh cells were in the range of levels observed in the leukaemic cells of children with *ETV6-RUNX1*+ ALL. Results showed that miR-125b, in combination with miR-99a or miR-100, induced resistance to VCR, whereas the same effect was not observed for each of the miRNAs individually. After combined overexpression of miR-125b/miR-100, miR-125b/miR-99a, or miR-125b/miR-99a/miR-100, the fold change in VCR resistance did not differ. The mature sequences of miR-99a and miR-100 differ only by one nucleotide and consequently have considerable overlap in the list of their predicted target genes. The rate of apoptosis and cell-cycle distribution in the absence of VCR did not vary between Reh cells expressing miR-125b together with miR-99a and/or miR-100, compared to those cells expressing miR-125b in combination with a scrambled miRNA control. These results suggest that for the development of VCR resistance, the combined overexpression of miR-125b, miR-100, and/or miR-99a may provide a specific trigger. Whether the target genes directly regulated by these miRNAs and subsequent effected pathways could provide a way in which VCR resistance in *ETV6-RUNX1*+ ALL could be modulated remains to be seen [53].

Glucocorticoids (GCs) are also widely used in the clinical treatment of paediatric ALL; the interindividual differences in GC therapy response, however, compromise their clinical application. Additionally, GC resistance is one of the major contributing factors of poor outcome in paediatric ALL. Recent studies have shown that miRNAs play a crucial role in GC sensitivity and may provide potential strategies to overcome GC resistance [54]. In a genome-wide miRNA analysis of paediatric ALL patients, a reduced miR-335 expression was identified as the most significant miRNA abnormality associated with poor outcome [42]. Overexpression of miR-335 significantly sensitized the ALL cells to PRED, across a range of concentrations, indicating that ectopic expression of miR-335 can confer an enhanced sensitivity. Since no enhancement of cell death was observed in cell lines overexpressing miR-335 when treated with other chemotherapy drugs, it was proposed that miR-335 downregulation could decrease the susceptibility of ALL cells to PRED treatment. To determine the functional role of miR-335 in PRED resistance, investigation of downstream pathways revealed that a low level of miR-335 results in higher MAPK1-mediated survival. Moreover, MEK/ERK inhibitor treatment enhanced PRED-induced cell death suggesting that reintroducing synthetic miR-335 and overriding MAPK1 activity and MEK/ERK pathway inhibition could provide a basis for the development of further

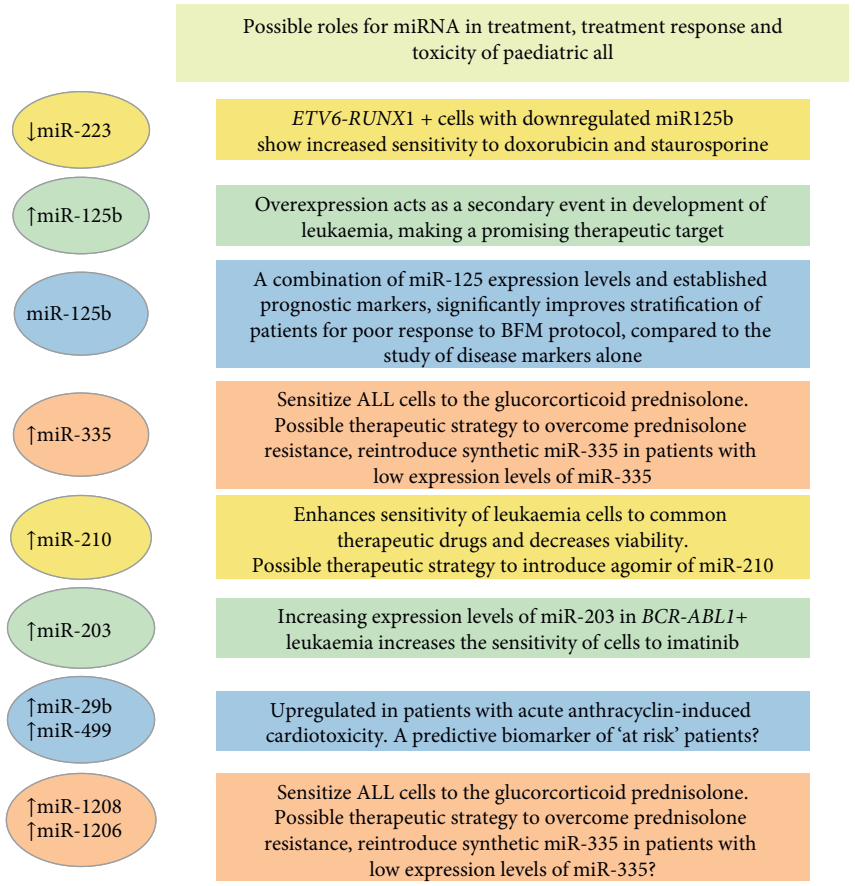

Figure 5: Potential applications of miRNAs as biomarkers of treatment response and toxicity in paediatric ALL. The figure summarises those miRNAs that are either upregulated or downregulated in the various subsets of ALL during therapy. Their potential use as a tool for treatment or marking treatment response is discussed in the text.

therapeutic strategies to overcome PRED resistance and so improve treatment outcome in ALL patients [42].

Clinical studies indicate that miR-210 is differentially expressed in several types of cancer. To establish its effect on treatment outcome in leukaemia, intracellular miR-210 levels were altered by transfecting two different paediatric ALL cell lines (*ETV6-RUNX1*+ Reh and *MLL-AF4*+ RS4;11) with an agomiR and antagomiR to miR-210 [55]. Twenty-four hours after transfection, both cell lines were treated for 48 hours individually with the four common therapeutic drugs (dexamethasone [DEX], VCR, DNR, and L-ASP) or in four drug combinations. The miR-210 agomiR produced an approximate 30-fold increase and antagomiR an 80% decrease, respectively, compared to the negative controls. In Reh cells, the half maximal inhibitory concentrations (IC50s) of DNR, DEX, and L-ASP were significantly decreased (agomiR) or increased (antagomiR) compared to those cells transfected with the negative control mimics. Similarly, in RS4, 11 cells, the IC50s of DNR, DEX, and VCR (but not L-ASP) were decreased and increased by the miR-210 agomiR and antagomiR, respectively. Based on these results, the use of agomiRs or antagomiRs of miRNAs could be a feasible alternative to overcome resistance to chemotherapy. It was, however, noted that safe and effective delivery of synthetic miRNAs to leukaemia cells still remains a great challenge [55]. As discussed later, it will also be paramount to study the off-target effects of using particular miRNA as a therapeutic target, either alone or with chemotherapy, since both treatments can regulate the expression of other critical miRNA.

Although quite rare in children, ALL patients that harbour the p190 variant of the *BCR-ABL1* (Ph+) fusion gene are treated with tyrosine-kinase inhibitors (TKI) such as imatinib, but the prognosis of these patients remains suboptimal. *ABL1* is a direct target of miR-203 and in malignancies expressing either *ABL1* alone or *BCR-ABL1*, miR-203 is silenced through genetic and epigenetic mechanisms. Restoration of the expression of miR-203 reduces levels of *ABL1* and *BCR-ABL1* and inhibits cell proliferation [56, 57]. To determine whether the inhibition of *BCR-ABL1* by miR-203 is sufficient to overcome resistance to TKI, cells expressing *BCR-ABL1*, as well as cells overexpressing both *BCR-ABL1* and miR-203 together, were treated with imatinib and cell viability determined. At all drug doses, the *BCR-ABL1*+ cells without miR-203 overexpression retained higher viability, suggesting that the sensitivity of *BCR-ABL1*+ cells to imatinib could be increased with the restoration of miR-203 [58].

2.6. miRNAs as Tools for Immunotherapy. In the main, studies of miRNA biology have focused on their role as

either oncogenes or tumour suppressors; however, recent advances in our understanding of the immune system suggest that miRNA also play an important role in immune regulation and can therefore act as both agents and targets of immunotherapy. Immune checkpoints play a significant role in cancer therapy; control of checkpoint receptors through miRNA may be through direct target of checkpoint genes or via other proteins that themselves are regulated by miRNA. Checkpoint inhibitors are able to block the proteins that prevent the immune system from attacking cancer cells. As discussed above, in the context of being able to restore normal miRNA levels, miRNA mimetics can be used to restore the expression of the various downregulated miRNAs that target immune checkpoint mRNAs and consequently can serve to reduce the levels of checkpoint proteins. A comprehensive role for miRNA in B-cell biology is rapidly being documented, but one important established mark is in the acceleration of bone marrow differentiation. Numerous miRNA appear expressed in a stage or disease-specific fashion in both early and late B-cells, suggesting functional and/or pathologic specificity. Koralov and colleagues [59] have shown that a deficiency in Dicer results in a significant defect in B-cell differentiation at the pro-B to pre-B-cell stage, and a number of miRNAs have been identified that are dynamically controlled during early B-cell lineage specification [60]. For example, the expression level of miR-155 is raised in murine BCP-ALL, suggesting that miR-155 can cause arrest and accumulation of pre-B-cells [61]. *Programmed cell death protein 1* (PD1) is a cell surface receptor checkpoint protein expressed on pro-B-cells (and T-cells) that down regulates the immune response [62]. The antitumor activity of PD-1 immune checkpoint blockade has been established in clinical trials [63] where solid tumour regression was observed. Targeting *PD-1*, or the genes of its ligands (*PD-L1* and *PD-L2*), has since identified a number of miRNAs (including miR-34a and miR-424) that can activate anticancer immunity, initiate apoptosis, and reverse chemoresistance by blocking the PD-1 immune checkpoint [64, 65]. In addition, miR-142-5p was found to down-regulate the expression of PD-L1 by directly binding to the 3′ UTR of *PD-L1* mRNA [66]. The let-7 family of miRNA is generally considered to be a good biomarker for the diagnosis and prognosis of ALL, but have also been shown to be involved in metabolic control and the normal immune response [67]. As discussed later, one exciting prospect for future treatment strategies is the knowledge that the gut microbiome in patients for whom PD-1 blockers work well differs considerably from those patients in whom they fail [68].

Chimeric antigen receptor-T (CAR-T) cell therapies have recently begun to show dramatic success in clinical trials that treat patients with BCP-ALL [69]. Analysis of the transcriptome profiles in patients with B-ALL before and after CAR-T therapy, in combination with miRNA-seq, revealed that many miRNAs (including let-7 family members) are involved in the immune process and could also regulate the crosstalk between the transcription factors and histone proteins involved in the response to CAR-T therapy [70]. For example, within an elegant regulatory network, the authors showed that FOS, JUN, and CEBP regulate the expression of histone genes *HIST1H4A* and *HIST2H4A*, which were targeted by miR-148a-3p. In addition, 15 upregulated and 7 downregulated miRNAs were also identified in patients in remission that may now become additional biomarkers of prognosis.

2.7. miRNA and Late Effects of Treatment. Anthracyclines such as doxorubicin are also used widely in the treatment of paediatric leukaemia. Anthracycline (AC)-related cardiotoxicity is one of the most significant long-term threats to survivors of cancer, with cardiac events being the most common cause of death in this population. It has been estimated that the prevalence of cardiomyopathy in 50-year-old survivors of childhood cancer exposed to cardiotoxic chemotherapy is 21% [71]. AC-induced cardiomyopathy is progressive, with no definitive treatment, and currently methods to detect cardiac injury early in the course of its progression are lacking. The specificity and sensitivity of detection that has been established by miRNAs in various cardiovascular disease states indicates a potential role for miRNAs in early detection of cardiotoxicity. To identify AC-induced alterations in the expression of plasma miRNA and to correlate these changes to known markers of cardiac injury, a prospective cohort study was performed on children receiving AC chemotherapy [71]. A key finding was that the overall dysregulation of a panel of plasma miRNAs (with cardiac relevance) was greater following AC therapy compared to the control group. Furthermore, plasma miR-29b and miR-499 were particularly upregulated in patients as demonstrated by increased troponin concentrations post-AC. The post-AC expression levels of miR-29b and miR-499 correlate significantly with cumulative AC dose, a known predictor of cardiotoxicity risk. Further studies are required to determine the mechanistic role of these miRNAs in AC-induced cardiac injury but miR-29b and miR-499 could be useful for identifying patients at high risk for developing AC-induced cardiomyopathy and subsequently in the early detection of cardiomyocyte injury [71].

Methotrexate (MTX), widely used in the consolidation phase of treatment for childhood ALL, is also considered to be a main cause of the hepatotoxicity observed in two-thirds of patients. Many genes involved in normal liver-specific signalling pathways are tightly controlled by miRNAs and shown to be open to modulation by variations in miRNA gene sequence. A significant association between high levels of transaminase toxicity during MTX treatment in the consolidation phase (but not induction phase) and the presence of a single nucleotide variation (rs264881) that effects stability in the pre-miRNA of miR-1208 has been identified [72]. As expected, targets of miR-1208 include genes of the MTX pharmacodynamic and pharmacokinetic pathways; among them, dihydrofolate reductase (*DHFR*) is a principal target of MTX. This finding suggests that a higher expression of miR-1208 could perhaps moderate the adverse toxic effects of MTX that arise through inhibition of DHFR [72]. Other similar miRNA-related SNPs have shown their worth as tools for the prediction of treatment-related

toxicity; for example, more than one study has confirmed the association between the rs2114358 variant in miR-1206 and MTX-induced oral mucositis, which occurs in 20% of MTX-treated paediatric ALL [73, 74]. The search continues to discover other beneficial miRNA with nucleotide sequence variations that may also become predictors of toxicity. To date, no miRNA drug candidates have entered into phase 3 clinical trials for the treatment of patients with ALL, and the long-term effects of such treatments remain to be evaluated. However, off-target effects of miRNA antagonists and mimics may trigger both neurotoxicity and immunotoxicity [75].

2.8. Future Perspectives: Gut miRNA. Implicated in host metabolism, immunity, and disease, the gut microbiota are a complex and diverse population of commensal bacteria, the composition of which in children is influenced by genetics, birth route, diet, and disease [76]. There is a significant interpersonal and interspecial variation between the gut microbiota of individuals, characterized by a balance and composition that is usually unique to and beneficial to the host. Disruption of this balance results in disease susceptibility; therefore, it is important to characterize the biological mechanisms through which the host can maintain the gut microbiota composition and to understand how this relationship is affected during pathological changes.

Recent studies have identified host gut or faecal miRNAs as a readily detectable and quantifiable normal component of human faeces [76]. Whilst the functional role of miRNAs in the communication between the host and microbes is only just beginning to be understood, recent findings indicate that miRNAs produced by the host intestinal epithelial cells can affect bacterial growth within the gut and hence participate in shaping the gut microbiota. Indeed, miRNA can enter bacteria and regulate bacterial gene expression and growth [76]. Furthermore, it has been shown that host miRNA expression levels can become dysregulated in the presence of a disturbed gut microbiota (for example, after chemotherapy or antibiotics) and that expression of host miRNA can in turn be influenced by the gut microbiota [77, 78]. A link between the gut microbiota and miRNA expression has been well studied in colorectal cancer (CRC) and confirms that certain miRNAs can mediate an interaction between the host and the microbiome. Novel mechanisms have also been implicated whereby miRNA may provide a possible target for therapeutic strategies in CRC patients [79]. Furthermore, numerous studies have investigated how faecal miRNAs can be exploited as potential noninvasive diagnostic and prognostic biomarkers. The faecal-based miR-20a and miR-221 are only two of the several faecal miRNAs that have so far been identified as promising diagnostic biomarkers of CRC [80, 81]. Adult survivors of childhood BCP-ALL often have health problems that continue for many years after the end of treatment, and chemotherapeutic agents and antibiotics clearly have an adverse effect on the gut and associated tissues. Reduced microbial diversity evidently exists in survivors of BCP-ALL and may be accompanied by an increase in biomarkers of inflammation (such as IL-6) and the activation of T-cells [82]. High levels of IL-6 are thought to play a role in expression of let-7a [83]. In accord with the "Greaves hypothesis" of an infectious origin for BCP-ALL [82], Bürgler and Nadal [84] suggest that memory T-helper (Th) cells may be attracted by preleukaemic B-cells and can then be activated through presentation of antigens. BCP-ALL cells can also respond to Th cell cytokines [84]. Correspondingly, miRNA have been shown to play a role in the regulation of cytokine genes and to be strongly regulated themselves during inflammation. Indeed, many downstream components of TGF-β signalling, eg, *SMAD* genes, contain miRNA binding sites and SMADS can induce positive or negative regulation of miRNA transcription [85]. SMAD3 has been shown to play a role in dysregulation of the TGF-β pathway in *ETV6-RUNX1*+ preleukaemia [86] and may contribute to both the persistence or maintenance of covert preleukaemic clones in healthy children and enhance their competitive positive selection in an inflammatory context. Determination of the "preleukaemic" gut miRNA profile in the faeces of healthy children with *ETV6-RUNX1* fusion remains an important goal.

There is now substantial evidence that the normal gut microbiome is acquired very early in life, particularly in the first year, and can be modified for up to three years, where it finally stabilises [87, 88]. The evidence that microbiome status is a key risk variable in ALL is indirect and based upon indirect measures made through epidemiological studies [89]. However, the important acquisition of the microbiome in early life involves the same features implicated in risk of BCP-ALL: birth route, breastfeeding, and social contacts. Microbial exposures earlier in life appear protective but, in their absence, later infections may trigger the critical secondary mutations and gene deletions necessary for overt leukaemia. Moreover, BCP-ALL has a worldwide incidence that tracks with socioeconomic development and there is now considerable evidence to indicate that children in developed countries have a gut microbiome that is lower in diversity from those living in more basic environments [89]. Currently, several groups are performing studies to determine the expression levels and functional role of faecal miRNA and gut bacteria in childhood leukaemia. Information obtained will provide insight into the pathogenesis of childhood ALL and intrinsically particular miRNA may be considered as tools for prevention of certain subtypes of ALL. It is currently unknown, in the context of ALL, which bacteria or viral species are most relevant to neonatal immune priming and which, if any, might have the capacity to prevent, or provide the infection trigger, for overt ALL to develop from a clinically silent preleukaemia. One way to assess whether bacterial species and relevant host miRNA are critical to either prevention or promotion of ALL will be to assess in young children the impact of antibiotics on erosion of the beneficial gut bacterial microbiome. Faecal miRNA transplantation has already been shown to be able to restore the gut microbiota [76] and will undoubtedly provide future therapeutic options. In humans, these biomarkers may help to provide candidate species for intervention with prebiotics and probiotics.

3. Conclusions

miRNAs, a class of noncoding RNAs, target mRNAs and regulate gene expression posttranscriptionally. Furthermore, miRNAs are differentially expressed in distinct stages of lymphopoiesis and influence the maturation process of lymphoid precursors. The aberrant miRNA signatures observed in ALL can be used to define biomarkers for diagnosis, classification, and prognosis of this disease. Circulating miRNAs can be detected with the use of sensitive and easily applicable methods such as RT-qPCR, allowing for easy detection and a minimally invasive approach for diagnosis of ALL. Indeed studies are currently being performed to establish a highly sensitive and specific set of 2-3 miRNAs that will allow for accurate diagnosis and classification of this haematological malignancy [90]. Specific profiles of miRNA expression have also been described for commonly used drugs, uncovering miRNAs that are associated with treatment response. Changes in expression levels of several miRNAs have been shown to play functional roles both in leukaemogenesis and in drug resistance; reversal of such expression profiles could improve drug sensitivity and subsequently give rise to better clinical outcomes [29, 91]. A number of miRNA have consistently been reported to be dysregulated in paediatric ALL, incorporating different cellular or molecular subgroups [27, 92]. Whilst the utilization of miRNA as diagnostic and predictive biomarkers is promising, there are still a few difficulties that need to be addressed [91]. There are inconsistencies in the methods being used for miRNA detection between different studies and comparable studies may share limited similarity within mRNA profiles when different stages of normal and aberrant cells are compared. There is therefore a need for uniformity in the collection of cells in both the experimental and control groups, as well as standardisation in the methods of detection [91, 93]. Currently, there are limited strategies to interrupt miRNA function; whilst the transfection of miRNA mimics or miRNA inhibitors *in vitro* allow for the increase or decrease of specific miRNA expression levels, safety concerns and degradation effects still limit their efficacy *in vivo*. The need for systemic delivery of miRNA as a therapeutic agent in the treatment of ALL itself raises the issue of unforeseen late effects of treatment.

Finally, albeit that survival of children with ALL has dramatically improved, there is still a need for defining novel sensitive, efficient, and reproducible biomarkers such as miRNA that can be used for early diagnosis, classification, prediction of treatment response, and ultimately perhaps even its prevention.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Review Article

Casein and Peptides Derived from Casein as Antileukaemic Agents

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Milk is a heterogeneous lacteal secretion mixture of numerous components that exhibit a wide variety of chemical and functional activities. Casein, the main protein in milk, is composed of α-, β-, and κ-caseins, each of which is important for nutritional value and for promoting the release of cytokines, also are linked to the regulation of haematopoiesis and immune response and inhibit the proliferation and induce the differentiation of leukaemia cells. It has been shown that the digestive process of caseins leads to the release of bioactive peptides that are involved in the regulation of blood pressure and the inhibition or activation of the immune response by serving as agonists or antagonists of opioid receptors, thus controlling the expression of genes that exert epigenetic control. Later, they bind to opioid receptor, block nuclear factor κ-beta, increase the redox potential, and reduce oxidative stress and the pro-inflammatory agents that favour an antioxidant and anti-inflammatory environment. Therefore, the bioactive peptides of casein could be compounds with antileukaemia potential. This review provides a summary of current knowledge about caseins and casein peptides on the immune system as well as their roles in the natural defence against the development of leukaemia and as relevant epigenetic regulators that can help eradicate leukaemia.

1. Introduction

Milk is a heterogeneous lacteal secretion mixture of numerous components (carbohydrates as oligosaccharides, lipids as long-chain polyunsaturated fatty acid, milk-specific microbiota, etc.) that exhibit a wide variety of chemical and functional activities. Milk is considered to be a functional food with direct and measurable influences on the health of the recipient [1], and it is now widely accepted that components of milk can influence and direct the physiological development of offspring.

In the traditional view, the major role of milk is to supply amino acids and nitrogen to young mammals, with use by adults being banned for most species; that is, humans are the only mammals known to consume the milk of another species, a unique behaviour that emerged during the Neolithic Revolution and that remains to this day. Thus, bovine milk has been an essential dietary staple for numerous human populations around the globe and an almost ubiquitous component of human nutrition [2, 3], regardless of the age of the consumer [4]. In this sense, among all mammals, bovine milk is the most studied; thus, we focus on it, especially the protein fraction of bovine milk that consists mainly of two major families of proteins, caseins (insoluble) and whey proteins (soluble), as well as other minor proteins and peptides, such as hormones.

We also discuss the available studies on breast milk and human caseins, since their data are relevant to the subject we address herein.

There are solid data indicating that caseins are linked to the immune system and to the generation of blood cells in mouse and rat models. Studies *in vitro* suggest that caseins, and the peptides resulting from the enzymatic hydrolysis of casein, have antitumour activity, which agrees with studies in humans that show that a lower frequency of breastfed infants develop leukaemia [5], and a similar effect has been described for older adults who consume milk of bovine origin [6], which suggests some factors that are transmitted

through breast milk may prevent the development of this disease [7].

This narrative review provides a summary of current knowledge about caseins and casein peptides on the immune system and how they might have biomedical relevance in the defence against the development of leukaemia and as relevant epigenetic regulators that can help eradicate leukaemia.

All research articles for this paper were obtained by searching Google Scholar and PubMed (https://www.ncbi.nlm.nih.gov/pubmed/).

2. Intake of Milk and Cancer Risk

The intake of milk during childhood is fundamental since it is the only source of macro- and micronutrients [8] and since breastfeeding has a protective effect against infection in infants, and there are studies that suggest that breastfeeding confers protection against childhood cancer [9]. Mathur et al. examined the relationship between the duration of total breastfeeding and exclusive breastfeeding and childhood cancer (58 % of lymphoma cases were non-Hodgkin's lymphoma). Their results suggest that breastfeeding has a protective effect against childhood cancer. Furthermore, they indicate that exclusive breastfeeding provides more beneficial immunological effects than breastfeeding that is supplemented by alternative feeding [10].

Shu et al. tested the hypothesis that breastfeeding decreases the risk of childhood leukaemia in two case-control studies of childhood acute myeloid leukaemia (AML) with the M0, M1, and M2 morphologic subtypes, and for childhood early pre-B-cell lymphoblastic leukaemia (ALL). They show a reduction in risk among breastfed infants, particularly those breastfed for more than 6 months [5]. Few studies have explored the association between diet and adult AML. It has been shown that consumption of whole milk increases lung and ovarian cancer risk [11, 12], but the role of dairy products such as milk in the risk of cancer is inconclusive [13]. Thus, in a hospital-based case-control study of 111 cases and 439 controls, regular milk intake was a factor associated with a significant decrease in the risk of AML in females with the highest weekly intake of milk compared with those in the lowest intake category [13]. A multicentre case-control study was conducted in south-eastern and northeastern China, and their findings suggest that diets rich in vegetables and an adequate amount of milk reduce the risk of adult leukaemia [6]. Additionally, milk intake has been related to a reduced risk of cancers of the distal colon and rectum [14]. These epidemiological data suggest that some component of milk has an antitumour effect but the composition of milk changes constantly throughout the lactation period and it has been shown that there are significant differences in milk composition between different species. Diet and the environment are important factors that influence the composition of milk. Some micronutrients may vary with nutritional status, and environmental toxins would differ according to the level of environmental exposure of chemicals specific to the region [10]. Further research is warranted to investigate the risk associated with milk intake.

3. Milk Composition

Milk contains specific proteins, fats designed to be easily digested, carbohydrates, minerals, vitamins, and other components [15]. Their composition reflects the nutritional requirements for the growth and development of each species. Thus, bovine milk is composed of approximately 3.2% protein, 4% lipid, 5% carbohydrates, and 0.7% mineral salts [16], whereas human milk consists of 1% protein, 4% lipid, 7% carbohydrates, and 1% mineral salts [17] (Table 1).

Milk protein has a high biological value, and milk is therefore a good source of essential amino acids; however, a wide array of milk proteins have biological activities that range from antimicrobial functions to the facilitation of nutrients absorption, and others act as growth factors, hormones, enzymes, antibodies, and immune stimulants [8].

Milk proteins can be broadly classified into 3 categories: caseins, whey proteins, and mucins, which are present in the milk fat globule membrane. In milk, caseins interact with calcium phosphate, forming large stable colloidal particles termed micelles. These micelles make it possible to maintain a supersaturated calcium phosphate concentration in milk, providing the newborn with sufficient calcium phosphate for the mineralization of calcifying tissues [21].

Milk proteins also facilitate the uptake of several important nutrients such as trace elements and vitamins and contain a group of proteins that provide a protective function, indicating their importance as multifunctional substances [22].

Bovine whey protein comprises immunoglobulins, α-lactalbumin, β-lactoglobulin, serum albumin, immunoglobulin, lactoferrin, proteose peptone fractions, and transferrin. Lower amounts of other minor proteins and peptides also exist with, for example, hormonal or other physiological activities [23]. In human milk, the whey proteins found in significant quantities are α-lactalbumin, lactoferrin, IgA, osteopontin, and lysozyme [18].

Bovine caseins, the most thoroughly studied, comprise αs1-, αs2-, β-, and κ-caseins. They are synthesized in the mammary gland under multihormonal control, and in the bovine genome, they are associated within a 200 kb region on chromosome 6, in the following order: αs1-, β-, αs2-, and κ-casein [24].

β-Casein has 209 amino acids. The presence of proline or histidine at the 67th position of β-casein allows the distinction between two types of milk, A1 and A2, and there are no other differences between these caseins. A1 β-casein is a major variant of β-casein in the milk of the common dairy cows of north European origin: Friesian, Ayrshire, British Shorthorn, and Holstein. A2 β-casein is predominantly found in the milk of Channel Island cows, Guernsey and Jersey, in Southern French breeds, Charolais and Limousin [25], and in the Zebu original cattle of African origin. The presence of proline or histidine at the 67th position of β-casein is associated with the major effects from bioactive peptide release by different gastrointestinal enzymes [26]; thus, a bioactive seven-amino-acid peptide, β-casomorphin-7 (BCM7) can be more easily released by digestion in the small intestine of A1 β-casein with pepsin, leucine

Table 1: Differences in the composition of human and bovine milk.

	Human	Bovine	Ref
Protein	Predominantly whey and β- and κ-caseins with lower concentrations of α-casein	The major protein fractions consist of α-, β-, and κ-casein	[17, 18]
Lipid	Cholesterol, palmitic and oleic acids, phospholipids, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, and long-chain polyunsaturated fatty acids (APGI-LC)	Triacylglycerols 98.3%, diacylglycerols 0.3%, monoacylglycerols 0.03%, free fatty acids 0.1%, phospholipids 0.8%, and sterols 0.3%	[19, 20]
Carbohydrate	Mainly lactose (6–8 g/100 ml) but at least 30 oligosaccharides, all of which contain terminal Gal-(β 1,4)-Glc and range from 3–14 saccharide units per molecule	In addition to lactose (4–6 g/100 ml), oligosaccharides, glycoproteins, and glycolipids	[15, 19]
Minerals	Calcium 25–35 mg/100 ml Phosphorus 13–16 mg/100 ml Sodium 15 mg/100 ml Potassium 156 mg/100 ml	Calcium 120 mg/100 ml Phosphorus 94 mg/100 ml Sodium 43 mg/100 ml Potassium 58 mg/100 ml	[20]
Vitamins	Retinol 58 μg/100 ml Vitamin E 0.34 mg/100 ml Biotin 0.7 μg/100 ml Riboflavin 0.03 mg/100 ml Vitamin B6 0.01 mg/100 ml Vitamin B12 trace	Retinol 19 μg/100 ml Vitamin E 0.04 mg/100 ml Biotin 3 μg/100 ml Riboflavin 0.24 mg/100 ml Vitamin B6 0.06 mg/100 ml Vitamin B12 0.9 mg/100 ml	[15, 20]

aminopeptidase, and elastase, but the alternative proline at position 67 prevents protein cleavage at this site [27]. There is a hypothesis that A1 (but not A2) β-casein may increase the risk of developing type I diabetes (DM-I) in genetically susceptible children [28], and it was suggested that A1 β-casein may also be a risk factor for coronary heart disease (CHD) [29].

αs2-Casein constitutes as much as 10 % of the casein fraction in bovine milk; it consists of 2 major and several minor components that exhibit various levels of post-translational phosphorylation [30], as well as minor degrees of intermolecular disulfide bonding [31].

αs2-Casein is the most calcium-sensitive member of the casein family, possibly because of its high ester phosphate content, which is derived from 10 to 13 phosphate groups on each peptide chain [32].

4. Caseins as Regulators of Haematopoiesis and the Immune System

Historically, sodium caseinate (SC), a bovine casein salt soluble in water with 65% proteins [33], provided the first evidence that milk proteins are linked to the biology of the immune system. SC used as a pro-inflammatory molecule induces chemotaxis of granulocytes and macrophages in the peritoneal cavity of mice [34, 35] and induces the accumulation of myeloid progenitor cells in mouse bone marrow [36]. Over time, it has been shown that SC accelerates the transition of band cells from bone marrow to polymorphonuclear cells, thus inducing macrophage colony-stimulating factor (M-CSF) [37]. Bone marrow progenitor cells of mice cultured with interleukin 3 (rmIL-3) as a growth factor in the presence of SC show increased cell numbers that exceed 50% [38]. Consequently, the administration of SC every 48 h for 6 days in BALB/c mice has been shown to increase myeloid cell proliferation and the number of total and mononuclear cells from bone marrow, events that are considered indices of medullary haematopoiesis activation [36]. SC also induces the proliferation of granulocytic lineage cells and increases the levels of both granulocyte colony-stimulating factor (G-SCF) and granulocyte macrophage colony-stimulating factor (GM-CSF) cytokines in serum and of G-CSF in bone marrow plasma, and the granulocytes generated have enhanced phagocytic activity [39]. This enhanced granulopoiesis and the subsequent reinforcement of innate immune system activation could explain why mice injected with lethal doses of bacteria survive after administration of casein [40].

Since SC consists of α-, β-, and κ-casein molecules, it seems logical to conclude that caseins would have biological effects similar to those of sodium salt. Most milk proteins are susceptible to the degradative effects of gastric processing, and extensive hydrolysis takes place upon exposure to enzymes in the gut; therefore, there are a few reports that show the biological effect of bovine and human caseins as complete molecules *in vitro*, without previous enzymatic degradation (Table 2).

Evidence has shown that caseins *in vivo*, through the production of cytokines, could be involved in the development of the mucosal immune system in neonatal mice [49], in erythropoiesis of mice [50], and in the restoration of haematopoiesis in rat models of myelosuppression [51].

5. Systemic Effect of the Peptides Derived from Milk Proteins

It has become increasingly evident that a consideration of the milk protein value must take into account the relationship between the protein structure and the amount and composition of the peptides derived from the proteins casein and lactalbumin during digestion in the gastrointestinal tract [52]. In addition, the relevant physiological activities of the

Table 2: Effect of caseins in haematopoietic cells *in vitro*.

Casein	Biological functions	Ref
Bovine α-, β-, and κ-caseins	Inhibit the proliferation of the 32D myeloid mice cell line and induce the expression of cfms and FcgRIIB1 and FcgRIIB22 receptors	[41]
Bovine α-, β-, and κ-caseins	Inhibit the proliferation of WEHI-3 leukaemic cells but induces cell differentiation, the expression of GM-CSF and its receptor GM-CSFR, as well as the isoforms FcgRIIB1 and FcgRIIB22	[42]
Human αS1-casein	Activates the secretion of pro-inflammatory cytokines such as GM-CSF, IL-1β, and IL-6 in human monocytes via the MAPK-p38 signalling pathway	[43, 44]
Human αS1-casein	Enhances the mitogen-stimulated proliferation of murine splenic T lymphocytes	[45]
Human αS1-casein	Pro-inflammatory properties throughout the TLR4 pathway	[46]
Human αS1-casein	May constitute an autogenous stimulus to uphold chronic TLR4 pathway inflammation	[47]
Bovine β-casein	Enhances mitogen-induced proliferation of bovine T and B lymphocytes in a dose-dependent manner	[48]
Bovine κ-casein CGP	Suppresses murine and rabbit lymphocyte proliferation induced by mitogens	[45]

cfms, M-CSF receptor; IL-1β, interleukin 1β; IL-6, interleukin 6; CGP, caseinoglycopeptide; MAPK-p38, mitogen-activated protein kinase p38.

infant during the breastfeeding and of children and adults consuming bovine milk should be considered.

Biologically active peptides derived from milk proteins are defined as fragments of 3–20 amino acid residues that have a positive impact on the physiological functions of the body. In general, these peptides are inactive within the sequence of the parent protein; thus, functional properties are revealed only after degradation of the native protein structure during gastrointestinal digestion or food processing. Once they become a bioactive, peptides may act as regulatory compounds with hormone-like activity [22], which ultimately affects the health of the living organism [53].

This gastrointestinal degradation may be a consequence of enzymatic hydrolysis, fermentation of milk by the starter cultures of proteolytic bacteria, and other processes used in dairy production [54]. In most cases, caseins are enzymatically degraded in the gut by endogenous enzymes, secreted by the digestive system, by enzymes of exogenous origin derived from actively metabolizing gut microflora [52] or, alternatively, by enzymatic degradation of granulocytes and macrophages [55, 56]. In any case, many of the peptides released by enzymatic hydrolysis have specific biological functions on their basis of their ability to bind to (and affect) the cellular function [57, 58].

Bioactive milk peptides were described for the first time after studies showed that the ingestion of casein-derived phosphorylated peptides led to enhanced vitamin D–independent calcification in rachitic infants. Since this discovery, several immunomodulatory peptides have been found in bovine and human milk [23, 59, 60]. Among the most studied peptides are α-lactalbumin derivatives, as well as α-, β-, and κ-caseins (Table 3). The following peptides have been studied: (1) Casein phosphopeptides, generated by the degradation of α- and β-caseins, are involved in promoting the absorption of calcium in the intestine to simulate the calcification of bones [76]. (2) Peptide inhibitors of angiotensin-1 converting enzyme, derived from α-lactalbumin, and α- or β-casein are crucial for regulating blood pressure [77]. (3) Opioid agonists derived from α- or β-casein and α-lactalbumin play an important roles in sleep patterns and are necessary for the development and gastrointestinal function on infants [78]. (4) Antioxidant peptides of β- or k-casein eliminate reactive oxygen species by reducing oxidative stress in newborns [79–81]. (5) Immunostimulatory peptides of β-casein and α-lactalbumin stimulate the phagocytic activity of macrophages [64, 82]. (6) The α-lactalbumin peptides exhibit bactericidal activity, since they have a high affinity for the iron in pathogens, which they use to exert a strong bacteriostatic effect [68, 78].

All these studies suggest to us that these systemic repercussions after milk intake in human beings, maternally sourced during the first months of life and of bovine origin in childhood and adulthood, could be of medical and clinical interest, and special attention might be directed to studies on opioid peptides.

6. Opioid Peptides from β-Casein, α-Casein, and κ-Casein

Opioid peptides are defined as peptides such as enkephalins that have both affinities for opiate receptors and opiate-like effects that are inhibited by specific antagonists of opiate receptors such as naloxone. The typical opioid peptides all originate from three precursor proteins: proopiomelanocortin (endorphins), proenkephalin (enkephalin), and prodynorphin (dynorphins) [83]. All of these typical endogenous opioid peptides have the same N-terminal sequence: Tyr-Gly-Gly-Phe [22]. On the other hand, food protein–derived opioid peptides are classified as exogenous opioids: while they possess a Tyr residue within their

Table 3: Immune activities of peptides and protein hydrolysates from caseins.

Casein	Derived peptide	Biological functions	Ref
αs1-Casein	Trypsin-derived f194-199 C-terminal	Promotes antibody formation and accelerated phagocytosis *in vitro* Provides protection against lethal bacterial infections *in vivo* Reduces *Klebsiella pneumoniae* infection in mice *in vivo* Protects mice against infection by *Staphylococcus aureus* prior to infection	[61, 62]
αs1-Casein	Chymosin-derived f1 ± 23 N-terminal	Stimulate a phagocytic response in mice infected with *Candida albicans* when injected intravenously Protection in cows and sheep against mastitis has also been observed following injection of the peptide into the udder	[63]
	Caseins digested by non-pretreated trypsin	Stimulate phagocytosis by murine peritoneal macrophages *in vitro* and consequently to exert a protective effect against *K. pneumoniae* challenge in mice after intravenous treatment	[64]
αs1-Casein	Pepsin/trypsin-derived peptides	Inhibit the proliferative responses of murine splenic lymphocytes and rabbit Peyer's patch cells *in vitro* Suppress mitogen-induced proliferation of human peripheral blood mononuclear cells *in vitro*	[65]
α-Casein	dPHLr	Decreases the production of IL-2 in activated T lymphocytes *in vitro*	[66]
αs1-Caseins	HLGG	Suppresses the proliferation of lymphocytes	[65]
κ-Casein*	Synthetic peptide Tyr-Gly	Enhances the proliferation of human peripheral blood lymphocytes *in vitro*	[67]
κ-Casein*	Chymosin-derived f106 ± 169 CGP	Inhibits LPS- and PHA-induced proliferation of murine splenic lymphocytes *in vitro*, and it also suppresses antibody production in murine spleen cell cultures *in vitro*	[48]
κ-Casein	Pepsin/trypsin-derived peptides	Enhances mitogen-induced proliferation of human lymphocytes *in vitro*	[68]
κ-Casein	Trypsin-derived f17 ± 21	Promotes antibody formation and accelerated phagocytic activity of murine and human macrophages *in vitro*	[69, 70]
κ-Casein	Synthetic peptide f383-389 (Tyr-Gly)	Immunomodulating peptide can pass across the intestine in quantitatively significant amounts to reach local lymphocytes Enhances cellular proliferation of human peripheral blood lymphocytes activated with concanavalin A *in vivo*	[71, 72]
β-Casein	FLAb	Immunomodulatory activity that might be related to interactions with monocytes-macrophages and T-helper cells, especially Th1-like cells *in vitro*	[65]
β-Casein	f54–59	Stimulates phagocytosis of SRBCs by murine macrophages *in vitro*; significantly enhance the resistance of mice to normally lethal infection with *K. pneumoniae*	[42]
β-Casein	f54-59 (Gly-Leu-Phe)	Stimulates phagocytosis of SRBCs and provides protection against infection by *Klebsiella pneumonia in vivo*	[73]
β-Casein	f191–193 (Leu-Leu-Tyr)	Fails to protect mice against infection but slightly but significantly stimulates antibody secretion against SRBCs by murine spleen cells *in vivo*	[73]
β-Casein	FLAb	Has immunomodulatory activity that might be related to interactions with monocytes-macrophages and T-helper cells, especially Th1-like cells	[74]

Table 3: Continued.

Casein	Derived peptide	Biological functions	Ref
β-Casein	f193-209	Upregulates MHC class II antigen expression on bone marrow-derived macrophages, increasing their phagocytic activity, and induces only a low level of cytokine release	[75]
β-Casein	HLGG	Suppresses the proliferation of lymphocytes	[65]
β-Casein	Pancreatin/trypsin-derived peptides	Inhibits mitogen-stimulated proliferative responses of murine splenic lymphocytes and rabbit Peyer's patch cells when included in cell culture *in vitro*	[48]

κ-Casein$*$, bovine κ-casein; HLGG, hydrolysed by *Lactobacillus GG*; dPHLr, derived peptides by hydrolysis with *Lactobacillus rhamnosus*; LPS, lipopolysaccharide; PHA, phytohaemagglutinin; SRBCs, sheep red blood cells; FLAb, fermented by lactic acid bacteria.

sequence, usually at the N-terminus or in the N-terminal region (except for αs1-CN-exorphin, casoxin 6, and lactoferroxin B and C), they differ from endogenous opioid peptides, which often feature Tyr-Gly-Gly-Phe as the N-terminal sequence [84], potentially with another aromatic residue, Phe or Tyr, at the 3rd or 4th position [85]. It is thought that, as in endorphins, this domain is important for the binding of peptides to the opioid μ-receptor (MOR) in the central nervous system, gastrointestinal tract, and some immune cells [86, 87]. In addition to its structural similarity, the activity of peptides is abrogated by naloxone, and therefore, it is accepted that these milk peptides affect the opioid receptor pathway [58, 88].

In most cases, these exogenous peptides were isolated and subsequently identified from enzymatic digests of their parent protein molecules. All the major milk proteins contain opioid ligands, which have been specifically termed exorphins and casoxin D when derived from α-casein.

Other milk opioid agonist peptides are α-casein-derived exorphins corresponding to bovine αs1-casein f90–95 (Arg-Tyr-Leu-Gly-Tyr-Leu) and f90 ± 96 (Arg-Tyr-Leu-Gly-Tyr-Leu-Glu), both of which have opioid-like properties that are inhibited with naloxone [1].

β-casomorphins (BCMs) are 4 to 11 amino-acid peptides encrypted in an inactive form and are released during digestion both *in vivo* and *in vitro*. Among them, the most active are BCM7 and BCM5, which represent fragments f60–66 and f60–64 of β-casein, respectively [89]. Both of these BCMs cross the intestinal barrier and reach the cerebrospinal fluid in normal individuals [90]. The physiological implications of this phenomenon have not yet been clarified, but it has been suggested that there is a relationship between BCMs and autism. β-casomorphin induces Fos-type immunoreactivity in brain regions relevant to autism, and elevated levels of BCM7 have been observed in patients with this condition in whom it exerts a relaxing effect [90]. Similarly, BCM7 and opioid receptors could be related to schizophrenia in people with few opioid receptors [91]. Thus, despite high levels of BCM7 in these patients [90], BCM7 cannot exert the relaxing effect it does in autistic patients.

BCMs were originally isolated from human and bovine β-casein following trypsin hydrolysis *in vitro* [92]. Pepsin and LAP are responsible for the release of the Tyr residue at the N-terminus of all types of pro-BCMs: pepsin cleaves the Leu58-Val59 peptide bond and LAP removes valine from the amino terminus. It should be noted that these peptides show strong opioid activities after the valine residue is removed [93].

BCM inhibits the proliferation of human lamina propria–derived lymphocytes *in vitro*. This antiproliferative effect is reversed by the addition of the opiate receptor antagonist naloxone to the culture [92]. However, BCM also enhances the resistance of mice to *Klebsiella pneumoniae*, likely by stimulating peritoneal macrophages. Additionally, the administration of an opioid antagonist in mice *in vivo* results in the suppression of this stimulatory effect, suggesting an active opioid receptor binding site for the biologically active peptide [42].

BCM7 f60 ± 66 and BCM10 f193 ± 102 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile and Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg, respectively) can exhibit bipolar modulatory effects on human peripheral blood lymphocyte proliferation. In *in vitro* cultures with mitogen-stimulated T lymphocytes, both peptides at low concentrations have been shown to suppress proliferation but enhance proliferation when administered at high concentrations [71].

7. Caseins and Peptides Derived from Caseins in the Regulation of Cancer

A wide variety of bioactivities for milk protein components has been reported, with one component having more than one type of biological activity, but here, we present only examples in which caseins and casein peptides have effects on different cancer cell lines or animal models. Then, we focus on the antileukaemic activities of these peptides. α-, β-, and κ-casein proteins all inhibit the migration *in vitro* of murine mammary tumour cells of the Met-1 cell line, the human breast cancer cell line MCF10A-H-Ras (G12V), and MDA-MB-231 cells, with α-casein being the most effective [94].

Casein hydrolysates generated using different commercially available food-grade enzyme preparations from mammalian, bacterial, and plant sources have an inhibitory effect on the viability and growth of both human Jurkat leukaemia T-cells and human epithelial colorectal adenocarcinoma Caco-2 cells lines, but SC had no significant effect on the viability and growth of Caco-2 cells [95].

Peptides derived from αs1-casein and β-casein digested by lactic acid bacteria inhibit the enzymatic activities of purified recombinant matrix metalloprotease (MMP)-2,

MMP-7, and MMP-9 in human HT-29 and SW480 colon carcinoma cells [96].

Lactaptin, the proteolytic fragment (f57 ± 134) of human κ-casein, induces apoptosis of MCF-7 adenocarcinoma cells [97]. Additionally, RL2, a recombinant analogue of lactaptin, induces apoptosis in MDA-MB-231 cells from an epithelial human breast cancer cell line and MCF-7 cells, and both downregulates Bcl-2 expression and induces p53-independent cell death [98]. On the another hand, it reduces the viability of A549 lung carcinoma cells and Hep-2 larynx epidermal carcinoma cells but is not accompanied by apoptosis, and in an interesting finding, nonmalignant human mesenchymal stem cells (MSC) are completely resistant to the action of RL2 [99].

In addition, 90-95 and 90-96 α-casomorphin, BCM7, BCM5, and the morphiceptin, the amide of β-Casomorphin-4, have an antiproliferative action on T47D cells, blocking cells in the G0/G1 phase [58].

Furthermore, 90–95 and 90–96 α-casomorphin, BCM5, and αs1-casomorphin amide inhibit the proliferation of human prostate DU145 and PC3 cells [100].

Moreover, f63-68 from β-casein inhibits the proliferation of SKOV3 human ovarian cancer cells partially by promoting apoptosis through suppression of the BCL2 pathway [101]. β-casein peptide f41-45 induces cytotoxicity in B16F10 melanoma cells [102].

Among the first findings of antitumour activity of casein *in vivo*, rats fed a diet rich in casein showed a marked decrease in colon carcinogenesis that had been induced by azoxymethane compared with the carcinogenesis in rats fed a low casein diet [103].

RL2, a recombinant analogue of lactaptin, significantly suppressed the growth of solid tumours in mouse xenografts bearing MDA-MB-231 breast cancer cells [98].

8. Caseins and Peptides Derived from Caseins Have Antileukaemic Properties

The first evidence of the antileukaemic activity of protein milks was shown *in vitro* by SC inhibiting the proliferation of leukaemia in mouse cells, such as those from the WEHI-3, J774, and P388 cell lines, even inducing apoptosis in one of them: the WEHI-3 myelomonocytic leukaemia cell line. However, in mononuclear normal cells from BALB/c mice (MNCs) bone marrow, SC induces a marked proliferation stimulus [38]. The evidence showed that normal tissues could be less sensitive to the biological effects of new molecules with potential antileukaemic properties [104, 105]; these data are significant since the usefulness of a potential anticancer compound depends not only on its ability to induce cytotoxicity in malignant cells but also on its relative lack of ability to induce toxicity in normal tissues and, in the case of SC, its ability to suppress the proliferation and induce the death of leukaemia cells. However, in addition to exerting no cytotoxicity towards nonleukaemia MNCs, SC induces their proliferation, which is a rare property among most drugs tested for use in the treatment of acute myeloid leukaemia. Then, it became clear that not only caseins but also casein peptides had an inhibitory effect on the proliferation of leukaemia cells when the casein hydrolysate inhibited the proliferation of the J774 and P388 leukaemia macrophage-like cell lines, although only in the latter was cytotoxicity confirmed [106]. Other evidence suggest that κ-casein f25-34 and f35-41 inhibit the proliferation of 32D normal cells and WEHI-3 myelomonocytic leukaemia cells and induce the differentiation of cells in the monocyte-macrophage and granulocyte-neutrophil lineages. κ-casein f35-41 reduces the proliferation of cells in both cell lines and induces 32D differentiation towards the monocyte-macrophage lineage, and WEHI-3 cell differentiation towards the granulocyte neutrophil lineage, whereas κ-casein f58-61 has no effect on the proliferation of any of the cells but induces their differentiation towards becoming granulocytes in both cell lines. This reduced proliferation is not due to a possible cytotoxic effect of the molecules [107].

β-casomorphin decreases the proliferation of 32D mouse cells by as much as 50% [108], and suppresses the proliferation of cells in the WEHI-3 myelomonocytic leukaemia cell line [109].

It was later shown that SC injected i.p. into mice inoculated lethally with WEHI-3 myelomonocytic leukaemia cells reduced the tumour burden and suppressed hepatomegaly, which collectively increased the survival of the leukaemic mice to a significant extent [38]. Similarly, in mice inoculated with cells from the J774 leukaemia macrophage-like cell line, a model of macrophage-like tumour M5 AML, SC significantly reduced splenomegaly, hepatomegaly, and the presence of solid tumours [110]. In both cases, the mechanisms of this antileukaemic action *in vivo* are unknown, but it has been observed that i.p. administration of SC in healthy mice induces the production of cytokines both in plasma and bone marrow [39]; therefore, the antitumour effects of SC might be the result of induction of profound inflammatory cell migration into the peritoneal cavity [34], either via the bioactive components of SC [111] or the secretion of growth factors, cell differentiation, or the effect of systemic inflammation [40]. Additionally, SC can activate mechanisms other that those associated with a simple inflammatory process because, although other agents, such as zymosan or thioglycolate, increase the levels of pro-inflammatory cytokines (IL-1β, TNF-α, MIP-2, and MCP-1/CCL2) [112, 113], they have no inhibitory effect on the proliferation of haematopoietic cells [114].

The available evidence for caseins, both in their complete form and in fragments resulting from their enzymatic degradation, reveal an enhancement of different aspects of the immune system, but their potential as antitumour agents has been scarcely explored. The use of caseins or their peptides to enhance the immune system to fight cancer is a rational strategy, as the immune system constantly works to keep us free of tumours. However, it is, of course, not always successful, with an estimated 19,520 new cases of AML diagnosed in the United States in 2018, accounting for approximately one-third of all new leukaemia cases [115]. Nevertheless, enhancing the immune system to eradicate cancer remains a valid and widely explored strategy against cancer. There are elements that suggest that caseins or casein peptides could eradicate leukaemia by functioning as

enhancers of the immune system and inducing cell death of malignant cells.

As we noted above, the mechanisms of the antileukaemic action of SC *in vivo* are unknown, but all these data on casomorphins, added to the fact that both granulocytes and macrophages are capable of hydrolysing caseins to release biologically active peptides [55, 56], suggest to us that these opioid peptides may be responsible for the antileukaemic effects observed for SC or caseins [116].

9. Mechanism of Action for Caseins and Derivate Peptides in Haematopoietic and Leukaemia Cells

It has been shown that in haematopoietic cells such as polymorphonuclear cells and monocytes there are specific receptors for caseins [117, 118], although little attention has been paid to this topic and it has been looked at whether another type of receptor in haematopoietic cells could be involuted in the biological effects of caseins has been explored.

Haematopoietic stem/progenitor cells (HSPCS) and their differentiated progeny express toll-like receptors (TLRs), which ensure an effective immune response in response to acute damage or infection. They are also responsible for promoting the recognition and elimination of tumour cells. Consequently, the recognition of TLR4 on antigen-presenting cells enhances antigen-specific antitumour immunity [119, 120], and an immunotherapeutic regimen capable of eliminating large, established mouse tumours has been developed using HMGN1, a DC-activating TLR4 agonist that is capable of inducing antitumour immunity [121]. It has recently been shown that α-casein binds to TLRs [47, 122]; thus, casein could exert immunomodulatory effects on leukocytes and even participate in the genesis of blood cells via TLRs (Figure 1), which could explain the antineoplastic effect of α-casein in WEHI-3 leukaemia cells [116].

However, overexpression or aberrant translation of TLR signalling is also associated with inefficient or malignant haematopoiesis, as in the case of leukaemia. Thus, overexpression of TLR-4 and TLR-2 has been observed in acute myeloid leukaemia and is more pronounced in acute promyelocytic leukaemia (Table 4), but it is reduced in the cells of patients treated with chemotherapy, suggesting the involvement of cellular signals that promote the development and prevalence of leukaemia [128, 131]. The activation of TLRs induces the production of interleukin 8 (IL-8), which attracts suppressor cells derived from the myelocytes (MDSCs) responsible for propitiating a tumorigenic microenvironment [132]. Additionally, leukaemia cells have been shown to stimulate bone marrow stromal cells of oneself to produce IL-8, a cytokine that supports the development of leukaemia cells [132]. During signal transduction, TLR activates nuclear factor κ-beta (NFκB) [133], the main pro-inflammatory promoter prevailing in the tumour microenvironment, leading to increases in pro-inflammatory cytokines such as TNF-α, IL-1β, and IFN-γ but reductions in anti-inflammatory molecules such as interleukin 10 (IL-10), SOD, CAT, and GPx (Figure 1(a)). Thus, the role of TLRs in the genesis and/or elimination of leukaemia is controversial; therefore, it is pertinent to suggest that more studies are needed to clarify the circumstances under which the TLRs are associated with the development of cancer and under what conditions these same receptors can serve as a therapeutic alternative against the development of leukaemia.

We know that SC administration in the peritoneal cavity promotes the survival of leukaemic mice [38, 110]. The possible mechanism of this antileukaemic effect may be due to the activation of TLRs by α-casein to exerting an antitumour activity (Figure 1(b)). Alternatively, these resident cells of the peritoneal cavity of mice could induce casein fragmentation to release casomorphins with opioid receptor activities, as indicated below.

It has been observed that the absence of MOR in mice enhances the genesis of haematopoietic progenitor cells [134], revealing a possible negative regulatory role of haematopoiesis for this type of receptor. In contrast, overexpression of this type of receptor has been observed in leukaemia cells (Table 4), and the use of opioid agonists has even been proposed for the treatment of different types of tumours, including those of leukaemia [135]; in this sense, methadone, a specific ligand of MOR, has been proposed for the treatment of cancer [135, 136] because it induces apoptosis and increases the sensitivity of leukaemia cells to the effect of doxorubicin in a mechanism that involves the reduction of cAMP, a promoter of cell proliferation [124].

In the framework of the development of leukaemia, in addition to high levels of reactive oxygen species (ROS), the expression and activation of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxides (GPx) are disturbed, in particular, both SOD and CAT activity are reduced in lymphocytes from ALL and CLL patients [137, 138]. It has been shown that BCM7, using epigenetic control, elevates the levels of glutathione S transferase (GST), a detoxifying enzyme of cancer-promoting agents [139], which is expressed at low levels in patients with leukaemia [140]. In fact, the GST gene is hypermethylated in the lung, breast, and liver cancers; leukaemia; and lymphomas [141].

After 15 days of oral administration of BCM7 to diabetic mice, the pancreatic malondialdehyde level was markedly reduced, with an increase in CAT activity and a reduction in NFκB and iNOS gene expression. Thus, BCM7 causes a pronounced decrease in oxidative stress and inhibits the NFκB-iNOS-NO signalling pathway [142]. Additionally, it has been shown that BCM7, when binding to MOR, increases the GSH/GSSG ratio and decreases the level of enzymes involved in SAM/SAH methylation, resulting in a reduced methylation of the CpG region [139], revealing its role as an epigenetic modulator of relevant genes in redox control.

All these elements suggest that BCM7 could decelerate leukaemogenesis via MOR, or, as an alternative, CN and αs1-caseins via TLR4. In any case, as a consequence of the activation of either receptor or both of them, the signalling of NFκB, the main pro-inflammatory promoter that prevails in

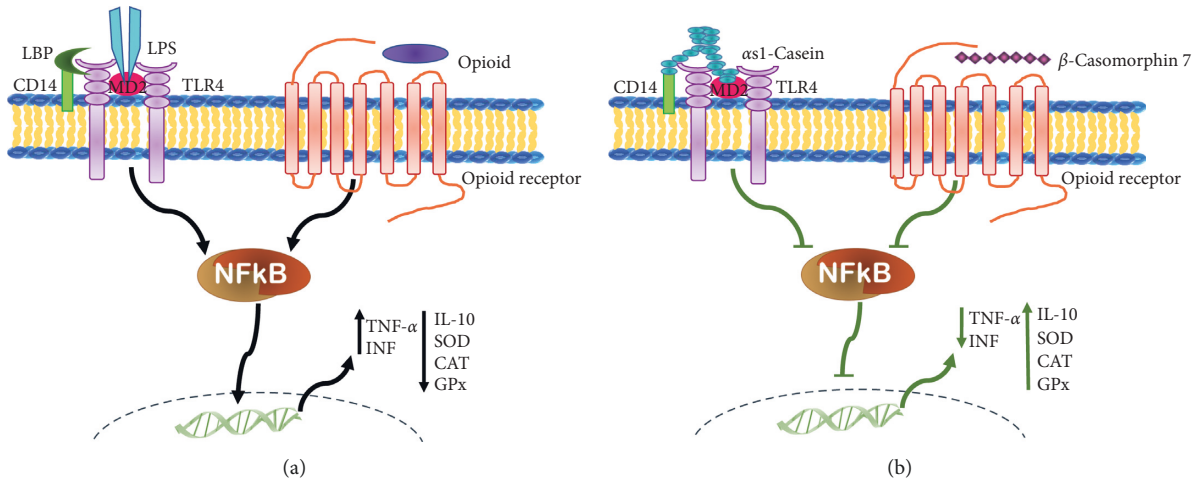

Figure 1: Mechanism of antineoplastic activity induced by casein or BCM7. (a) OPRs or TLR4 ultimately activates the nuclear factor κ-beta (NFκB) and the main proinflammatory promoter that prevails in the tumour microenvironment and increases in proinflammatory cytokines such as TNF-α, IL-1β, and IFN-γ but reduces anti-inflammatory molecules such as IL-10, SOD, CAT, and GPx. (b) BCM7 activates the MOR or αs1-casein, activates TLR4, and reduces the activation of NFκB, reduces the levels of TNF-α and IFN-γ, and increases IL-10, SOD, CAD, and GPx, contributing to a weakened leukaemogenic environment.

Table 4: Types of TLRs and OPRs in leukaemia cells.

Cell type	Opioid receptor	TLR receptor	Ref
Jurkat leukaemia cell line	MOR	—	[123]
Acute lymphoblastic leukaemia	MOR	—	[124]
HL60 leukaemia cell line, T-cell lymphoblastic leukaemia cells	MOR	—	[125]
AML M4 and M5	—	TLR4	[126]
Jurkat, K562 and HL-60 leukaemia cell lines	—	TLR4	[127]
AML M3	—	TLR4 y TLR2	[128]
THP-1 and HL-60 leukaemia cell lines	—	TLR4	[129, 130]

AML, acute myeloid leukaemia; MOR, μ-opioid receptor; TLR, toll-like receptors.

tumour microenvironments, could be blocked, thus reducing the levels of pro-inflammatory cytokines but increasing anti-inflammatory molecules, which could reduce the leukaemogenic environment (Figure 1(b)).

10. Future Perspectives

It is undeniable that leukaemia cells overexpress TLRs and opioid receptors that bind caseins and casomorphins, respectively. In both cases, the interaction leads to a reduction in the pro-inflammatory microenvironment prevalent in the development of tumours, so it would be interesting to evaluate whether by effectively reducing the oxidative stress, the production of anti-inflammatory cytokines is favoured over the production of pro-inflammatory cytokines; such information would support the potential antioncogenic use of caseins and casomorphins.

Caseins and some casomorphins inhibit proliferation and induce the differentiation of leukaemic but not normal cells, and caseins promote proliferation and differentiation of cells and even prolong the survival of leukaemic mice. It would be very interesting to determine whether the cause of these biological effects depends on the presence of TLRs and/or MOR.

Given the relevance of the physiological effect of the peptides derived from casein, it is reasonable to consider that they can have a relevant role as micronutrients and that their absence can cause the development of not only leukaemia but also of another types of cancers.

It should not be overlooked that αS1-casein is expressed in cells distinct from the mammary gland, mainly in patients with autoimmune diseases, which makes it necessary to analyse with caution the role of this compound as an antineoplastic agent.

11. Conclusions

There is evidence that caseins, both in their complete form and in fragments produced by their enzymatic degradation, enhance different aspects of the immune system, such as the proliferation of lymphocytes and generation of antibodies. They can also regulate normal haematopoiesis *in vitro* and *in vivo* via the secretion of cytokines, thereby inducing differentiation and enhancing proliferation. In leukaemia cells, however, they induce apoptosis and negatively regulate proliferation. This phenomenon highlights the potential of milk proteins as antitumour agents, but further research is needed to fully understand the mechanisms underlying the effects of the bioactive peptides of milk. Thus far, we have been shown that the TLR and OPR are involved in the transduction of signals from casein peptides in leukaemia and normal haematopoietic cells. Although humans consume milk over a much longer period than other mammals, we do not yet understand the complete scope of the administration of casein or its peptides as an antileukaemia

therapeutic regimen. However, the ultimate proof that a milk-derived product will or will not benefit human health will only be obtained in clinical trials.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Review Article

Targeting Leukemia Stem Cell-Niche Dynamics: A New Challenge in AML Treatment

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One of the most urgent needs in AML is to improve the disease cure rate as relapse still occurs in 60–80% of patients. Recent evidence suggests that dismal clinical outcomes may be improved by a better definition of the tight interaction between the AML cell population and the bone marrow (BM) microenvironment ("the niche"); the latter has been progressively highlighted to have an active role in the disease process. It has now been well established that the leukemic population may misinterpret niche-derived signals and remodel the niche, providing a shelter to AML cells and protecting them from the cytotoxic effects of chemoradiotherapy. Novel imaging technological advances and preclinical disease models have revealed that, due to the finite number of BM niches, leukemic stem cells (LSCs) and normal hematopoietic stem cells (HSCs) compete for the same functional areas. Thus, the removal of LSCs from the BM niche and the promotion of normal HSC engraftment should be the primary goals in antileukemic research. In addition, it is now becoming increasingly clear that AML-niche dynamics are disease stage specific. In AML, the niche has been linked to disease pathogenesis in the preleukemic stage, the niche becomes permissive once leukemic cells are established, and the niche is transformed into a self-reinforcing structure at a later disease stage. These concepts have been fostered by the demonstration that, in unrelated AML types, endosteal vessel loss occurs as a primary AML-induced niche alteration, and additional AML-induced alterations of the niche and normal hematopoiesis evolve focally and in parallel. Obviously, this endosteal vessel loss plays a fundamental role in AML pathogenesis by causing excessive vascular permeability, hypoxia, altered perfusion, and reduced drug delivery. Each of these alterations may be effectively targeted by various therapeutic procedures, but preservation of endosteal vessel integrity might be the best option for any future antileukemic treatment.

1. Introduction

In AML, there is an urgent need to develop more effective treatments as the current standard of care chemotherapy cures only 40–45% of younger and 10–20% of older patients [1]. The cytogenetic/genomic categorization of the disease has allowed the precise definition of distinct AML molecular subsets but has not translated into an increased number of drugs inspired by genomics. In addition, current evidence suggests that AML clinical outcomes might be improved not only by more selective and better tolerated treatments that target the AML cell population but also specifically through the development of novel strategies that target the interaction between the AML population and the BM microenvironment ("the niche") whose critical role in AML has been progressively defined [2–4]. Despite the complex and sometimes conflicting data, various seminal studies have highlighted that normal hematopoietic stem and progenitor cells (HSPCs) and leukemic stem cells (LSCs) utilize bidirectional cross talk with their neighboring niche cells to create specific reciprocal dependencies [5, 6]. An early study reported that AML homing occurs around vessels of a specialized E-selectin and CXCL12 positive endothelium [7], two subsequent studies reported that leukemic engraftment occurs in areas enriched with osteoblasts [8, 9], and another has shown that LSCs are themselves able to generate walled-off abnormal niches [10]. In addition, an increase in microvascular density, altered innervation [11], and the loss of

osteoblasts [12] were reported to occur in the AML niche, but no correlations with the disease stage or type were established. Instead, current intravital imaging and genetic mouse models have revealed that the interactions between malignant cells and the BM microenvironment are disease subtype and stage specific [13, 14], and this assumption has also been validated in AML. Osteoblasts halt the expansion of BCR-ABL CML-like disease but promote the propagation of MLL-AF9-driven AML [4]. The niche, which is necessary for leukemogenesis in a preleukemic stage, becomes permissive once leukemia is established [2], and it is transformed into a self-reinforcing leukemic compartment at a later disease stage [3]. However, the most relevant finding provided by intravital imaging and genetic mouse models is that the primary alteration induced by the AML cell population affects the vascular architecture and function of the niche [13, 15]. The loss of osteoblasts and the suppression of normal hematopoiesis occur at a later disease stage, and these events evolve focally and in parallel and end in niche collapse that is no longer reversible. These data are of pivotal relevance as they further improve our knowledge regarding the mutual cellular influences that allow the development of AML through the adjustment of secure signaling pathways involved in self-renewal, proliferation, survival, and immune evasion. More importantly, these vascular alterations that consist of increased vascular permeability, hypoxia, altered perfusion, and reduced drug delivery may become potential targets of novel candidate interventions that might restore the normal niche ecology making AML cells more susceptible to chemotherapy. This review focuses on possible interventions that might restore normal hematopoiesis and normalize niche function.

2. Strategies to Dislodge LSCs from Functional Niches

The demonstration that the number of functional niches within the BM is limited and that niche occupancy is the limiting factor in transplantation of normal HSPCs is the basis for the notion that LSCs and HSCs might share and compete for the same BM niches [8, 9, 16, 17]. In addition, functional niches within both the BM and extramedullary compartments may protect LSCs from the toxic effects of chemotherapy and the graft-versus-leukemia (GvL) effect by creating "sanctuary sites" that provide LSCs with survival signals and improve immune escape by limiting antileukemic T-cell responses [18]. Colmone et al. were the first to propose the existence of a competition between HSCs and LSCs. In the BM of a severe combined immunodeficiency (SCID) mouse model with a pre-B acute lymphoblastic leukemia (ALL), they demonstrated that both normal HSCs and leukemic stem cells (LSCs) localize to specific vascular niches enriched with stromal cell-derived factor-1 (SDF-1)/CXCL-12 [7]. They also found that leukemic cells alter the BM microenvironment by creating malignant areas that prevent CD34 + cell engraftment through the release of stem cell factor (SCF). Thus, therapeutic targeting of SCF may increase the hematopoietic reserve and improve engraftment of allogeneic and autologous stem cells [10]. Subsequently, other studies reported that, in experimental mouse models, the cotransplantation of increasing doses of normal HSCs or cord blood hematopoietic stem/progenitor cells (CB-HSPCs) together with AML cells prolonged animal survival by inhibiting disease progression and resulted in distinct foci of either normal HSCs or AML cells in the early post-transplant period [19, 20]. In addition, in these cotransplantation models, high doses of normal HSPCs were able to decrease leukemic cell cycling, confirming the clinical observation that, in human transplantation, leukemia relapse is significantly lower in patients who receive high than in those who receive low CD34 + cell doses [21]. This result that does not seem to be due to a more robust graft-versus-leukemia (GvL) effect is provided by the higher CD34 + cell doses. These observations strengthened the idea that normal HSCs might effectively outcompete LSCs for niche occupancy especially after some particular cellular manipulations. The most impressive results were obtained when the infusion of CB-HPSCs occurred soon after (i.e., one hour after) the mobilization of AML cells, in comparison with infusions made one day after their mobilization. In order to explain these results, the potential return of AML cells to their BM niches was suggested based on the observation that, in the context of preestablished leukemic disease, poor CB-HPSC engraftment occurred. Other studies have hypothesized that the targeting of adhesion molecules overexpressed by AML cells (e.g., CXCR4, very late antigen 4 (VLA4, also known as $\alpha 4\beta 1$ integrin), CD44, E-selectin, and CD98) together with the exploitation of the aforementioned competition between normal and leukemic stem cells for the same BM niches might be an effective therapeutic strategy in patients with minimal residual disease (MRD) at the time of hematopoietic stem cell transplantation (HSCT). Among the adhesion molecules responsible for normal and leukemic stem cell homing to BM niches, CXCR4 which binds to CXCL12 appears to be the most relevant one as it controls migration, mobilization, homing, and retention of LSCs within the BM and activates apoptosis that is prevented by an as-yet-undefined soluble factor produced by osteoblasts [22–24]. The CXCR4-CXCL12 axis activates PI3K/Akt and MEK/ERK pathways and downregulates microRNA let-7a. In AML, increased expression of CXCR4 on LSCs has been associated with a shorter survival and an increased relapse rate [25–27]. Thus, as demonstrated in xenograft models, targeting the CXCR4-CXCL12 axis might be an effective treatment strategy that would achieve more profound disease eradication by mobilizing LSCs [28, 29] from their "sanctuary sites" rendering them more susceptible to both the toxic effects of chemotherapy and the antileukemic T-cell response [28, 30]. This explanation provides the rationale for combining the principal inhibitor of the CXCR4-CXCL12 axis (i.e., plerixafor) with conventional chemotherapy [31]. Phase I/II clinical trials based on this combination have provided beneficial results [32]. Importantly, the addition of plerixafor to the myeloablative regimen for allogenic HSCT for AML patients in their first complete remission is a safe and well-tolerated therapeutic procedure [33]. Thus, further

studies in larger cohorts are warranted to investigate the impact of plerixafor on the relapse rate and survival of transplanted patients.

Mobilization of LSCs might also be achieved by targeting the VCAM1/VLA4 axis, which mediates the binding of AML cells to endothelial cells with the development of a quiescent leukemic cell phenotype. These AML cells are physically and functionally integrated within the endothelium but may still cause relapse [34]. However, disrupting the VCAM1/VLA4 axis may be effective only in patients with minimal residual disease, whereas in those with active disease, this strategy needs to be combined with conventional chemotherapy. In addition, natalizumab, a humanized VLA4 monoclonal antibody, which causes prolonged HSC mobilization [35], has limited clinical utility because it may cause JC virus-associated progressive multifocal leukoencephalopathy [36]. E-selectin is also involved in AML cell binding to the vascular niche with activation of Wnt signaling and might be another potential target of mobilizing treatments. AML cell binding to E-selectin is associated with increased localization of leukemic cells within the BM niche and their increased survival during chemotherapy. Thus, clinical trials with GMI-1271, a small E-selectin inhibitor, which in a mouse xenograft model enhanced the effects of chemotherapy, are currently ongoing [37]. In addition, leukemic cell binding to endothelial cells is also increased by the glycoprotein CD98 that binds integrin β1. Recent *in vitro* studies have revealed that a CD98 antibody significantly reduces adhesion and the colony-forming ability of primary AML cells, and in experimental murine models, it significantly reduces the AML tumor burden [38].

Sympathetic nervous system (SNS) neuropathy is another mechanism that in the MLL-AF9 AML model may lead to leukemia progression [1]. These leukemic cells decrease the density of the SNS network through the alteration of β2 adrenergic signaling with loss of niche quiescence. In particular, this loss is due to the expansion of mesenchymal stromal progenitor cells committed to osteoblastic differentiation and the reduction of arteriole-associated NG2+ niche cells. Thus, a potential therapeutic approach could be to circumvent this β2 adrenergic receptor damage with receptor agonists. However, to date, these last drugs have provided contradictory results.

The BM vascular niche and the major interactions between its cellular components are summarized in Figure 1; the mechanisms by which different drugs mobilize HSC and LSC are also presented.

3. Targeting Hypoxia

Evidence coming from rat models has sufficiently demonstrated a close relationship between BM hypoxia and AML cells. In particular, it was demonstrated that AML cells preferentially infiltrate BM hypoxic areas, which expand on leukemia engraftment and disease progression [41]. In addition, AML cells from leukemic rats are more hypoxic than BM cells obtained from healthy rats [42, 43]. These hypoxic BM niches have a reprogrammed metabolism. A recent study showed that glycolysis was higher in the femurs of leukemic mice than in the femurs of healthy control mice. In addition, in samples from AML patients, leukemic blasts overexpressed genes defining a "hypoxia index" [44]. Interestingly, in these xenograft leukemia models, exposure to the hypoxia-activated prodrug TH-302 depleted hypoxic cells, prolonged survival, and reduced the leukemia stem cell pool especially when this hypoxia-activated prodrug was combined with sorafenib. More relevantly, TH-302 prolonged survival in a syngeneic AML model by eliminating residual hypoxic leukemic BM cells that had persisted after chemotherapy [44]. These results confirm that leukemic cells preferentially home to hypoxic niches where the compromised blood flow reduces their exposure to chemotherapy and immune effector cells [41–43, 45] and that these BM areas contain true quiescent and chemoresistant LSCs endowed with self-renewal capacity. Hypoxia activates the hypoxia-inducible factors 1α and 2α (Hif-1α and Hif-2α) and the PI3K/Akt/mammalian target of the rapamycin (mTOR) signaling pathway, which provides environmental prosurvival cues to leukemic cells. It was demonstrated that Hif-1α and Hif-2α through their interaction with the "hypoxia responsive elements" (HREs) of various genes (e.g., TGFβ, c-Kit, FGF-2, VEGF, and Notch-1) may upregulate the expression of CXCR4 and CXCL12 on AML cells and endothelial cells, thereby promoting LSC maintenance and disease aggressiveness [46–48] (Figure 2). However, the most relevant effect of Hif-1α, which is stabilized by the AML-associated mutations of the isocitrate dehydrogenase (IDH) 1 and IDH2 genes, is to make leukemic cells completely dependent on mitochondrial oxidative phosphorylation to fulfill their low energy requirements as they are unable to employ glycolysis [49]. More importantly, Bcl-2, one of the most relevant mediators of mitochondrial respiration, is overexpressed in AML especially in patients with IDH1 and IDH2 mutations. Thus, Bcl-2 therapeutic targeting is expected to be highly effective in AML. This suggestion was confirmed by various studies. AML cells are exquisitely sensitive to the pharmacological blockade of Bcl-2 pathways since leukemic cells assemble the apoptotic machinery on the mitochondrial membrane kept in check by inhibitory Bcl-2 proteins [50]. In addition, it was demonstrated that ABT199 at nanomolar concentrations induced apoptosis in various AML cell lines in *in vitro* cultures of chemosensitive and chemoresistant AML stem and progenitor cells and inhibited leukemic progression in *in vivo* murine models [51]. A phase II clinical trial of venetoclax monotherapy in relapsed/refractory AMLs showed only modest activity; however, it identified the lack of myeloblast dependence on BCL-X_L or MCL-1 and the presence of IDH1/IDH2 mutations as the best predictors of response [52, 53]. In contrast, venetoclax was much more effective when combined with cytarabine and idarubicin [54], PI3K inhibitors [55], 5-azacytidine [56–58], and mTOR inhibitors [59]. Moreover, the combination of venetoclax with low-dose cytarabine which is currently being evaluated in a randomized phase III trial has up to now provided impressive results with a CR/CRi rate of 62% and a median overall survival of 11.4 months [60]. In addition, an ongoing phase Ib trial enrolling elderly patients with relapsed/

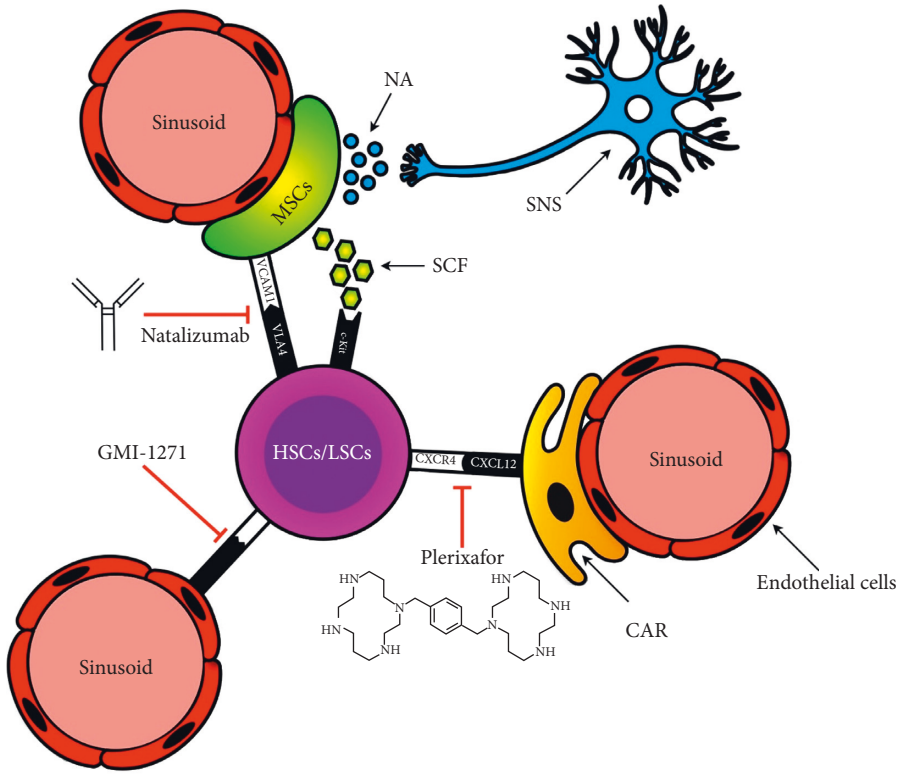

Figure 1: Bone marrow vascular niche. The vascular niche is composed of sinusoidal endothelial cells and mesenchymal stem cells (MSCs), both of which express adhesion molecules such as VCAM1, which bind to the corresponding receptor VLA4 (an $\alpha 4\beta 1$ integrin) expressed on both HSCs and LSCs, and the soluble stem cell factor (SCF), which binds c-Kit to the surface of HSCs and LSCs. Natalizumab, an anti-VLA-4 monoclonal antibody, acts by preventing the VLA4-VCAM1 interaction. CXCL-12 is produced mainly by CXCL-12-abundant reticular cells (CARs). The binding of CXCL-12 to CXCR4 on HSCs and LSCs plays an important role in HSC and LSC homing and retention within the bone marrow. Plerixafor, which acts by inhibiting this link, promotes the HSC/LSC mobilization from the bone marrow vascular niche. E-selectin is expressed on endothelial cells and is involved in HSC/LSC retention within the bone marrow vascular niche through its interaction with sialylated carbohydrate expressed on both the HSC and LSC surfaces. GMI-1271, which is an E-selectin inhibitor, promotes HSC and LSC displacement by weakening this link. Finally, a close interaction between MSCs and adrenergic fibers has also been demonstrated. Release of noradrenaline by the sympathetic nervous system (SNS) induces metalloproteinase expression and activity, which then act to cleave other adhesion molecules (CXCR4, VLA4, VCAM1, and SCF), thereby promoting HSC release from the bone marrow [39, 40].

refractory AML and employing venetoclax in combination with the MDM2 inhibitor idasanutlin has reported interesting results [61]. The rationale for combining these drugs was based on the demonstration that p53 activation through MDM2 inhibition reduces Ras/Raf/MEK/ERK signaling with GSK3β activation and MCL-1 degradation, hence overcoming venetoclax resistance [62]. Recent data have revealed that mitochondrial apoptosis can also be induced by glutaminase inhibitors that cause an increase in glutaminase levels and act synergistically with ABT compounds [63].

Hypoxia can also be targeted by hypoxia-activated prodrugs [41, 64]. TH-302 is one of these compounds that under hypoxic conditions release the DNA alkylating agent bromo-isophosphoramide mustard. A study reported that chemoresistant AML cells after exposure to TH-302 developed reduced HF-1α expression, DNA strand breaks, cell cycle arrest, and apoptosis [41, 64]. This observation provided the rationale for initiating phase I clinical trials on refractory AML, but these studies have achieved only modest results: most patients developed an early but transient cytoreduction that was not maintained until the next cycle and only two responded [65].

4. Targeting Leukemic-Stromal Cell Interactions

Additional treatment strategies aimed at hampering leukemic niche dynamics are based on the demonstration that leukemic cells may alter stromal cell activities and the BM microenvironment in order to favor disease development and progression. One of the mechanisms that AML cells use to remodel the niche is represented by exosome (i.e., membranous nanosized vesicles derived from the endocytic compartment) or microvesicle (larger vesicles produced by the shedding of the plasma membrane) release [66, 67]. miR150, miR210, and various transcripts contained within distinct exosomes/microvesicles may affect AML prognosis, treatment, and niche function by altering the behavior of bystander cells [67, 68]. Currently, Rab27a has been identified as one protein involved in exosome release and is a potential therapeutic target in experimental models. Recent

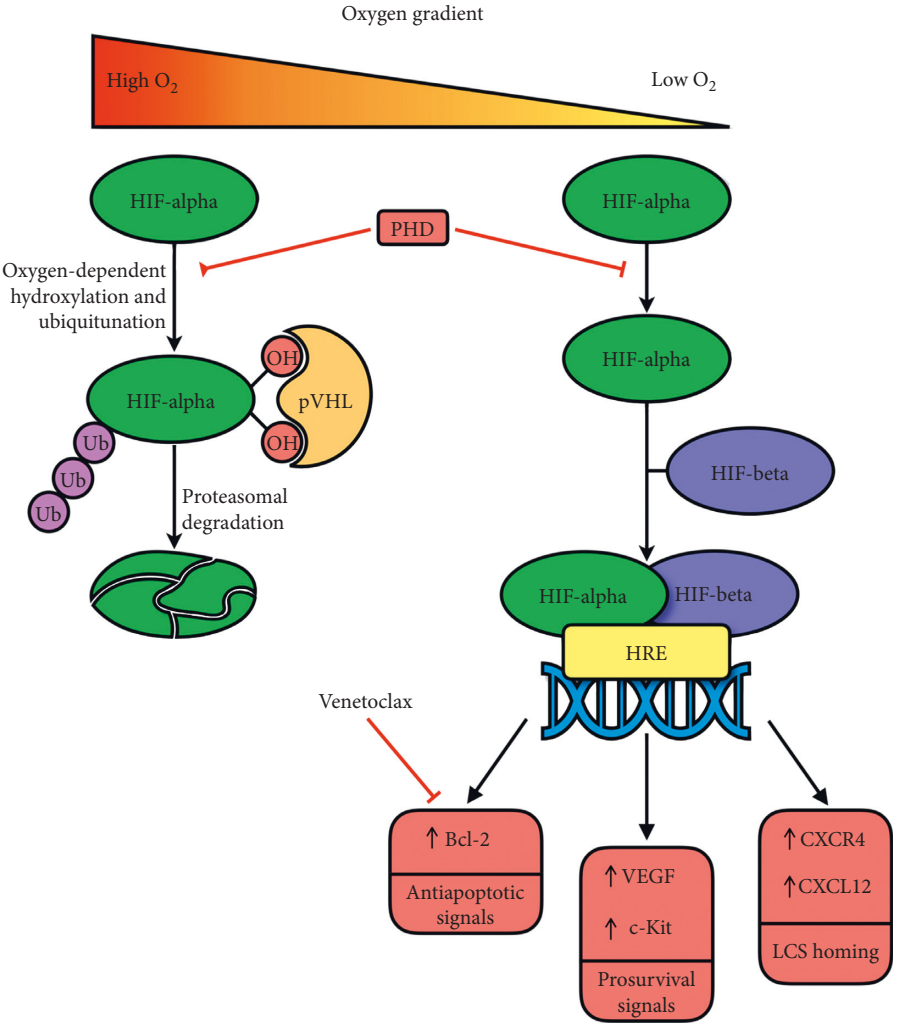

Figure 2: Role of hypoxia in leukemic stem cell maintenance. Under normoxia conditions, the alpha-subunit of hypoxia inducible factor (HIF-alpha) undergoes an oxygen-dependent hydroxylation by the enzyme prolyl hydroxylase (PHD). The hydroxylated form of HIF-alpha is recognized by the von Hippel–Lindau protein (pVHL); this interaction allows for ubiquitination and proteasomal degradation of HIF-alpha. Under hypoxic conditions, the hydroxylation and subsequent ubiquitination and degradation of HIF-alpha do not take place. Therefore, HIF-alpha is able to interact with the beta-subunit of hypoxia inducible factor (HIF-beta), forming a heterodimeric complex that can promote transcription of specific genes called hypoxia responsive elements (HREs). These genes encode for proteins with antiapoptotic (e.g., Bcl-2) and prosurvival (e.g., c-Kit and VEGF) functions. HREs also encode for CXCR4 and CXCL12, which play an important role in LSC homing and preservation. The ability of venetoclax, a potent Bcl-2 inhibitor, to induce LSC apoptosis is explained by the high level of Bcl-2 expression on LSCs.

data show that Rab27a knockdown reverses the ability of AML exosomes and AML cells to alter the BM composition of wild-type recipient mice. In particular, Rab27a knockdown eliminates the increase of SCA1+ CD146+ stromal cells and the block of osteoblast differentiation induced by *Dkk1* upregulation and significantly prolongs the survival of wild-type recipient mice [69]. Another intriguing therapeutic strategy might be to target the reciprocal contact-dependent transfer of functional mitochondria between mesenchymal stem cells (MSCs) and AML cells [70]. This transfer, which increases during chemotherapy providing a better leukemic cell survival rate, thanks to a 1.5-fold increase in energy production [70], might be targeted by cytochalasin, a finding that supports the potential involvement of nanotubes in this process [71]. Reciprocal mitochondrial transfer from AML cells to MSCs also occurs in leukemic patients [72]. These data, along with the observation that LSCs from tyrosine kinase inhibitor-resistant (TKI-res.) CML patients are also completely dependent on mitochondrial transfer, have led to the approval of tigecycline in combination with imatinib for TKI-res. CML [73]. Current evidence suggests that the AML cell metabolism also might be a promising treatment target. Recently, *in vitro* screening revealed that LSC growth is suppressed when these cells are cocultured with MSCs in the presence of lovastatin, a cholesterol-lowering drug, an effect that was not achieved when AML cells were cultured alone. In addition, infusions of cells obtained from LSC-stromal cell cocultures pretreated with lovastatin prolonged mice survival [74]. Even more recently, it was reported that etomoxir, an inhibitor of

carnitine O-palmitoyltransferase 1 (CPT-1), a protein involved in the fatty acid oxidation (FAO) pathway, was able to sensitize an LSC subset to chemotherapy [75, 76]. These cells that express CD36 (a marker of poor prognosis) at high levels [77] are able to highjack the lipolytic process in the gonadal adipose tissue to fuel their metabolic needs and evade chemotherapy-induced apoptosis [78]. An LSC subset that was exposed to etomoxir was no longer able to use FAO, an alternative source of energy which interfered with BAX/BAK oligomerization in response to apoptotic stimuli [79, 80].

5. Maintaining Vascular Integrity

Various studies have reported that, in murine AML models and AML patients, the number of sinusoidal vessels within the BM central region (the so-called "BM microvessel density" (BM-MVD)) is increased [81–83]. It has been suggested that this alteration was induced not only by MSCs and megakaryocytes but also by the AML cells themselves [84, 85]. Nowadays, it is well known that leukemic cells may generate autocrine and paracrine loops through the release of vascular endothelial growth factor (VEGF) and angiopoietins 1 and 2 and the expression of their respective receptors [84, 86]. However, despite these data, AML patients have always had a disappointing response in clinical trials that employed antiangiogenic drugs, a result that can be explained by two very recent studies that identified endosteal vessel alterations as the primary events in AML pathogenesis [13, 15]. One study described a heterogeneous distribution of endothelial cells (ECs) with a reduction of those associated with sinusoids *versus* a significant increase of those associated with arterioles, a scenario that leads to the generation of several hypoperfused BM areas. At an early AML stage, these hypoxic areas, which appeared leakier than areas nonengrafted by AML cells, were localized near AML cells, whereas at a later disease stage, they were scattered throughout the entire BM [15]. Another study reported that the endosteum of the metaphysis, but not that of the diaphysis, was the main site of this vessel loss [13]. Moreover, with low BM infiltration (i.e., 5–15% of BOM infiltration), the appearance of the perivascular and endosteal stroma was normal; but with high infiltration, the appearance of these stromal areas was abnormal with depleted perivascular and endosteal stromal areas. In low-infiltrated areas, blood vessels appeared narrower than those of control mice and far from the endosteal surface. In contrast, in high-infiltrated areas, the formation and retraction of blood vessel sprouts were observed, and these lead to a less-effective angiogenic process. Finally, the detachment of ECs from the vascular wall leads to their accumulation within the vascular lumen and to their potential uptake by AML cells. EC depletion occurs as the primary event of AML-induced niche alterations and is followed by the concomitant loss of osteoblastic and HSCs. In addition, these alterations of the niche and normal hematopoiesis evolved focally and in parallel. Moreover, when the vascular niche had collapsed, no treatment strategy was able to induce its restoration [13]. Thus, it was suggested that the best therapeutic strategy is to prevent the AML-induced endosteal vessel loss in order to prevent AML progression due to the generation of BM hypoxic areas (real "sanctuaries" of chemoresistant cells) [9] and to favor leukemic cell targeting by cytotoxic agents [8, 87]. In order to achieve this goal, leukemic mice were treated with the iron chelator deferoxamine (DFO) that also enhances HIF-1α stability and activity, thereby promoting the expansion of endosteal vessels and increasing the number of HSCs within the trabecula-rich metaphysis. However, DFO did not modify the number of BM AML cells and did not improve the survival and disease progression rate unless it was combined with chemotherapy. Interestingly, recent evidence suggests that DFO by modulating reactive oxygen species (ROS) pushes the differentiation of leukemic blasts and normal BM precursors towards monocytes/macrophages. This observation points to a strong role of ROS in endosteal vascular niche remodeling [88]. In normal BM, NADPH oxidase 4 (NOX4), an endothelial isoform of NADPH oxidase, regulates the production of ROS and the activation of nitric oxide synthase 3 (NOS3) with the consequent generation of nitric oxide (NO) [15] in response to oxidative stress and hypoxia. The gene-encoding NOX4 is overexpressed by BM-derived ECs and in the BM of AML patients and patient-derived xenograft (PDX) mice. This NOX4 overexpression has been associated with NOS3 overexpression and ROS/NO overproduction. Of note, a persistent elevation of NO levels has been associated with an increased rate of treatment failure in AML patients. This evidence supports the results obtained with PDX mice, in which NO inhibition combined with Ara-C treatment reduced NOS3 activation, vascular leakiness, and BM hypoxia, leading to reduced AML progression in the BM and spleen and a "remission-like" phase longer than that of control mice. Furthermore, NO inhibition combined with Ara-C treatment was more efficient than chemotherapy alone in reestablishing not only the number but also the function of BM SLAM + cells allowing them to outcompete leukemic cells during the relapse process. Unfortunately, even though NOS2 is the preferential target of available NO inhibitors, the mechanisms of NOS3 regulation and NO production have not been clarified yet. Several preclinical studies have suggested that the most promising strategy leading to NO inhibition might rely on the targeting of NOS gene regulators.

6. Prevention of Immune Escape

HSCT, which remains the only curative treatment for most AML patients, can be considered an immunological procedure since it causes the potential eradication of recipient LSCs through various immunological mechanisms (i.e., activation of donor T lymphocytes and natural killer (NK) cells and production of an inflammatory milieu) that constitute the so-called graft-versus-leukemia (GVL) effect. Peptides presented by self-HLA are recognized as targets by T cells, whereas activation of NK cells depends on the absence or downregulation of self-HLA class I molecules. These two different types of recognition have evolved as complementary strategies to ensure immunological

recognition of virus-infected cells in which downregulation of self-HLA class I molecules represents an important mechanism to avoid immunological recognition by virus-specific T cells and promote viral persistence [89, 90]. However, LSCs may effectively escape these immunological mechanisms by altering the expression of target genes and acquiring new selective mutations that eventually determine a posttransplant relapse, still one of the major transplant complications. Currently, the elimination of HLA alleles without the reduction of the overall expression levels of HLA class I molecules achieved through a copy-neutral loss of heterozygosity has been identified as a mechanism of relapse in about one-third of HSCTs, especially those from haploidentical donors, while the downexpression of HLA class II molecules is one of the principal mechanisms of relapse in both HLA-matched and HLA-mismatched HSCTs. Accordingly, recent studies have shown that a higher expression of HLA class II molecules predicts a better prognosis. Currently, there are no therapeutic strategies that can counteract the downexpression of HLA molecules. However, from a clinical point of view, this information explains why the infusion of donor lymphocytes should be avoided in posttransplant relapse with genomic HLA loss, as the infusion of these donor cells might be associated with a less effective GvL effect and may be associated with the development of GVHD.

In addition, LSCs can upregulate the expression of coinhibitory molecules in order to block T-cell activation [91]. Physiologically, the interaction between these molecules (CD80/86 for CTLA-4 and PD-ligand-1/2 (PD-L1/2) for PD-1/2) expressed on antigen-presenting cells (APCs) and various immune-checkpoint molecules (inhibitory molecules (e.g., T lymphocyte-associated antigen-4 (CTLA-4) and programmed death-1 (PD-1)) expressed on activated T cells) leads to controlled T-cell inhibition, which is of primary importance to prevent immune-mediated diseases. Data from several clinical trials support the main role of this T-cell inhibition in AML relapse, in both the transplant and the nontransplant setting. AML patients who relapse after allogenic HSCT present a deregulated expression of PD-L1 and other immune-regulatory molecules (e.g., B7-H3 and CD155/PVRL2). In these patients, various clinical trials have revealed the clinical effectiveness of CTLA-4 blockage in disease control [92–94]. The role of PD-L1 expression has also been shown in $JAK2^{V617F}$ myeloproliferative neoplasms. In these disorders, STAT3 and STAT5 phosphorylation by the oncogene $JAK2^{V617F}$ enhances PD-L1 expression by increasing PD-L1 promoter activity. T cells that interact with these $JAK2^{V617F}$ mutant cells have reduced cell-cycle progression and metabolic activity, explaining why these myeloid cells can escape immune responses [95, 96]. A similar mechanism has also been demonstrated in Myc-driven lymphomas. In these neoplasms, the oncogenic activity of Myc leads to PD-L1 and CD47 overexpression. CD47 is a transmembrane protein that by binding to signal regulatory protein-α (SIRPα) expressed on APCs limits their activity and blocks their antigen uptake [97]. In these neoplastic cells, Myc inactivation leads to a reduction in PD-L1 and CD47 expression and reverses their phenotype [98]. However, retrospective studies have shown that the administration of anti-PD-L1 in patients with Hodgkin's lymphoma relapsed after allogenic HSCT is associated with high GVHD rates, a potential limitation of immune-checkpoint inhibition in the transplant setting [99]. A higher incidence of both acute and chronic GVHDs has also been reported in other studies that used higher anti-PD-L1 dosages. Ipilimumab is an anti-CTLA-4 antibody whose safety and efficacy were tested in a phase 1/1b multicenter study that enrolled twenty-eight patients with hematological neoplasms relapsed after allogenic HSCT. The overall response rate was 32%, including 23% complete remissions, and responses were long-lasting [94]. In patients who responded to ipilimumab, tissue analysis revealed higher $CD8^+$ T-cell and lower Treg counts. Thus, further studies with ipilimumab in AML relapsed after transplant are currently ongoing to establish the effectiveness of this therapeutic strategy in preventing posttransplant immune escape.

Another mechanism of LSCs to evade immune control relies on the production of anti-inflammatory cytokines that create an anti-inflammatory microenvironment able to block the immune response by hindering leukemic cell recognition and destruction. Several studies performed in the nontransplant setting have shown that CML cells can produce IL-4 and TGF-β that reduce the expression of MHC class II molecules, an event that renders leukemic cells less immunogenic [100–103]. IL-4 and IL-10 are also secreted by AML and chronic lymphocytic leukemia (CLL) cells, and these cytokines support the CLL immune escape program [104, 105]. Proinflammatory cytokines, including interferon-γ (IFN-γ), IL-15, IL-1β, and granulocyte colony-stimulating factor (G-CSF), are produced by normal myeloid and lymphoid progenitors and improve leukemic cell recognition and immune cell activation. Therefore, it is reasonable for leukemic cells to block the production of these proinflammatory cytokines, as demonstrated by laboratory and clinical data. In a clinical study performed in 393 patients with B-lineage ALL, lower IFN-γ levels were associated with a younger age at diagnosis and a high-risk profile defined by prednisone response, cytological remission, and minimal residual disease. This finding supports the role of IFN-γ in immunosurveillance and its effect on the early response to steroid therapy [106]. IL-15 is produced by healthy myeloid progenitors and promotes leukemia control by stimulating the generation of human memory stem T cells from naive precursors and by expanding NK cells, thereby boosting the alloimmune effect to eliminate residual LSCs after allogenic HSCT [107–110]. IL-15 also enhances T-cell antitumor activity by reprogramming their mitochondrial metabolism [111–113]. Based on these data, various studies have employed IL-15 to eliminate residual leukemic cells after allogenic HSCT [109]. Apart from HSCT, IL-15 secretion by healthy myeloid precursors stimulates AML cell recognition and elimination by $CD8^+$ T cells and NK cells. More importantly, AML cells carrying the internal tandem duplication of the FLT3 gene (FLT3-ITD) are able to block IL-15 production. In animal studies, FLT3 inhibition together with T-cell transfer promoted AML cell elimination

and helped achieve long-term disease control [111]. IL-15 production stimulated by sorafenib and midostaurin enhances the immunogenicity of leukemic cells and the activation of T cells. This result supports the role of FLT3 activity in the regulation of IL-15 secretion.

Moreover, it can be speculated that other mechanisms of immune escape may rely on the block of several proinflammatory cytokines, such as G-CSF (which is involved in APC maturation) and IL-1β. Further studies are needed to clarify the mechanisms by which these inflammatory cytokines help the immune system recognize and eliminate leukemic cells in order to develop therapeutic strategies. However, it is known that these cells can also evade immune surveillance by producing various immunosuppressive enzymes. One of these, indoleamine 2,3-dioxygenase 1 (IDO1), is responsible for the first step of tryptophan degradation and subsequent kynurenine production. Both the lack of tryptophan and the presence of kynurenine negatively affect T-cell functions and reprogram Treg activity [114]. IDO1 is expressed by leukemic cells, and its production has been linked to an unfavourable prognosis [115]. Arginase, another enzyme produced by AML cells, is required for the degradation of arginine that is needed for T-cell proliferation and polarization of monocytes toward an inflammatory M1-like phenotype. In laboratory studies, T-cell proliferation and NK-cell proliferation were inhibited when AML cell supernatants were added to lymphocytes cultures [116, 117]. Ectonucleotidase (CD73) is an enzyme required for enzymatic cleavage of adenosine monophosphate (AMP), which results in an adenosine with an immunosuppressive effect. Thus, CD73 inhibition could be a promising therapeutic target as it might enhance leukemia control. CD39, another ectonucleotidase that produces adenosine diphosphate (ADP) and AMP from adenosine triphosphate (ATP), might also represent a logical target to prevent the immunosuppressive activity of AML cells [118, 119].

7. Conclusions

AML treatment has remained unchanged over the past twenty-five years and is frequently associated with dismal outcomes. This has not changed with the development of molecular drugs that are mutation specific, as they do not address the genetic heterogeneity of the disease. Thus, there is a need for the development of novel treatments that target the cellular and molecular mechanisms controlling dynamic AML-niche interactions and resolve niche-mediated drug resistance. Current studies have continued to highlight the role of the niche in AML development and progression, and potential targets of niche-directed treatments are now starting to emerge. AML xenograft models have revealed that severe vascular BM damage consisting of increased vascular leakiness occurs as the primary AML-induced niche alteration and leads to mobilization of healthy HSCs to the periphery. This experimental finding may seriously limit the success of allo-HSCT by reducing the outcompetition effect of patient residual and donor-derived HSCs on the AML cell population. Therefore, studies aimed at keeping HSCs in their BM niches are warranted [120]. This goal might be achieved by considering studies that aim to reveal very specific requirements of the neoplastic cell to alter their microenvironments. For example, a current study has suggested that the main determinant of LSC or HSC physical localization might be their metabolic status (i.e., glycolytic level) [121]. Using a metabolic imaging system with a highly responsive genetically encoded metabolic sensor (SoNar), this study identified pyruvate dehydrogenase kinase 2 (PDK2) as the enzyme that fine tunes glycolysis, homing, and symmetric division of LSCs. SoNar-high cells, which prefer homing to the endosteal niche and symmetric division to maintain their leukemogenic activities, are more glycolytic, enriched for higher LSC frequency, and develop leukemia much faster than SoNar-low counterparts. Thus, these recent findings have pinpointed novel relevant niche alterations that are expected to be the basis for the development of innovative strategies aimed at eradicating LSCs and sparing normal HSCs.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Research Article

Mutated *WT1*, *FLT3-ITD*, and *NUP98-NSD1* Fusion in Various Combinations Define a Poor Prognostic Group in Pediatric Acute Myeloid Leukemia

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Acute myeloid leukemia is a life-threatening malignancy in children and adolescents treated predominantly by risk-adapted intensive chemotherapy that is partly supported by allogeneic stem cell transplantation. Mutations in the *WT1* gene and *NUP98-NSD1* fusion are predictors of poor survival outcome/prognosis that frequently occur in combination with internal tandem duplications of the juxta-membrane domain of *FLT3* (*FLT3-ITD).* To re-evaluate the effect of these factors in contemporary protocols, 353 patients (<18 years) treated in Germany with AML-BFM treatment protocols between 2004 and 2017 were included. Presence of mutated *WT1* and *FLT3-ITD* in blasts (n=19) resulted in low 3-year event-free survival of 29% and overall survival of 33% compared to rates of 45-63% and 67-87% in patients with only one (only *FLT3-ITD; n=33,* only *WT1* mutation; n=29) or none of these mutations (n=272). Including *NUP98-NSD1* and high allelic ratio (AR) of *FLT3-ITD* (AR ≥0.4) in the analysis revealed very poor outcomes for patients with co-occurrence of all three factors or any of double combinations. All these patients (n=15) experienced events and the probability of overall survival was low (27%). We conclude that co-occurrence of *WT1* mutation, *NUP98-NSD1,* and *FLT3-ITD* with an AR ≥0.4 as triple or double mutations still predicts dismal response to contemporary first- and second-line treatment for pediatric acute myeloid leukemia.

1. Introduction

Pediatric acute myeloid leukemia (AML) is a rare and heterogeneous disorder, for which continuous improvement of risk-adapted treatment approaches over the last 30 years has led to overall survival rates of approximately 70% [1, 2]. In current pediatric AML treatment protocols, cytogenetic abnormalities of the leukemic blasts at initial diagnosis are important indicators for risk group stratification and treatment assignment [1, 2]. Approximately, 25% of pediatric patients have AML blasts with a normal karyotype, but even these cases often harbor somatic mutations in genes such as *WILMS TUMOR 1* (*WT1*), *NPM1*, *NRAS, KRAS*, *Fms-like tyrosine kinase 3* (*FLT3),* and/or *c-KIT/CD117* [1, 2].

The *WT1* gene is located on chromosome 11, has ten exons and four zinc finger domains, and functions as a transcription factor and master regulator of tissue development [3]. Within normal hematopoiesis, *WT1* has two distinct roles: in early

stages, it mediates quiescence of primitive progenitor cells, and later, WT1 expression is important for differentiation towards the myeloid lineage [4]. In AML, *WT1* mutations are present in approximately 10% of patients and predominantly located in exons 7 and 9, which contain the DNA-binding zinc finger domains of the protein. The majority of these mutations are out-of-frame deletion/insertions or premature termination codons that will lead to truncated proteins with altered functional consequences for the cells [5]. If these truncated proteins are stable, they might have dominant negative effects by partially blocking the wild-type WT1 protein; if unstable, the diminished WT1 protein levels may lead to haploinsufficiency [5]. Nevertheless, it has been clearly established that the occurrence of *WT1* mutations in AML blasts with normal karyotypes is associated with adverse clinical outcomes in adult [6–9] as well as pediatric patients [10, 11].

Somatic *WT1* mutations in AML blasts often co-occur with other genetic aberrations, most frequently with an internal tandem duplication in the juxta-membrane domain of the tyrosine kinase receptor *FLT3* (*FLT3-ITD*) [5]. Classified as type-I or proliferating mutation, *FLT3-ITDs* are present in 10-15% of pediatric AML cases and lead to poor clinical outcomes [12–14]. We previously demonstrated in a cohort of 298 pediatric patients with *de novo* AML treated before 2004 on AML-BFM protocols that the combination of *FLT3-ITD* and mutated *WT1* is associated with even worse survival [10]. Comparably, an independent study from the Children's Oncology Group (COG) in a cohort of 842 children with *de novo* AML showed that the poor prognostic impact of *WT1* mutations depends on the *FLT3-ITD* status [11]. These two pediatric studies confirmed earlier findings in adults that first established the adverse prognostic impact of both *WT1* and *FLT3-ITD* mutations [15, 16].

Two additional prognostic indicators in *FLT3-ITD*-positive AML cases established in the last few years are the mutational burden in each patient defined as the ratio between mutant and wild-type *FLT3-ITD* alleles (allelic ratio, AR) [12, 17, 18] and the co-occurrence of *FLT3-ITD* with a cytogenetically cryptic translocation of chromosomes 5 and 11 or t(5;11)(q35;p15) [19]. This translocation leads to fusion of the *nucleoporin* (*NUP98*) gene on chromosome 11 and the gene for nuclear receptor binding SET-domain protein 1 (*NSD1*) of chromosome 5 (*NUP98-NSD1*). As the breakpoints for the NUP98 gene are often not detected by classical cytogenetic due to its terminal localization at 11p15, it has been described in AML cases with a "normal" karyotype [20]. Importantly, this rare recurrent aberration is mutually exclusive with other recurrent translocations and more prevalent in pediatric AML, in which it is associated with the presence of *FLT3-ITD* and poor survival outcomes [21, 22].

In the present study, we re-evaluated the role of mutations in *WT1*, *FLT3-ITD,* and the *NUP98-NSD1* translocation as prognostic factors in two contemporary pediatric treatment protocols by analyzing their association with co-occurring genetic and cytogenetic aberrations and by determining their clinical significance and influence on treatment outcome. Thereby, we were able to define a group of high-risk patients for which the efforts for salvage/second line treatment largely failed.

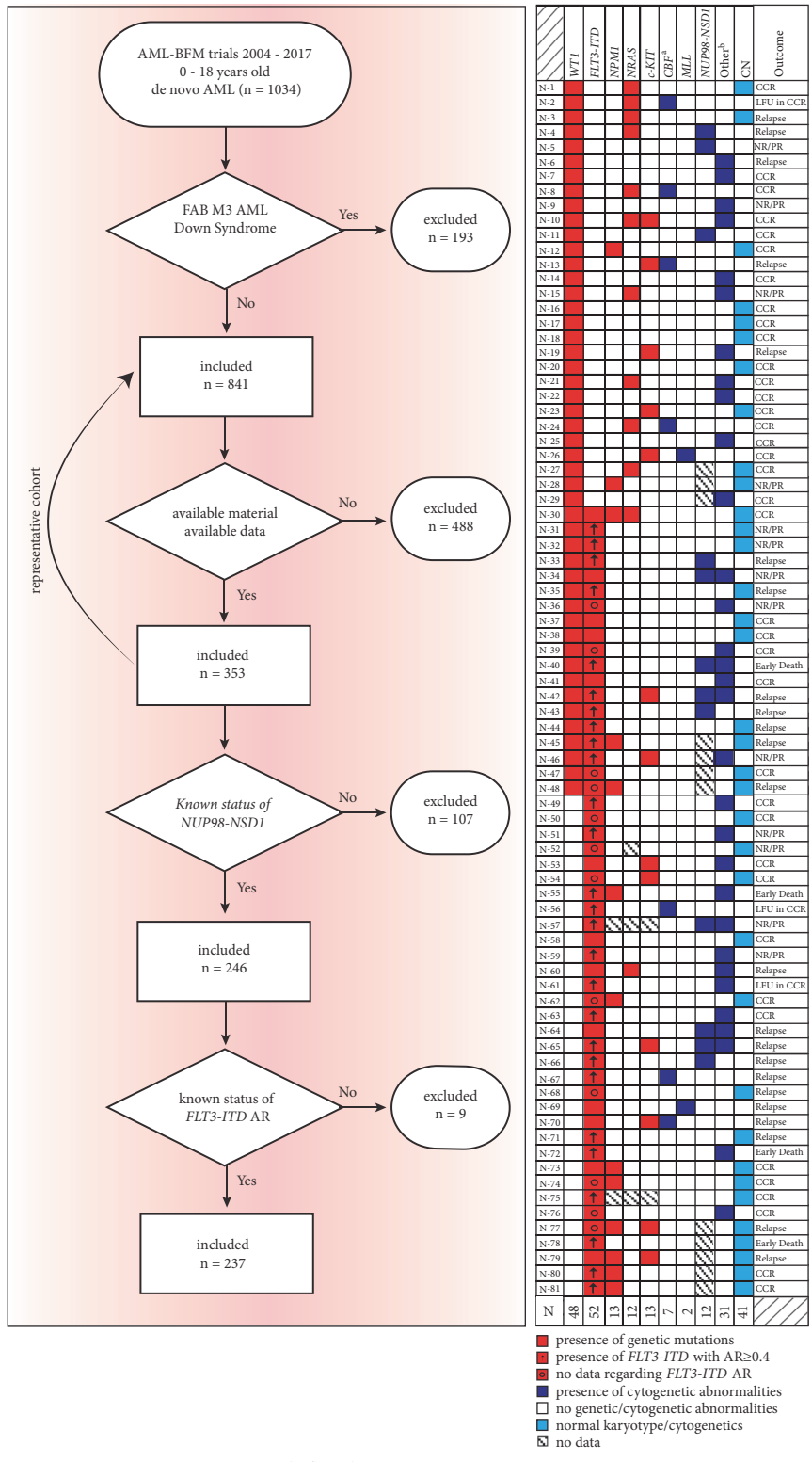
2. Materials and Methods

From April 2004 to May 2017, 841 patients aged 0–18 years with *de novo* AML (excluding FAB M3 and Down Syndrome) were treated in Germany according to the AML-BFM 04 trial (ClinicalTrials.gov Identifier: NCT00111345) or the AML-BFM 2012 registry and trial (EudraCT number: 2013-000018-39) (Figure 1(a)). Both trials were approved by the ethical committees and institutional review boards of university hospitals of Münster and Hannover and an informed consent was obtained from each patient or their legal guardians before the beginning of treatment. Standard procedures for the diagnosis of AML were carried out by the German AML-BFM reference laboratory as previously described [23–25]. This included mutation analysis in *WT1*, *FLT3-ITD*, *NPM1*, *NRAS,* and *c-KIT* by Sanger and/or next-generation sequencing or GeneScan analysis. In 353 patients (42%), sufficient material and clinical data were available for further analysis. As a confirmation, material from *WT1* and/or *FLT3-ITD* positive and negative cases was re-analyzed by next-generation sequencing (NGS) using the TruSight Myeloid Panel (Illumina)[26] with median read counts for *WT1* and *FLT3-ITD* of around 4,200 and 6,000 reads, respectively, as we described previously [27]. In addition, the allelic ratio of *FLT3-ITD* to *FLT3* wild-type was calculated via GeneScan analysis [13] and the expression of *NUP98-NSD1* was analyzed in 246 out of 353 patients with available material by real-time quantitative PCR using previously described primers [19]. Initial analysis demonstrated that the selected cohort was representative for all patients treated between 2004 and 2017 on the AML-BFM protocols for features such as gender, age, AML subtype, initial cytogenetics, and preliminary, early response to treatment (data not shown).

Clinical end-points were defined as previously described [28, 29] and survival rates were calculated via Kaplan-Meier analysis and compared by log-rank test. Multivariate analysis was performed using Cox regression model evaluating the hazard ratio (HR) of each covariate with 95% confidence interval (CI). Stem cell transplantation was included in the Cox regression model as a time-dependent variable. Differences with a p value less than 0.05 were considered as significant. Data were analyzed using the Statistical Analysis System software version 9.4 (SAS Institute, Cary, NC). Data acquisition was stopped at June 30, 2018, with a median follow-up of 3.6 years.

3. Results

3.1. Study Cohort and Patient Characteristics. In this study, we included 353 patients treated on either the AML-BFM 2004 or AML-BFM 2012 protocol for whom sufficient material and information were available (Figure 1(a)). As shown in Table 1, 48 (14%) patients had *WT1* and 52 (15%) *FLT3-ITD* mutations in their leukemic blasts at diagnosis. Mutations in *NPM1*, *NRAS,* and *c-KIT* were present in the blasts of 9%, 17%, and 12% of patients, respectively. Most patients with mutated *WT1* (n=35, 73%) harbored at least one co-occurring mutation in the AML blasts, with the most common being *FLT3-ITD* (n=19, 40%) followed by *NRAS* mutations (n=11,



(a) Study flowchart

(b) Features

Figure 1: *Study flowchart and patient characteristics.* (a) Study flowchart outlining the process of patient recruitment in the data analysis. (b) *WT1* mutations often co-occurred with *FLT3-ITD* and other genetic aberrations. AML-BFM, acute myeloid leukemia-Berlin-Frankfurt-Muenster; n, number; *WT1*, *Wilms Tumor 1*; *FLT3-ITD*, fms-related tyrosine kinase 3-internal tandem duplication; *NPM1*, *nucleophosmin 1*; *NRAS*, *neuroblastoma RAS viral oncogene homolog*; *c-KIT*, *KIT proto-oncogene*; CBF, core binding factor; MLL, rearrangements of *MLL* gene; *NUP98-NSD1*, *Nucleoporin-Nuclear Receptor Binding SET Domain Protein 1* fusion gene; CN, cytogenetic-normal AML; AR, allelic ratio; CCR, continued complete remission; LFU, lost to followup; NR, non-response; PR, partial remission. [a]CBF aberrations include translocation of chromosomes 8 and 21 and inversion or translocation of chromosome 16. [b]Other cytogenetic aberrations such as trisomy 8, various chromosomal translocations, and complex karyotype alterations.

Table 1: Patient Characteristics.

FEATURES		All patients		*WT1* wild-type		*WT1* mutated		p*	*FLT3-ITD* neg.		*FLT3-ITD* pos.		p*
		n	%	n	%	n	%		n	%	n	%	
Study Population													
Number (%)		353	100	305	100	48	100		301	100	52	100	
Age (years), median (range)		9.09 (0 -18)		7.97 (0 - 18)		10.68 (0.8 - 17.8)		0.03	7.8 (0 -18)		12.95 (2.7 - 17.9)		0.0001
Gender													
male		183	52%	159	52%	24	50%	0.783	153	51%	30	58%	0.36
female		170	48%	146	48%	24	50%		148	49%	22	42%	
WBC count at diagnosis													
median x 10^9 cells/L (range)		24.5 (0.019 - 475)		24 (0.23 - 475)		42.85 (0.019 - 324)		0.1	20.1 (0.019-475)		73.5 (1.8 - 324)		0.0001
Morphological Classification													
FAB	M0	9	3%	7	2%	2	4%	0.008	5	2%	4	8%	0.0001
	M1/M2	134	38%	107	35%	27	56%		100	33%	34	65%	
	M4Eo+	31	9%	27	9%	4	8%		29	10%	2	4%	
	M4Eo-/M5	132	37%	125	41%	7	15%		121	40%	11	21%	
	M6	3	1%	2	1%	1	2%		3	1%	0	0%	
	M7	22	6%	21	7%	1	2%		22	7%	0	0%	
	AUL/other	22	6%	16	5%	6	13%		21	7%	1	2%	
Cytogenetics													
	t(8;21)	38	11%	38	12%	4	8%	<0.0001	36	12%	2	4%	0.0001
	inv(16)	32	9%	28	9%	0	0%		31	10%	1	2%	
	MLL rearr.	61	17%	60	20%	1	2%		60	20%	1	2%	
	others	117	33%	99	32%	18	38%		97	32%	20	38%	
	normal	97	27%	72	24%	25	52%		69	23%	28	54%	
	no data	8	2%	8	3%	0	0%		8	3%	0	0%	
NUP98-NSD1													
	positive	15	4%	7	2%	8	17%	<0.0001	6	2%	9	17%	<0.0001
	negative	231	65%	198	65%	33	69%		197	65%	34	65%	
	no data	107	30%	100	33%	7	15%		98	33%	9	17%	
Co-mutations													
FLT3-ITD	negative	301	85%	272	89%	29	60%	<0.0001					
	positive	52	15%	33	11%	19	40%						
WT1	wildtype	305	86%						272	90%	33	63%	0.0001
	mutated	48	14%						29	10%	19	37%	
NPM1	wildtype	316	90%	273	90%	43	90%	0.69	277	92%	39	75%	0.0004
	mutated	31	9%	26	9%	5	10%		20	7%	11	21%	
	no data	6	2%	6	2%	0	0%		4	1%	2	4%	
nRAS	wildtype	282	80%	245	80%	37	77%	0.26	235	78%	47	90%	0.008
	mutated	59	17%	48	16%	11	23%		57	19%	2	4%	
	no data	12	3%	12	4%	0	0%		9	3%	3	6%	
KIT	wildtype	308	87%	267	88%	41	85%	0.51	266	88%	42	81%	0.31
	mutated	41	12%	34	11%	7	15%		33	11%	8	15%	
	no data	4	1%	4	1%	0	0%		2	1%	2	4%	
CEBPA	wildtype	163	46%	121	40%	42	88%	<0.0001	122	41%	41	79%	<0.0001
	single	6	2%	4	1%	2	4%		6	2%	0	0%	
	double	10	3%	9	3%	1	2%		8	3%	2	4%	
	no data	174	49%	174	57%	0	0%		165	55%	9	17%	
HSCT													
HSCT in Its CR		64	18%	56	18%	8	17%	0.78	53	18%	11	21%	0.54
Chemotherapy only		289	82%	249	82%	40	83%		248	82%	41	79%	
Patient Status													
	alive	251	71%	221	72%	30	63%	0.14	225	75%	26	50%	0.0001
	deceased	73	21%	58	19%	15	31%		51	17%	22	42%	
	LFU	29	8%	26	9%	3	6%		25	8%	4	8%	

n, number; *WT1*, *Wilms tumor 1*; *FLT3-ITD, fms related tyrosine kinase 3*-internal tandem duplication; WBC, white blood cell; FAB, French-American-British; M4Eo+, AML M4 subtype with the presence of atypical eosinophils; M4Eo-, AML M4 subtype without the presence of atypical eosinophils; AUL, acute undifferentiated leukemia; t, translocation; inv, inversion; MLL rear., rearrangement of MLL gene; *NUP98-NSD1*, *Nucleoporin-Nuclear Receptor Binding SET Domain Protein 1* fusion gene; *NPM1*, *nucleophosmin 1*; *NRAS, neuroblastoma RAS viral oncogene homolog; c-KIT*, *KIT* proto-oncogene; *CEBPA*, *CCAAT/enhancer binding protein (C/EBP) alpha*; HSCT, hematopoietic stem cell transplantation; CR, complete remission; HSCT, hematopoietic stem cell transplantation; LFU, lost to follow-up. *p-values derived from Pearson's Chi-squares test.

23%, Table 1 and Figure 1(b)). Comparably, the majority of patients with *FLT3-ITD* had additional mutations in other genes (n=32, 62%), most commonly in *WT1* (n=19, 37%) and *NPM1* (n=11, 21%). Patients with mutated *WT1* or *FLT3-ITD* were older compared to the rest of the study cohort, and AML FAB M1/M2 was the most common morphologic subtype in both groups (Table 1). In addition, the AML blasts of more than half of patients with *WT1* (n=25/48, 52%) and *FLT3-ITD* (n=28/52, 54%) mutations had a normal karyotype at diagnosis; these percentages were significantly higher than those in patients without mutations in each of the two genes ($p<0.0001$, Table 1).

3.2. Characteristics of WT1 Mutations. We identified 64 different *WT1* sequence alterations in 48 patients (Table 2). These alterations were frequently located in exon 7 (n=55, 86%) and predominantly resulted in frameshifts producing premature termination codons (PTCs). In total, nine single nucleotide variants (SNVs) were found, mostly in exon 9 (n=7, 78%). Only three of the nine SNVs were not previously reported as pathogenic (Table 2). Using NGS, we characterized multiple distinct *WT1* mutations with highly diverse variant allele frequencies in 13 patients (11 patients had two and 2 patients, three distinct mutations). We then analyzed the heterozygosity of these mutations via the integrative genomic viewer (Broad Institute, MA, USA) and determined that they were all located on individual/different alleles/reads (Table 2).

3.3. Survival Significance of the Genomic Aberrations. Next, we analyzed the impact of each mutation on the clinical outcomes. Our analysis identified *WT1* and *FLT3-ITD,* but not *NRAS*, *NPM1,* or *c-KIT* mutations as single factors that significantly increased the chance of relapse or treatment failure and reduced the probability of 3-year overall survival (OS) in our patient cohort (Figures 2(a), 2(b), and 3). In addition, *FLT3-ITD* but not *WT1* mutations significantly decreased the 3-year probability of event-free survival (EFS, Figure 2(b)). When we grouped the two mutations together, the survival analysis revealed a 3-year EFS of 29±11% for patients with both *WT1* and *FLT3-ITD* mutations compared to 63±3% for patients with none of these mutations ($p=0.0004$) and 61±11% or 45±9% for patients with only *WT1* mutation ($p=0.016$) or *FLT3-ITD* ($p=0.16$), respectively (Figure 2(c)). Corresponding to this low EFS, co-occurrence of these two mutations was associated with an increased cumulative incidence of relapse (CIR) of 65±12% compared to 32±12% for patients with none of these mutations ($p=0.002$) and 39±11% or 46±9% for patients with only *WT1* mutation ($p=0.05$) or *FLT3-ITD* ($p=0.08$), respectively (Figure 2(c)). Furthermore, we identified a low 3-year OS probability of 33±12% in patients with co-occurrence of *WT1* and *FLT3-ITD*, which was significantly lower than those of patients without these mutations (81±3%, $p<0.0001$), patients with only mutated *WT1* (87±7%, $p=0.0007$), and patients with only *FLT3-ITD* (67±9%, $p=0.017$, Figure 2(c)). Comparing the curves for EFS and OS clearly demonstrated that our second line treatment was not able to rescue any patient with co-occurrence of *WT1* and *FLT3-ITD* mutations, while the OS rates increased by more than 20% for the other three subgroups (Figure 2(c)).

3.4. Impact of NUP98-NSD1 Fusion. To further characterize the prognostic significance of *WT1* and *FLT3-ITD* mutations, we analyzed the expression of *NUP98-NSD1* fusion in our patient cohort (Figure 1(a)). From 246 patients with available material for this retrospective real-time quantitative PCR analysis, 15 (6%) of them were identified to have the *NUP98-NSD1* translocation. Most of these patients (12/15, 80%) harbored additional *WT1* or *FLT3-ITD* mutations: 3 patients carried both *WT1* and *NUP98-NSD1*, 4 had a co-occurrence of *FLT3-ITD* and *NUP98-NSD1*, and 5 patients carried all three genetic alterations (Figure 1(b)). Only 1 of these 15 patients had a previous known status of *NUP98-NSD1* by conventional karyotyping: 2 others were previously diagnosed with deletion of chromosome 5, 1 carried an inversion of chromosome 16 (no other mutations and still in continuous complete remission), 4 carried complex karyotypes or rare aberrations, and 7 had no other cytogenetic abnormalities (data not shown).

We then analyzed the prognostic significance of *NUP98-NSD1* in the cohort of 246 patients with the known status of this fusion gene (Figure 1(a)). As a single factor, the presence of *NUP98-NSD1* in AML blasts of patients at diagnosis was associated with a significant increase in CIR (81%) in addition to decreased probabilities of 3-year EFS and OS (Figure 4(a)). Combining *NUP98-NSD1* with *WT1* and *FLT3-ITD* mutations in our multifactor survival analysis revealed that patients with all three or either two of these mutations had worse survival outcomes. These patients had a higher CIR of 73±11% compared to the CIR of 30±4% for patients with none of these aberrations or *NUP98-NSD1* alone ($p<0.0001$) and the CIR of 37±13% or 38±10% for patients with only mutated *WT1* ($p=0.0078$) or *FLT3-ITD* ($p=0.013$), respectively (Figures 4(a) and 4(b)). The increased CIR translated into a lower 3-year EFS probability of 23±10% for patients with triple or double mutations compared to the EFS of 62±4% for patients with none of these mutations or only *NUP98-NSD1* ($p<0.0001$) and the EFS of 63±13% or 54±10% for patients with only *WT1* ($p=0.003$) or *FLT3-ITD* ($p=0.036$) mutations, respectively (Figure 4(b)). Moreover, co-occurrence of all three or any double mutations resulted in a significantly lower 3-year OS probability of 42±12% compared to 80±8% for patients with none of the mutations or only *NUP98-NSD1* ($p=0.0003$) and 88±8% or 73±10% for patients with only *WT1* ($p=0.0007$) or *FLT3-ITD* ($p=0.049$) mutations, respectively (Figure 4(b)).

3.5. Survival Significance of the FLT3-ITD Allelic Ratio. We have previously established the prognostic significance of an *FLT3-ITD* allelic ratio of ≥0.4 in pediatric AML [12]. Therefore, to determine the impact of the mutational burden of *FLT3-ITD* on treatment outcomes in the present cohort, we calculated the *FLT3-ITD* AR in patients with available data/material. As indicated in Figure 1(b), 27 patients had an AR ≥0.4 at diagnosis. Analyzing the survival impact of

Table 2: Characteristics of *WT1* Variants.

UPN	exon	seq. read	mutation sequence[a]	amino acid alteration	VF (%)	dbSNP or COSMIC ID	published	previously reported sample	outcome
missense substitutions									
8	9		c.1333C>T	p.Arg445Trp	19.1	rs121907900, COSM21417	Yes	WT	CCR
15	9		c.1345C>A	p.Leu449Met	5.49		No		CCR
20	9		c.1385G>A	p.Arg462Gln	47.21	rs121907903, COSM4191067	Yes	AML, colon cancer, adenocarcinoma	CCR
21	9		c.1343A>G	p.His448Arg	33.12	COSM7335365	Yes	AML, mesothelioma	CCR
23	9		c.1333C>T	p.Arg445Trp	72.42	rs121907900	Yes	WT, DDS	CCR
26	7		c.1097C>G	p.Ser366Cys	2.57		No		CCR
35	9	different	c.1334G>A	p.Arg445Gln	3.12	rs121907903, COSM4191067	Yes	AML, colon cancer, adenocarcinoma	Relapse
	9	different	c.1307G>A	p.Cys436Tyr	44.21	COSM21438	Yes	AML	
nonsense substitutions/insertions, deletions or duplications									
1	7		c.1090_1093dupTC	p.Ala365Valfs∗4	43	COSM5487332	Yes	AML	CCR
2	7		c.1048-4_1056dupGCAGGATGTGCGA	p.Arg353Alafs∗19	30.25		No		LFU in CCR
3	7		c.1087_1161dup74	p.Lys387Asnfs∗44	n.d.		No		Relapse
4	7	different	c.1087_1091dupCGGTC	p.Ala365Glyfs∗69	5.08	COSM28954	Yes	AML, T-ALL	Relapse
4	7	different	c.1091C>A	p.Ser364∗	28.38	COSM27307	Yes	AML, WT	
5	7		c.1083_1098delTGTACGGTCGGCATCT	p.Val362Argfs∗65	46.82		No		NR/PR
6	7		c.1059dupT	p.Val354Cysfs∗14	35.9	COSM1317324	Yes	AML	Relapse
7	7		c.1179dupG	p.His394Alafs∗8	25		No		CCR
9	7	different	c.1078_1079insGCCGA	p.Thr360Serfs∗74	38.7		No		NR/PR
	7	different	c.1084_1085insGC	p.Val362Glyfs∗71	52.9		No		
10	7		c.1074_1077dupCCCG	p.Thr360Profs∗9	9.9		No		CCR
11	7		c.1079_1090delCTCTTGTACGGTinsTGGG	p.Thr360Metfs∗5	55.23		No		CCR
12	7		c.1058_1059insGA	p.Val354Metfs∗5	31.6		No		CCR
13	7	different	c.1058_1059insGGTG	p.Pro355Cysfs∗14	5.6		No		Relapse
	7	different	c.1078_1084dupACTCTTG	p.Val362Aspfs∗8	8.3	COSM5879281	Yes	AML	
14	7		c.1090_1093dupTCGG	p.Ala365Valfs∗4	22.81	COSM21392	Yes	AML	CCR
16	7		c.1054_1084dup	p.Val362Alafs∗16	7.3		No		CCR
17	7		c.1087delCinsGGG	p.Arg363Glyfs∗70	24.3		No		CCR
18	7		c.1054_1055insT	p.Arg352Leufs∗16	67.2	COSM5751511	Yes	T-ALL	CCR
19	7		c.1077_1078insTGTTTCTTCCGCCCAG	p.Thr360Cysfs∗13	36.95		No		Relapse
22	7		c.1087delCinsGG	p.Arg363Glyfs∗5	41.88		Yes	AML	CCR
24	7		c.1083_1090dupTGTACGGT	p.Ser364Leufs∗71	3.8	COSM27309	Yes	AML	CCR

Table 2: Continued.

UPN	exon	seq. read	mutation sequence[a]	amino acid alteration	VF (%)	dbSNP or COSMIC ID	published	previously reported sample	outcome
25	9	different	c.1323_1338dupAAAGTTCTCCCGGTCC	p.Asp447Lysfs∗18	40.1		No		CCR
	9	different	c.1322_1332dupGAAAGTTCTCC	p.Arg445Glufs∗9	40.5		No		
27	7	different	c.1077_1078insGTTG	p.Thr360Valfs∗9	43.71		No		CCR
	7	different	c.1089dupG	p.Ser364Valfs∗4	49.24	COSM28966	Yes	AML	
28	7	different	c.1058delGinsCCA	p.Arg353Profs∗6	19.72		No		NR/PR
	7	different	c.1054_1055insAAAAAGATT	p.Arg352delins4	19.55		No		
29	7		c.1179dupG	p.His394Alafs∗8	25		No		CCR
30	7		c.1048_1057delGATGTGCGACinsAAGG	p.Asp350_Arg353	46.34		No		CCR
31	7		c.1093dupG	p.Ala365Glyfs∗3	44.49		Yes	AML	NR/PR
32	7		c.1048-8_1055dupGCCTGCAGGATGTGCG	p.Arg353Profs∗20	2.5		No		NR/PR
33	7		c.1090_1091dupTC	p.Ala365Argfs∗68	44.25	COSM28955	Yes	AML	Relapse
34	7	different	c.1087delCinsGA	p.Arg363Glufs∗5	4.1		No		NR/PR
	7	different	c.1086dupA	p.Arg363Thrfs∗5	5.33	COSM1166631	Yes	AML	
	7	different	c.1090_1093dupTC	p.Ala365Valfs∗4	36.29	COSM5487332	Yes	AML	
36	7	different	c.1090_1093dupTCGG	p.Ala365Valfs∗4	6.94	COSM5487332	Yes	AML	NR/PR
	7	different	c.1091dupC	p.Ala365Glyfs∗3	39.42	COSM27304	Yes	AML	
37	7	different	c.1057delCinsGG	p.Arg353Glyfs∗15	42.78		Yes	AML	CCR
	7	different	c.1087delCinsGGG	p.Arg363Glyfs∗70	52.11		No		
38	7		c.1068_1076delAGTAGCCCCinsGACGGTCGTTATTA	p.Val357Thrfs∗77	42.14		No		CCR
39	7		c.1087delCinsGG	p.Arg363Glyfs∗5	47.54		Yes	AML	CCR
40	7	different	c.1058_1059insGGTGCCGCTCG	p.Gly356Leufs∗6	48.49		No		Early Death
	7	different	c.1082_1091dupTTGTACGGTC	p.Ala365Cysfs∗6	41.83	COSM27303	Yes	AML	
41	7	different	c.1123dupA	p.Met375Asnfs∗9	44.5		Yes	AML	CCR
	7	different	c.1057_1058insTA	p.Arg353Leufs∗6	45.8		No		
42	7		c.1051_1055dupGTGCG	p.Arg353Cysfs∗7	34.73		No		Relapse
43	7		c.1058delGinsCC	p.Arg353Profs∗15	44.78	COSM28946	Yes	AML	Relapse
44	7	different	c.1079_1101delinsGAA	p.Thr360Argfs∗4	20.37		No		Relapse
	7	different	c.1088_1089insCTCGG	p.Ala365Glyfs∗69	10.69		No		
45	7		c.1090_1091insAGGT	p.Ser364∗fs∗1	42.97		No		Relapse
46	7		c.1058delGinsCC	p.Arg353Profs∗15	51.08	COSM28946	Yes	AML, T-ALL	NR/PR
47	7	different	c.1048-3_1055dupCAGGATGTGCG	p.Val354Metfs∗8	2.49		No		CCR
	7	different	c.1053dupG	p.Arg352Alafs∗16	3.83	COSM28980	Yes	AML	
	7	different	c.1054delCinsGG	p.Arg352Glyfs∗16	35.86	COSM28970	Yes	AML, T-ALL	
48	7		c.1089_1090insGGCCTCTTGTACGG	p.Ser364Glyfs∗73	40.49		No		Relapse

UPN, unique patient number; Seq. read, sequence read; VF, variant allele frequency; dup, duplication; ins, insertion; indel, Insertion-deletion; fs, frame-shift; ∗termination codon; WT, Wilms tumor; DDS, Denis-Drash syndrome; T-ALL, T-cell acute lymphoblastic leukemia; CCR, continued complete remission, NR, non-response; PR, partial response; LFU, lost to follow-up.

[a]Transcript ID: NM_000378 was used to describe all alterations.

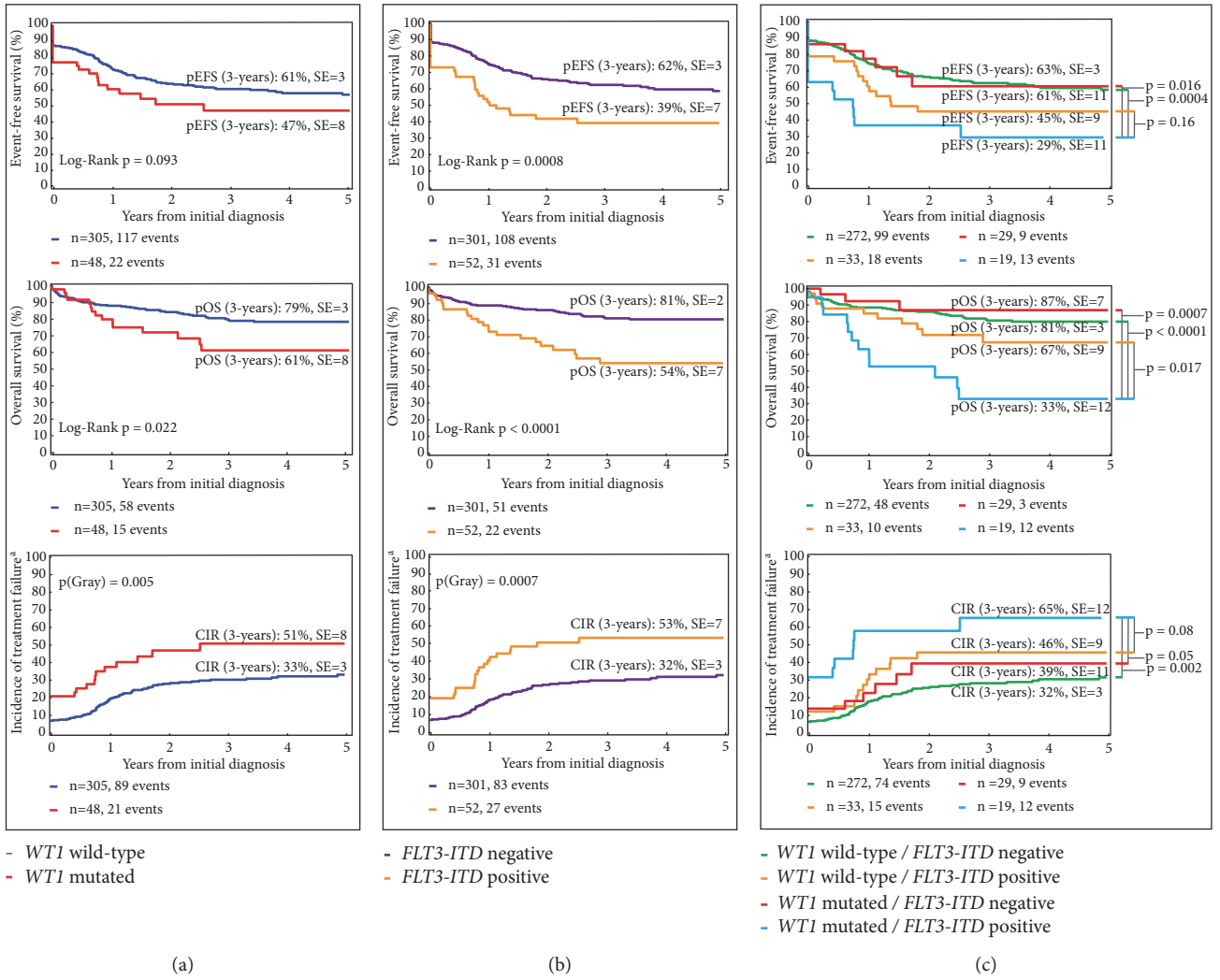


Figure 2: *Co-occurrence of WT1 and FLT3-ITD mutations at initial diagnosis of pediatric AML predicts poor survival outcomes.* (a) *WT1* mutation as single factor increased the incidence of relapse, reducing the probability of survival. (b) The presence of *FLT3-ITD*, individually, leads to an increased chance of relapse and decreased patient survival. (c) Clinical consequences of *WT1* mutations and *FLT3-ITD* were dependent on each other. *WT1*, *Wilms Tumor 1*; *FLT3-ITD*, fms related tyrosine kinase 3-internal tandem duplication; pEFS, probability of event-free survival; pOS, probability of overall survival; CIR, cumulative incidence of relapse; SE, standard error; n, number. [a]No response to treatment was considered as the occurrence of an event at time zero.

the *FLT3-ITD* AR ≥0.4 revealed that as a single factor, it was associated with an EFS of only 25±8% and an OS of only 47±10%, respectively (Figure 5(a)). Remarkably, the co-occurrence of *FLT3-ITD* AR ≥0.4, *WT1*, and *NUP98-NSD1* as triple or double mutations significantly increased the CIR to 93±15% compared to the CIR of 31±4% for patients with no mutations or only *NUP98-NSD1* or *FLT3-ITD* AR <0.4 ($p<0.0001$) and to the CIR of 31±11% or 36±15% in patients with only *WT1* ($p<0.0001$) or *FLT3-ITD* AR ≥0.4 ($p=0.001$) mutations, respectively (Figure 5(b)). The probability of 3-year EFS was zero in patients with double or triple *WT1*, *FLT3-ITD* AR ≥0.4, and *NUP98-NSD1* mutations as opposed to 61±4% in patients with no mutations or only *NUP98-NSD1* or *FLT3-ITD* AR <0.4 ($p<0.0001$) and 69±11% or 45±15% for patients with only mutated *WT1* ($p<0.0001$) or *FLT3-ITD* AR ≥0.4 ($p=0.019$), respectively (Figure 5(b)). Finally, the co-occurrence of double or triple mutations resulted in a 3-year OS probability of 27±13%, which was significantly lower than the 3-year OS of 79±3% in patients with no mutations or only *NUP98-NSD1* or *FLT3-ITD* AR <0.4 ($p<0.0001$) and 90±7% or 73±13% in patients with only *WT1* ($p=0.0003$) or *FLT3-ITD* AR ≥0.4 ($p=0.06$) mutations, respectively (Figure 5(b)). By multivariate analysis including *WT1* mutation, *FLT3-ITD* AR ≥0.4, core-binding factor aberrations, early bone marrow response to treatment, and stem cell transplantation as covariables, we confirmed that the interaction of these three factors, and not each of the aberrations individually, was a significant predictor of poor prognosis for EFS ($p=0.008$, HR: 3.88, 95% CI: 1.42 – 10.6) and OS ($p=0.042$, HR: 3.42, 95% CI: 1.04 – 11.21, Table 3). Importantly, none of the patients with triple mutations survived and the only patients who could be rescued harbored

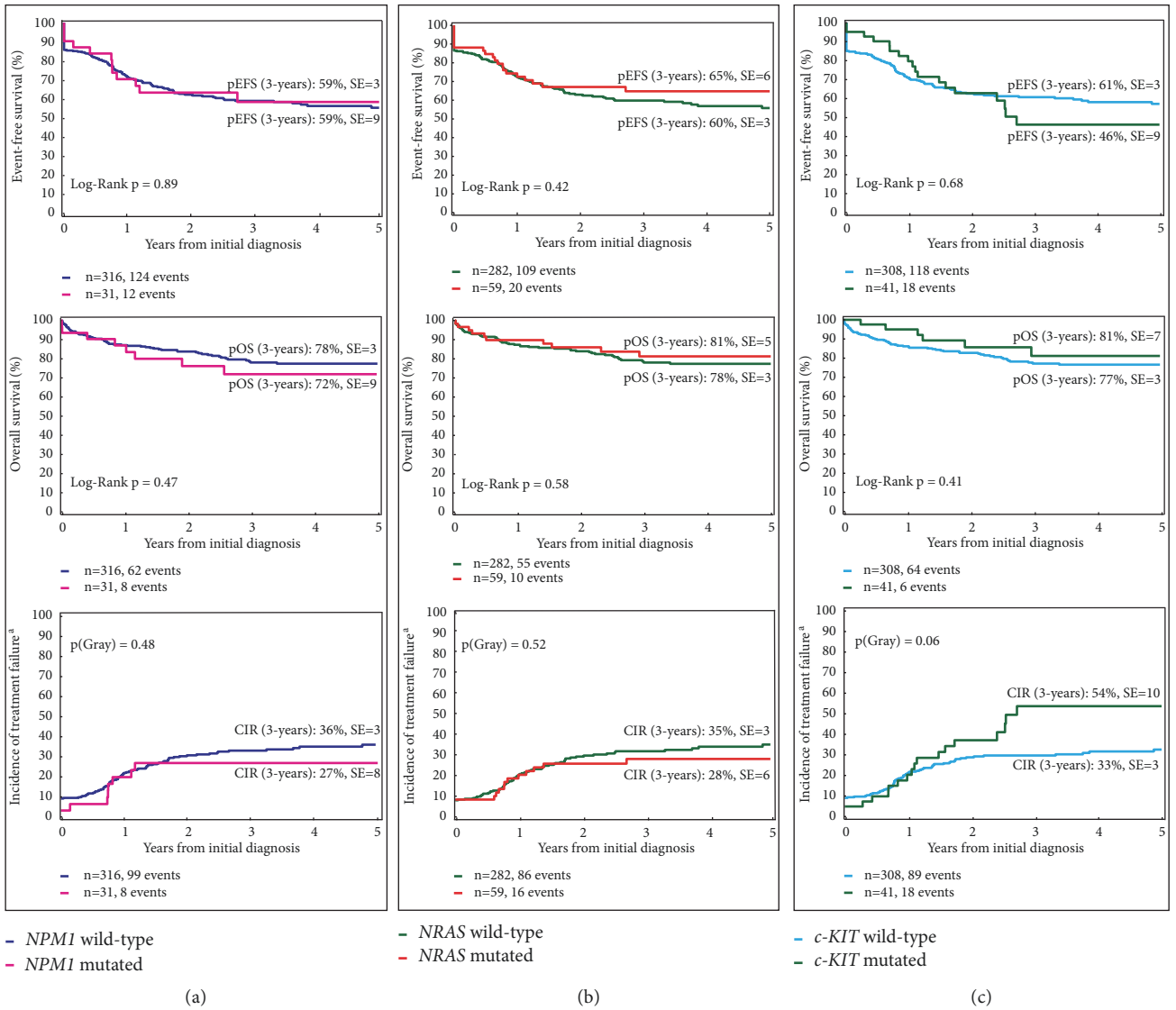


Figure 3: *Mutations in NPM1, NRAS, and c-KIT had no impact on survival.* (a) Prognostic impact of mutated *NPM1* on EFS, OS, and CIR. (b) Prognostic impact of mutated *NRAS* on EFS, OS, and CIR. (c) Prognostic impact of *c-KIT* mutation on EFS, OS, and CIR. *NPM1*, *nucleophosmin 1*; *NRAS*, *neuroblastoma RAS viral oncogene homolog*; *c-KIT*, *KIT* protooncogene; pEFS, probability of event-free survival; pOS, probability of overall survival; CIR, cumulative incidence of relapse; SE, standard error; n, number. [a]No response to treatment was considered as the occurrence of an event at time zero.

double *NUP98-NSD1* and *WT1* or *NUP98-NSD1* and *FLT3-ITD* mutations (Figure 1(b)), thus resulting in an OS of 27±13% (Figure 5(b)).

4. Discussion

Treatment of pediatric AML has significantly improved over the past three decades due to the development of intensified first-line treatments, efficient second-line therapies, and optimized supportive care [2, 30]. The success is, at least partly, achieved by more efficient risk group stratification using factors such as somatic mutations and cytogenetic aberrations of AML blasts at diagnosis as well as considering the primary response to treatment to optimize the allocation of patients to standard or enhanced treatment options [1]. In the present study, we analyzed the influence of three parameters, mutations in *WT1* and *FLT3* and the translocation of *NUP98-NSD1*, on the outcome of pediatric patients in the German AML-BFM 2004 and 2012 protocols. Although all three parameters have been established by us and others as important prognostic factors in both pediatric and adult patients [8–14, 20–22], their combined utility to identify high-risk patients likely to experience dismal treatment results has not yet been reported in a contemporary pediatric AML trial.

In a cohort of 237 patients treated within the AML-BFM 2004 and 2012 protocols and with sufficient material for re-analysis, we observed favorable outcomes for 3-year EFS of 61% and 69% and OS of 79% and 90% in patients

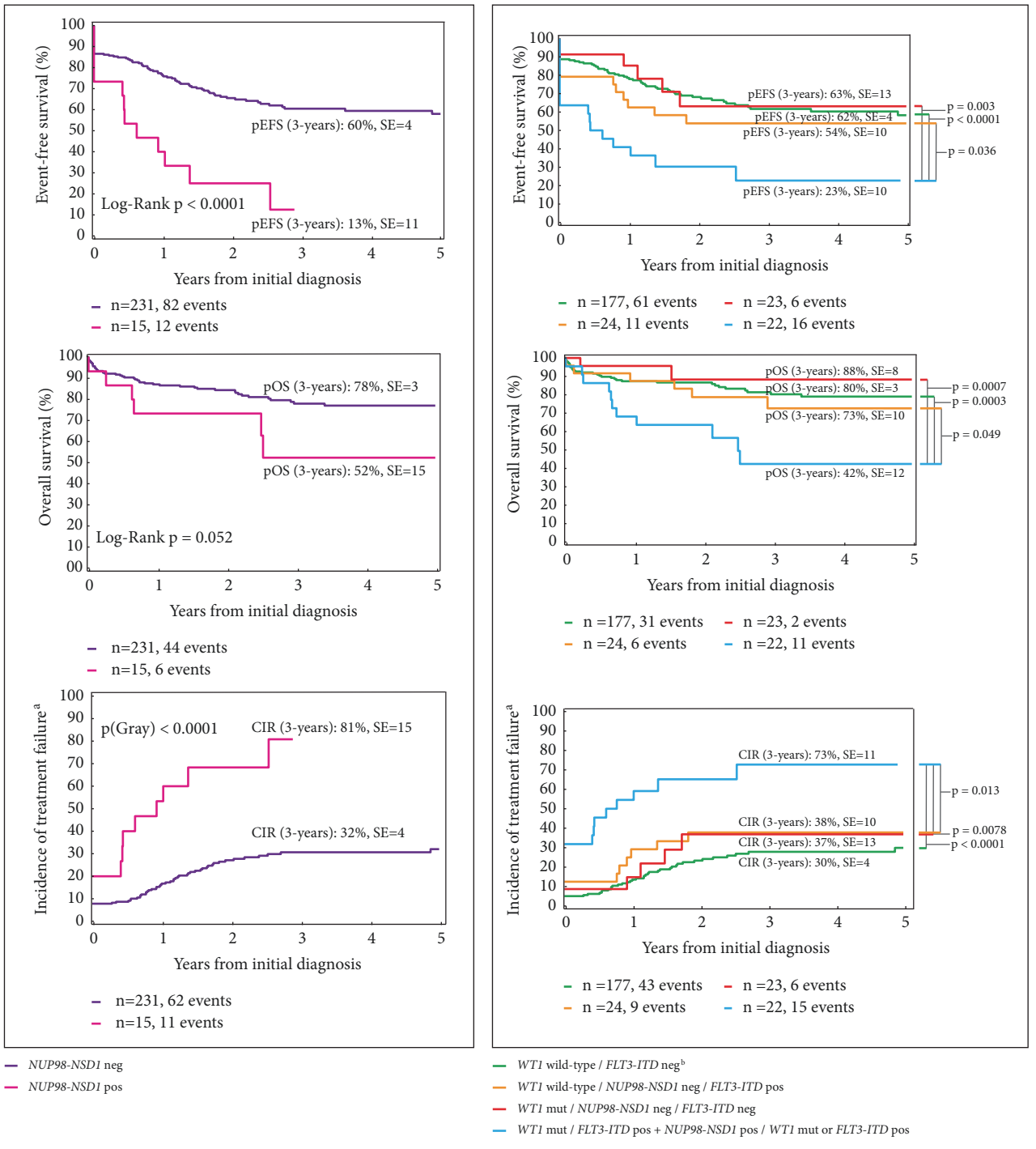


Figure 4: *Prognostic significance of NUP98-NSD1 fusion.* (a) *NUP98-NSD1* as single factor predicted poor outcomes. (b) Inclusion of *NUP98-NSD1* as poor prognostic factor with *WT1* mutation and *FLT3-ITD*, predicted poor outcomes for patients harboring all three factors in addition to patients with *NUP98-NSD1* and *WT1* mutation or *FLT3-ITD*. Patients with unknown status of *NUP98-NSD1* fusion were excluded from this analysis. W*T1*, *Wilms Tumor 1*; *FLT3-ITD*, fms related tyrosine kinase 3-internal tandem duplication; *NUP98-NSD1*, *Nucleoporin-Nuclear Receptor Binding SET Domain Protein 1* fusion gene; pEFS, probability of event-free survival; pOS, probability of overall survival; CIR, cumulative incidence of relapse; SE, standard error; mut, mutated; pos, positive; neg, negative. [a]No response to treatment was considered as the occurrence of an event at time zero. [b]Three patients with *NUP98-NSD1* are included in this group.

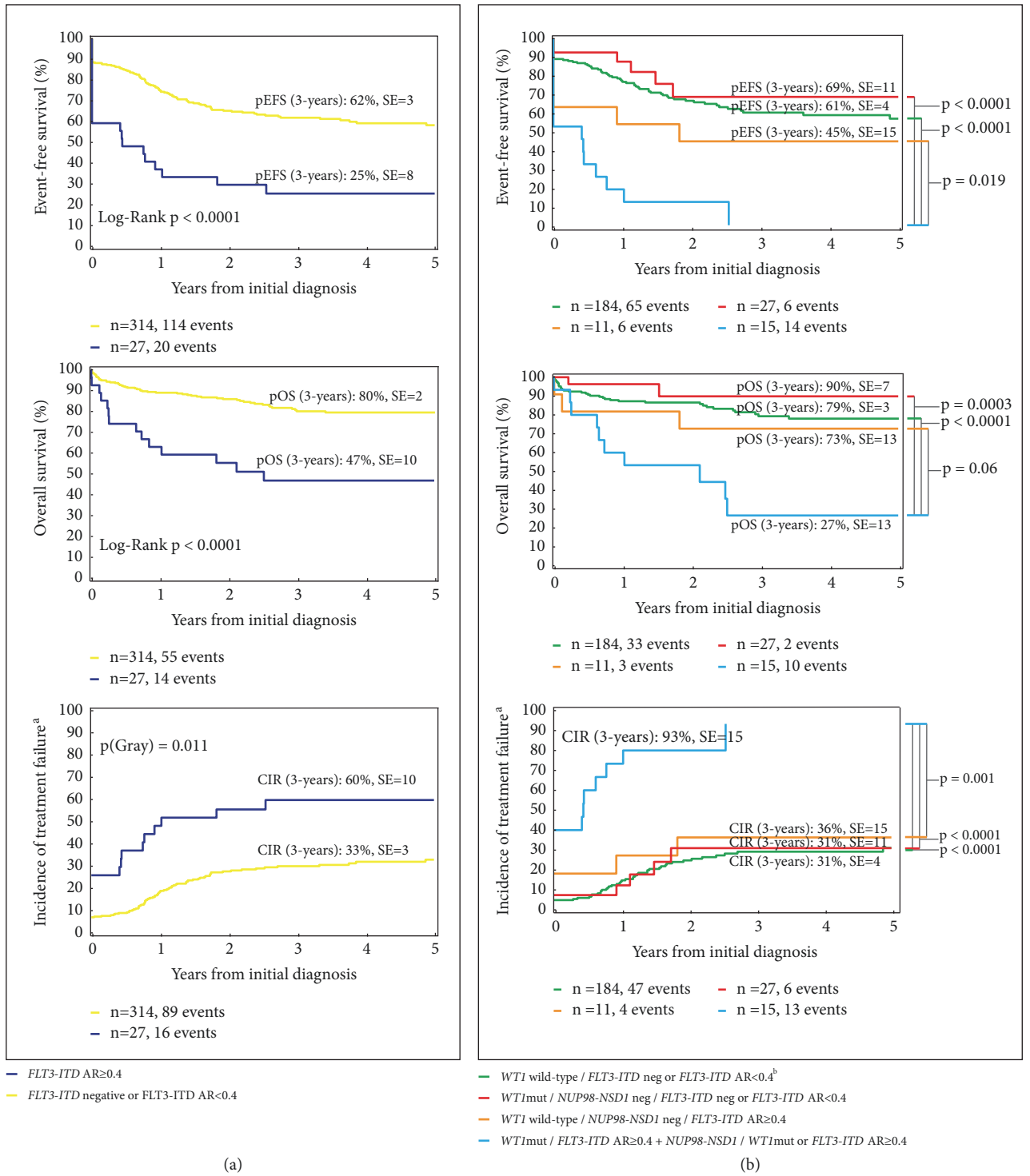


Figure 5: *Prognostic significance of mutational burden of FLT3-ITD.* (a) *FLT3-ITD* with an allelic ratio ≥0.4 as a single factor predicted poor outcomes. (b) High mutational burden of *FLT3-ITD* was another predictor of poor prognosis when it occurred with *WT1* and/or *NUP98-NSD1*. Patients with an unknown *FLT3-ITD* AR were excluded from this analysis. *NUP98-NSD1*, *Nucleoporin-Nuclear Receptor Binding SET Domain Protein 1* fusion gene; *FLT3-ITD*, fms related tyrosine kinase 3-internal tandem duplication; pEFS, probability of event-free survival; pOS, probability of overall survival; CIR, cumulative incidence of relapse; AR, allelic ratio; SE, standard error; n, number. [a]No response to treatment was considered as the occurrence of an event at time zero. [b]Three patients with *NUP98-NSD1* are included in this group.

Table 3: Multivariate analysis.

Cox regression analysis - Event-free survival				
Parameters	Hazard ratio	95% confidence interval		p value
		Lower limit	Upper limit	
WT1 mutation	0.79	0.41	1.53	0.479
FLT3-ITD AR ≥ 0.4	1.55	0.69	3.51	0.288
WT1 mutation, *FLT3-ITD* ≥ 0.4 and *NUP98-NSD1* interaction	3.88	1.42	10.66	0.008
t(8;21) and/or inv(16)	0.51	0.27	0.96	0.037
Unsatisfactory early response to treatment[a]	1.31	0.79	2.18	0.294
HSCT[b]	0.25	0.1	0.64	0.004
Cox regression analysis - Overall survival				
WT1 mutation	0.84	0.35	2.06	0.710
FLT3-ITD ≥ 0.4	1.51	0.57	4.02	0.404
WT1 mutation, *FLT3-ITD* ≥ *0.4* and *NUP98-NSD1* interaction	3.42	1.04	11.21	0.042
t(8;21) and/or inv(16)	0.45	0.16	1.31	0.143
Unsatisfactory early response to treatment[a]	1.21	0.61	2.42	0.589
HSCT[b]	1.18	0.51	2.73	0.700

WT1, *Wilms tumor 1*; *FLT3-ITD*, *fms related tyrosine kinase 3*-internal tandem duplication; *NUP98-NSD1*, *Nucleoporin-Nuclear Receptor Binding SET Domain Protein 1* fusion gen; t, translocation; inv, inversion; HSCT, hematopoietic stem cell transplantation.

[a]Unsatisfactory early response to treatment was defined as persistence of >5% blasts in bone marrow at day 15 and/or 28 after treatment. [b]hematopoietic stem cell transplantation events at first complete remission or after no-response to other treatments were included in the multivariate analysis as a time-dependent variable.

without *WT1* mutations or *NUP98-NSD1* fusion or with only one of these factors. Patients with leukemic blasts that were *FLT3-ITD* positive but negative for *WT1* and *NUP98-NSD1* mutations and that had an *FLT3-ITD* AR ≥0.4 still achieved an EFS of 45% and an OS of 73%. Surprisingly, our data therefore suggests that without *WT1* and *NUP98-NSD1* mutations, the negative impact of *FLT3-ITD* even with an AR≥0.4 might not be as severe as previously published [12, 17]. However, all patients positive for at least two of the three risk factors and with an *FLT3-ITD* AR ≥0.4 had events within the first three years and only 27% could be rescued by our salvage therapies. These unfavorable results in our double or triple mutated group unequivocally demonstrate that our current first-line treatment strategies for these patients are still insufficient/inadequate and urgently need improvement.

Of the three risk factors, currently only the *FLT3-ITD* mutation can be specifically targeted with inhibitors [31]. Although the first generations of these drugs only achieved limited and often transient efficacy due to intrinsic and extrinsic adaptations in the AML blasts and/or the environment [31], combination therapies of newer tyrosine kinase inhibitors such as Quizartinib with standard chemotherapy seem to be relatively well tolerated and in initial studies have demonstrated survival improvement in relapsed or refractory AML patients [32–34]. Due to the important role of FLT3 pathway activation in AML, numerous combinations of FLT3 inhibitors with other drugs are currently being tested. Whether these results will also be helpful for the treatment of pediatric AML will need to be carefully determined in future studies, especially considering the clonal heterogeneity of *FLT3-ITD* and the additional survival burden that it causes by increasing drug resistance through clonal evolution or selection and further expansion of resistant AML clones [35, 36]. Nevertheless, it is tempting to speculate that the simple addition of a newer FLT3 inhibitor to our standard therapy might be a feasible, well-tolerated, and effective approach for all patients with blasts that are positive for the *FLT3-ITD* mutation, regardless of the status of alterations in *WT1* or *NUP98*.

The role of WT1 in patients with AML is still controversial [4]. Although *WT1* is overexpressed in the majority of leukemias and can be used as a marker for minimal residual disease and maybe even vaccination attempts, the prognostic and therapeutic relevance of high or absent WT1 expression levels is not unequivocally accepted [37–39]. In contrast, mutations in *WT1* are clearly identified as determinants of poor prognosis and, as we showed here, confer a dismal prognosis especially in combination with *FLT3-ITD* or *NUP98-NSD1* fusion. In the present study, we identified 64 monoallelic *WT1* sequence alterations in exon 7 or exon 9 in the leukemic blasts of 48 patients. The majority of these alterations leads to frameshifts and/or premature terminations codons and thus shortened proteins. These mutant proteins can act in a dominant negative manner [40], which may contribute to a myeloid differentiation block present in AML blasts [41]. However, similar mutations have also been described in the context of Wilms tumors as gain-of-function mutations promoting proliferation [42]. Here, we show a favorable prognosis for patients with single *WT1* mutations, with 26 out of 29 cases reaching continued complete remission (CCR) (Figure 1(b)). Therefore, based on a 3-year EFS of 69% and an OS of 90%, the development of

new treatment approaches is not as urgently needed for these patients with *WT1* mutated blasts that do not harbor *FLT3-ITD* or *NUP98-NSD1* mutations.

Among the 31 different fusion gene partners of *NUP98* identified so far, the *NUP98-NSD1* t(5:11) translocation is the most frequent and present in 4-7% of patients in pediatric AML patients [20–22]. Importantly, the *NUP98* translocations that occur in AML all share the N-terminus of the protein and are thought to initially lead to epigenetic dysregulation of different leukemia-associated genes including *HOXA7*, *HOXA9,* and *HOXA10* in myeloid precursor cells [20]. Additional somatic mutations in other genes occur as secondary events and promote malignant transformation and uncontrolled cell growth [20]. As also shown in our patient data set, these secondary alterations often include activating mutations in *FLT3 (FLT3-ITD)* or truncating mutations *in WT1* [21]. Strikingly, only three patients in our study had a *NUP98-NSD1* translocation without mutations in *FLT3* or *WT1*; two of these patients achieved and remained in first CCR at the end of data acquisition. The third patient had no other genetic risk factors but a very high initial white blood cell count of almost 400,000 cells/μl. Complete remission induction was delayed, and the patient relapsed a year later but was successfully treated by allogeneic stem cell transplantation with a follow-up of 10 years. Therefore, as also described previously [21], our patients with *NUP98*-rearranged blasts with *WT1* and/or *FLT3-ITD* mutations had a poor prognosis, especially in contrast to patients with only *WT1* and *FLT3-ITD* mutations, who could at least partially be rescued by allogeneic transplantation. However, due to the high risk of failure of the first-line treatment, stem cell transplantation already in first CCR seems to be an attractive option for cases of *NUP98*-rearranged AML [21, 22]. Nevertheless, it should be noted that even allogeneic stem cell transplantation is not always effective in improving the treatment outcome in patients with a high probability of treatment failure based on risk stratification. Thus, introducing novel treatment approaches such as the use of small inhibitors, e.g., venetoclax and isadanutlin [43] or cellular therapies with allogeneic NK-cells or engineered T-cells with chimeric antigen receptors (CARs) [44] targeting leukemic blasts harboring *NUP98* rearrangement or *WT1* mutations should be taken into consideration in future clinical studies.

Recent analysis from a collaborative study between the American and Dutch children oncology groups (COG and DCOG) included patients from three clinical COG/DCOG trials and also young adults less than 39 years of age in the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) AML initiative [45]. Analysis of the different cohorts revealed similarly unfavorable outcomes with an EFS of 14-25% and an OS of 15-40% for patients with *FLT3-ITD* and *WT1* mutations and/or the *NUP98-NSD1* translocation [45]. In contrast to our findings however, the authors reported an EFS range of 15-35% in patients with *FLT3-ITD* only, which is lower than that achieved with current protocols, for which an EFS of 45% and an OS of 73% were found for patients with *FLT3-ITD* only. Notably, in the American-Dutch study, patients with co-occurrence of *NPM1* mutations and *FLT3-ITD* (and without *WT1* and *NUP98-NSD1*) were separated from patients with *FLT3-ITD* only and had a slightly increased, albeit probably not statistically significant, survival. Similarly, we have previously observed favorable outcomes for patients with *NPM1* mutations in their AML blasts with normal karyotype and proved this impact was not affected by the presence of *FLT3-ITD* [46]. In the current cohort, five patients were positive for mutations in *FLT3-ITD* and *NPM1* and negative for *WT1* and *NUP98* alterations. At present, four patients with a normal karyotype are still in first CCR, and the fifth patient with a complex karyotype and an *FLT3-ITD* AR >11 experienced early death. In summary, the principle findings of this American-Dutch study and the present study are very similar. However, the treatment outcomes for our patient groups are superior, most likely due to the fact that we included only patients between 0 and 18 years of age treated in Germany according to two contemporary protocols from the AML BFM study group.

5. Conclusion

Despite the fact that our study was partly based on data collected prospectively since 2004 and partly on data assessed *de novo* on stored material by either NGS or PCR, we can safely conclude that co-occurrence of the three factors, mutated *WT1* and *FLT3-ITD and/or NUP98-NSD1* translocation, still defines a subgroup of AML patients with devastating EFS and OS outcome, even with our current treatment protocols. Although the number of pediatric AML patients available for analysis of these three risk factors was limited and therefore not all interesting factors could be assessed in multivariate analysis, it is obvious that patients with double or triple mutations benefitted very little from the improved EFS and OS in our AML-BFM studies in recent years. Thus, for these pediatric patients, new and more targeted approaches are urgently needed for both first- and second-line treatments.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflicts of interest regarding the current work. Dirk Reinhardt has consulting or advisory roles for Roche, Celgene, Hexal, Pfizer, Novartis, Boehringer and receives research funding from Celgene. Dirk Reinhardt received travel grants from Jazz Pharmaceuticals and Griffols. Naghmeh Niktoreh and Christine von Neuhoff received travel grants from Jazz Pharmaceuticals. The other authors have nothing to declare.

Authors' Contributions

Helmut Hanenberg and Dirk Reinhardt contributed equally. Naghmeh Niktoreh and Christiane Walter collected and assembled data; Martin Zimmermann, Naghmeh Niktoreh,

Christiane Walter, Helmut Hanenberg, and Dirk Reinhardt analyzed and interpreted data; Naghmeh Niktoreh and Helmut Hanenberg wrote the manuscript; and all authors gave final approval of manuscript.

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Review Article

Not Only Mutations Matter: Molecular Picture of Acute Myeloid Leukemia Emerging from Transcriptome Studies

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The last two decades of genome-scale research revealed a complex molecular picture of acute myeloid leukemia (AML). On the one hand, a number of mutations were discovered and associated with AML diagnosis and prognosis; some of them were introduced into diagnostic tests. On the other hand, transcriptome studies, which preceded AML exome and genome sequencing, remained poorly translated into clinics. Nevertheless, gene expression studies significantly contributed to the elucidation of AML pathogenesis and indicated potential therapeutic directions. The power of transcriptomic approach lies in its comprehensiveness; we can observe how genome manifests its function in a particular type of cells and follow many genes in one test. Moreover, gene expression measurement can be combined with mutation detection, as high-impact mutations are often present in transcripts. This review sums up 20 years of transcriptome research devoted to AML. Gene expression profiling (GEP) revealed signatures distinctive for selected AML subtypes and uncovered the additional within-subtype heterogeneity. The results were particularly valuable in the case of AML with normal karyotype which concerns up to 50% of AML cases. With the use of GEP, new classes of the disease were identified and prognostic predictors were proposed. A plenty of genes were detected as overexpressed in AML when compared to healthy control, including *KIT*, *BAALC*, *ERG*, *MN1*, *CDX2*, *WT1*, *PRAME*, and *HOX* genes. High expression of these genes constitutes usually an unfavorable prognostic factor. Upregulation of *FLT3* and *NPM1* genes, independent on their mutation status, was also reported in AML and correlated with poor outcome. However, transcriptome is not limited to the protein-coding genes; other types of RNA molecules exist in a cell and regulate genome function. It was shown that microRNA (miRNA) profiles differentiated AML groups and predicted outcome not worse than protein-coding gene profiles. For example, upregulation of *miR-10a*, *miR-10b*, and *miR-196b* and downregulation of *miR-192* were found as typical of AML with *NPM1* mutation whereas overexpression of *miR-155* was associated with *FLT3*-internal tandem duplication (*FLT3*-ITD). Development of high-throughput technologies and microarray replacement by next generation sequencing (RNA-seq) enabled uncovering a real variety of leukemic cell transcriptomes, reflected by gene fusions, chimeric RNAs, alternatively spliced transcripts, miRNAs, piRNAs, long noncoding RNAs (lncRNAs), and their special type, circular RNAs. Many of them can be considered as AML biomarkers and potential therapeutic targets. The relations between particular RNA puzzles and other components of leukemic cells and their microenvironment, such as exosomes, are now under investigation. Hopefully, the results of this research will shed the light on these aspects of AML pathogenesis which are still not completely understood.

1. Introduction

Acute myeloid leukemia (AML), the most frequent leukemia in adults, is a severe myeloproliferative disorder with the high risk of relapse and high mortality rate [1, 2]. Random genetic alterations sequentially acquired by hematopoietic stem and progenitor cells disrupt hematopoiesis by differentiation blockades, uncontrolled growth and proliferation, and inhibition of apoptosis [3]. Immature, partially differentiated blast cells with self-renewal capacity first accumulate in bone marrow (BM) and then infiltrate peripheral blood (PB) and organs, impairing their functions [4]. Despite similar symptoms, blast morphology, and clinical implications, AML is a very heterogeneous disease presenting a wide spectrum of subtypes with different molecular features and outcomes [5, 6]. A number of chromosomal rearrangements and small mutations have been detected in AML and associated with the pathogenesis, diagnosis, and prognosis of the disease

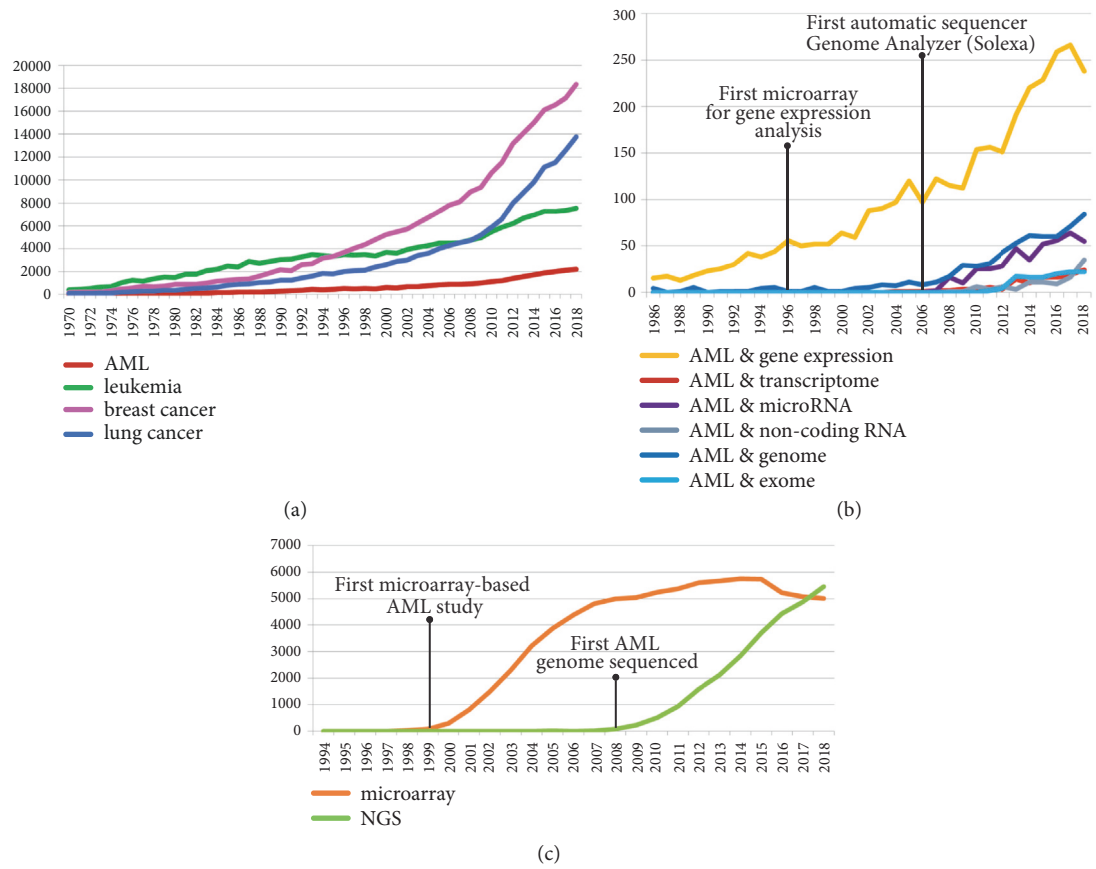

Figure 1: The number of publications found in PubMed, devoted to (a) AML, leukemia, and the two most common human cancers; (b) transcriptome and genome-based AML studies; (c) two the most common high-throughput technologies used in AML studies, microarrays, and next generation sequencing (NGS). The search terms and exact numbers of publications are noted in Supplementary Table 1.

[5, 7, 8]. AML heterogeneity is also reflected in its classification, first established in 1976 by French-American-British (FAB) cooperative groups, based on morphological and cytochemical criteria [9] and revised nine years later [10]. In 2001, an alternative classification system, combining the leukemic cell lineage and maturation stage with genetic aberrations, was proposed by World Health Organization (WHO) and improved in 2008 and 2016 [11–13].

Although AML accounts for not more than 1% of all cancer diseases, it belongs to one of the most extensively studied human tumors, which has been confirmed by the ever-growing number of scientific reports (Figure 1(a)). In the Cancer Genome Atlas (TCGA), a landmark cancer genomics program (https://www.cancer.gov/tcga), AML is one of 33 cancer types collected hitherto, being represented by 200 cases. Availability of the tumor cells, which can be easily and in extensive amounts extracted from BM aspirates or even PB, makes AML a perfect model for cancer studies. In AML, the existence of a cancer stem cell was first demonstrated, proving the rightness of the tumor stem-cell concept [14–16]. Since then, our knowledge about cancer stem cells started to increase [6, 17]. Progress in the development of high-throughput methods such as microarrays and next generation sequencing (NGS) advanced our understanding of AML and other cancers [18, 19] (Figures 1(b) and 1(c)). Figure 2 presents the timeline and milestones of AML research intertwined with the milestones of the Human Genome Project (HGP). The first application of global gene expression profiling (GEP) for cancer classification was demonstrated in 1999 on the example of two acute leukemias arisen from different lineages, myeloid (AML) and lymphoid (acute lymphoblastic leukemia, ALL) [20]. The first cancer genomes sequenced derived from AML patients [21, 22]. In 2013, TCGA Research Network published the sequences of 50 whole genomes and 150 exomes of AML patients [23]. Three years later, targeted resequencing of 111 genes in 1540 AML patients revealed more than 5 thousands of driver mutations [24]. In 2018, functional genomic landscape was drawn based on the exome sequencing, gene expression, and the analysis of *ex vivo* drug sensitivity in a cohort of over 500 AML patients [25]. Genome-wide studies revealed that the number of driver mutations in AML (on average, 13 somatic variants per patient) is lower than in solid tumors [23, 25]. New AML entities of diagnostic and prognostic significance have been identified and potential therapeutic targets have been indicated [26, 27]. Despite the tremendous effort put into research, AML (except for acute promyelocytic leukemia, APL) still lacks effective medical treatment [28, 29]. However,

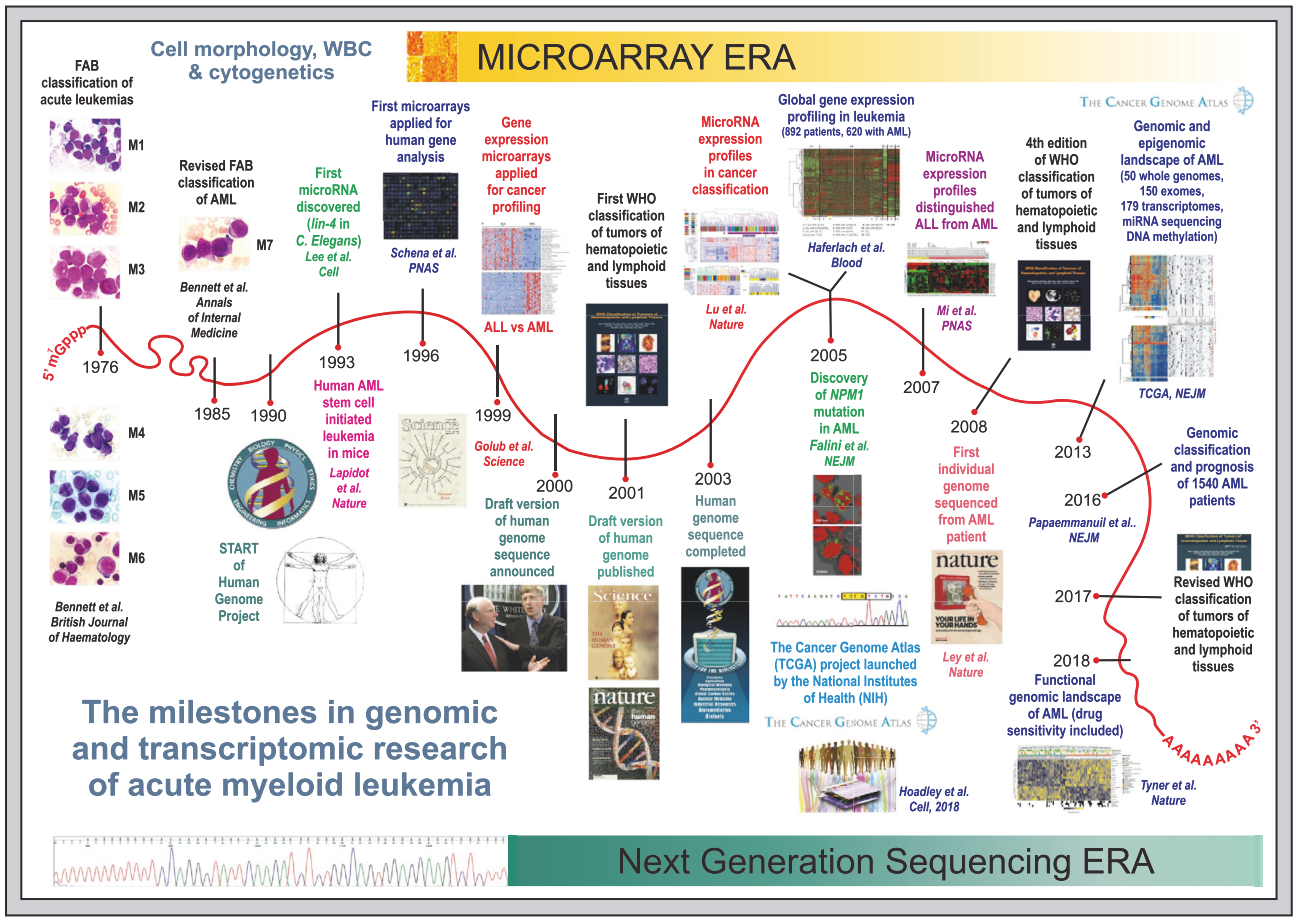

FIGURE 2: The milestones in genomic and transcriptomic research of acute myeloid leukemia. A symbolic mRNA molecule serves as a timeline on which the most important papers and events are marked, starting from the first FAB classification of AML in 1976 [9] and its revised version published in 1985 [10]. The microscopic images of M1-M7 FAB AML types come from the private collection of Prof. John M. Bennett and were used thanks to the courtesy of the Professor. The original pictures from the following publications were used with the permission of the authors and magazine publishers: Schena et al., PNAS 1996 [47] (Copyright 1996 National Academy of Sciences); Golub et al., Science 1999 [20] (reprinted with permission of AAAS); Lu et al., Nature 2005 [51] (reprinted by permission from Springer Nature, Nature, Copyright 2005); Falini et al., NEJM 2005 [52] (Copyright 2005 Massachusetts Medical Society, reprinted with permission from Massachusetts Medical Society); Haferlach et al., Blood 2005 [53] (reprinted by permission from American Society of Hematology, Copyright 2005); Mi et al. PNAS 2007 [54] (Copyright 2007 National Academy of Sciences); TCGA paper from NEJM 2013 [23] (Copyright 2013 Massachusetts Medical Society, reprinted with permission from Massachusetts Medical Society); Tyner et al. Nature 2018 [25] (reprinted by permission from Springer Nature, Nature, Copyright 2018); Hoadley et al., Cell 2018 [55] (reprinted by permission from Elsevier, Cell, Copyright 2018). Two Nature journal covers were reprinted by permission from Springer Nature, Nature, Copyright 2001 and 2008. Two Science journal covers, from 1999 and 2001, were reprinted with permission of AAAS. WHO publication cover images were reproduced with permission from Jaffe, E.S., Harris. N.L., Stein, H., Vardiman, J.W., Eds. WHO Classification of Tumours, Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues, IARC, Lyon, 2001 [11]; Swerdlow, SH, Campo, E, Harris, NL, Jaffe, ES, Pileri, SA, Stein, H, Thiele, J, Vardiman, JW. World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues. IARC, Lyon, 2008; Swerdlow, SH, Campo, E, Harris, NL, Jaffe, ES, Pileri, SA, Stein, H, Thiele, J, Arber DA, Hasserjian RP, Le Beau MM, Orazi A, Siebert R. World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues, revised 4th edition. IARC, Lyon, 2017. The photograph of Francis Collins and Craig Venter, made by Chuck Kennedy in 2000 (krtphotos001229) was used with the license of Newscom (https://www.newscom.com). Vitruvian man image was downloaded from Wikimedia Commons under a free license (https://commons.wikimedia.org/wiki/File:Vitruvian_man.jpg). Graphics representing the Human Genome Project (HGP) were used thanks to the courtesy of National Human Genome Research Institute ((NHGRI, https://www.genome.gov). The TCGA logo was used with the permission of National Cancer Institute (NCI, https://www.cancer.gov).

some promising therapeutic strategies are currently under investigation [30].

Despite the fact much attention has recently been devoted to genetic alterations occurring in AML, it should be remembered that the state of the cell is largely reflected by its transcriptome, which is the product of genomic activity. In fact, transcriptome-level analyses preceded whole genome sequencing and still serve as supplementary approaches in

AML studies. In this review, I tried to show to what extent deeper insight into AML transcriptomes helped to unravel the mysteries of the disease.

2. The Early Beginnings: Studies of Single Protein-Coding Genes

At the turn of the 1980s and 1990s, more and more reports documenting gene expression in AML started to appear. Expression of several protooncogenes, encoding transcription factors (*MYC*, *MYB*, and *FOS*) and tyrosine kinases (*ABL1*, *FES*, *KIT*, and *PIM*) with essential roles in the regulation of hematopoiesis, cell proliferation, differentiation, cell cycle, and apoptosis was demonstrated in AML cells extracted from patients [31–34]. Erythroid progenitors and HEL erythroleukemia cells presented amplification of another transcription factor involved in cell proliferation, *E2F1* [35]. Overexpression of *RUNX1* gene, previously known as *AML1* or *CBF2A*, regulator of hematopoiesis, particularly of myeloid lineage, suppressed granulocytic differentiation and stimulated cell proliferation in murine cells [36]. Carow et al. [37] showed that expression of the hematopoietic growth factor receptor-encoding *FLT3* gene was not limited to normal stem/progenitor cells but was even elevated in leukemic blasts. In AML M2 with t(8;21), high percentage of CD34+ cells was correlated with high expression of *CD34* gene [38]. *BMI1*, member of the polycomb complex, implicated in maintenance of normal and leukemic stem cells, was found to be expressed in AML M0 at a higher level than in other AML subtypes [39]. High expression of some genes was associated with adverse AML prognosis, e.g., *WT1* [40], *MN1* [41], *BAALC* [42], *ERG* [43], and *EVI1* (ecotropic viral integration site 1, at present known as *MECOM*, from *MDS1* and *EVI1* complex locus) [44]. Genes which were renamed within the last years are listed in Table 1, together with the most commonly used abbreviations.

3. Microarray-Based Gene Expression Profiling: A Tool for Disease Classification

Technological progress enabled transition from single gene analysis to whole transcriptome scale at the end of the previous millennium [45]. The milestone was the invention of the microarray, chip-format tool which allowed for simultaneous analysis of thousands of genes in one test [46, 47]. Golub et al. [20] and Alizadeh et al. [48] were the first who showed that global gene expression profiling (GEP) could be a tool for cancer research and classification. Each group used a different type of microarrays: Golub et al. used commercially available high-density GeneChips made of short oligonucleotides synthesized in situ (Affymetrix) whereas Alizadeh et al. [48] constructed their own cDNA array, Lymphochip, dedicated to analysis of gene expression in normal and malignant lymphocytes. Golub et al. [20] proved that the distinction between two acute leukemias, AML, and ALL could be performed in a single test. Out of 6,817 human genes measured, expression of 50 genes was selected as the most closely correlated with AML-ALL class distinction. The 50-gene predictor was successfully validated in an independent collection, including samples from PB instead of BM, samples from childhood AML patients and samples collected by various laboratories. Among the most informative genes overexpressed in AML were known genes encoding cell surface proteins, e.g., *CD33* and *CD11c* (currently *ITGAX*), and transcription factors, including *HOXA9* oncogene, whose high expression level was noted in AML patients with poor outcome. In fact, *HOXA9* seemed to be a single gene capable of predicting treatment failure in AML. The study revealed also novel AML markers, such as gene coding for leptin receptor with antiapoptotic activity, or zyxin gene encoding protein with cell adhesion function. The above results showed the power of GEP in disease classification and class discovery and encouraged other investigators to implement DNA microarray technology in their laboratories.

4. Protein-Coding Transcriptomes of Cytogenetically Defined AML Subtypes

Distinction between AML and other hematologic disorders seemed to be trivial. The question appeared whether gene expression profiles could successfully differentiate AML subgroups with cytogenetic and genetic abnormalities. Starting from AML with the most common chromosomal aberrations, different authors demonstrated that each of AML subgroups possessed its own gene expression signature and could be easily distinguished from one another. Below, the exemplary studies are described in more detail.

Virtaneva et al. [49] compared AML with isolated trisomy 8 (+8) to cytogenetically normal AML (CN-AML) and revealed fundamental biological differences between these two types of the disease. Common feature of both AML types was downregulation of genes encoding hematopoietic transcription factors (*STAT4*, *FUS*, *MCM3*, and *MCM5*) and myeloid markers (*ELANE* and *MPO*) in comparison to normal *CD34*+ bone marrow cells. Genes encoding complement factor D (*CFD*), proteins involved in cell growth and differentiation (*NDRG1* and *BTG1*), transcription factors *KLF6* and *ATF3*, and transcription coactivator *TAF10* were upregulated in both AMLs in relation to control *CD34*+ fraction. Two the most differentiating genes between AML +8 and CN-AML were *MLLT2* (present *AFF1* (*AF4/FMR2* Family Member 1), upregulated in CN-AML) encoding regulator of transcription and chromatin remodeling, and *FABP5* (upregulated in AML +8), encoding protein involved in fatty acid metabolism. Unsurprisingly, AML +8 blasts presented general overexpression of genes encoded on chromosome 8. The effect of genomic gains and losses on expression levels of genes located within the affected regions was further confirmed in a study of AML with trisomy 8, 11, 13, monosomy 7, and deletion 5q [50]. Surprisingly, in a study of Virtaneva et al. [49] protooncogene *MYC*, also encoded on chromosome 8, was downregulated in AML +8. On the one hand, in comparison to CN-AML, decreased expression level of proapoptotic genes (e.g., *CRADD*, *BAD*) was noted for AML +8 whereas *TP53* gene, encoding tumor suppressor and also apoptosis inducer, was increased in AML +8. On the other hand, only CN-AML

Table 1: The list of the genes renamed within the last years and the most commonly used abbreviations.

Renamed Genes			
Previous Name	Previous Description	Current Name	Current Description
AML1	Acute Myeloid Leukemia 1	*RUNX1*	Runt Related Transcription Factor 1
Ang-1	Angiopoietin-1	*ANGPT1*	Angiopoietin 1
BRN3A	Brain-3A	*POU4F1*	POU Class 4 Homeobox 1
CD11c	CD11c Antigen	*ITGAX*	Integrin Subunit Alpha X
CD133	CD133 Antigen	*PROM1*	Prominin 1
ELA2	Elastase-2	*ELANE*	Elastase, Neutrophil Expressed
ETO	Protein ETO	*RUNX1T1*	RUNX1 Translocation Partner 1
EVI1	Ecotropic Viral Integration Site 1	*MECOM*	MDS1 And EVI1 Complex Locus
FLJ14054	Homo sapiens cDNA FLJ14054 fis, clone HEMBB1000240	*NPR3*	Natriuretic Peptide Receptor 3
IL8	Interleukin-8	*CXCL8*	C-X-C Motif Chemokine Ligand 8
MADH1	Mothers Against Decapentaplegic Homolog 1	*SMAD1*	SMAD Family Member 1
MDR1	Multidrug Resistance Protein 1	*ABCB1*	ATP Binding Cassette Subfamily B Member 1
MEL1	MDS1/EVI1-Like Gene 1	*PRDM16*	PR/SET Domain 16
MLL	Mixed Lineage Leukemia	*KMT2A*	Lysine Methyltransferase 2A
MLLT2	Myeloid/Lymphoid Or Mixed-Lineage Leukemia (Trithorax (Drosophila) Homolog); Translocated To, 2	*AFF1*	AF4/FMR2 Family Member 1
MLLT4	Myeloid/Lymphoid Or Mixed-Lineage Leukemia Translocated To, 4	*AFDN*	Afadin, Adherens Junction Formation Factor
NICAL	NEDD9-Interacting Protein With Calponin Homology And LIM Domains	*MICAL1*	Microtubule Associated Monooxygenase, Calponin And LIM Domain Containing 1
OPN	Osteopontin	*SPP1*	Secreted Phosphoprotein 1
PTRF	Polymerase I And Transcript Release Factor	*CAVIN1*	Caveolae Associated Protein 1
PU.1	Hematopoietic Transcription Factor PU.1	*SPI1*	Spi-1 Proto-Oncogene
Abbreviations			
AML	acute myeloid leukemia		
ALL	acute lymphoblastic leukemia		
APL	acute promyelocytic leukemia		
BM	bone marrow		
CBF	core binding factor		
circRNAs	circular RNAs		
CLL	chronic lymphocytic leukemia		
CML	chronic myeloid leukemia		
CN-AML	cytogenetically normal AML		
CR	complete remission		
DEGs	differentially expressed genes		
DFS	disease-free survival		
FLT3-ITD	*FLT3*-internal tandem duplication		
FAB	French-American-British (classification system)		

Table 1: Continued.

GEP	gene expression profiling
HH	Hedgehog
HSCs	hematopoietic stem cells
HSPCs	hematopoietic stem-progenitor cells
lncRNAs	long noncoding RNAs
LSCs	leukemic stem cells
MDS	myelodysplastic syndromes
MRD	minimal residual disease
MSC	mesenchymal stem cells
NGS	next generation sequencing
NK-AML	normal karyotype AML
NPMc+	NPM-cytoplasmic positive
OS	overall survival
PB	peripheral blood
PBMCs	peripheral blood mononuclear cells
SAGE	serial analysis of gene expression
snoRNAs	small nucleolar RNAs
TCGA	the Cancer Genome Atlas
TEs	transposable elements
WBC	white blood cell
WHO	World Health Organization

showed upregulation of antiapoptotic gene *DAD1*. Therefore, the authors suggested different mechanisms of cell death escape for the two studied leukemia types and associated it with AML +8 resistance to cytarabine-based chemotherapy, which should induce apoptosis [49].

AML subtypes with three reciprocal rearrangements, t(8;21)(q22;q22), inv(16)(p13q22), and (15;17)(q22;q12), corresponding to the morphological FAB subtypes M2, M4eo, and M3/M3v, respectively, were the subject of research of Schoch et al. [56]. Principal component analysis (PCA) of microarray data, performed with the use of 1000 most informative genes, clearly separated AML samples according to chromosomal aberration. The minimal set of 13 genes (*PRKAR1B, GNAI1, PRODH, CD52, KRT18, CLIP3, CLU, PTGDS, HOXB2, CLEC2B, CTSW, S100A9, and MYH11*) was sufficient to distinguish one AML subtype from another on the basis of gene expression solely. Expression levels of 36 genes enabled accurate classification of all three studied AML subtypes. Another set of 82 genes allowed for distinction of M3 and M3v, two phenotypically different AML types with t(15;17). In addition, the study showed that AMLs with alterations involved core binding factor (*CBF*) complex, t(8;21) and inv(16), were more related to each other than to AML with t(15;17). The authors explained the overexpression of *MYH11* in inv(16) and *RUNX1T1* (former *ETO*) in t(8;21) as a consequence of high expression of fusion transcripts affecting these genes. A new marker of t(8;21) was identified by Debernardi et al. [57], who found that a level of transcription factor-coding gene, *POU4F1* (former *BRN3A*), was 43-fold higher in t(8;21) AML than in other AML samples.

Verhaak et al. [58], by gene expression analysis in two independent cohorts of AML patients under 60, each exceeding 200 cases, perfectly distinguished three favorable cytogenetic AML subtypes, t(8;21), t(15;17) and inv(16). For AML with *NPM1* or *CEBPA* mutations, GEP-based classifiers were less accurate. The distinction of AML with other mutations (e.g., *FLT3* and *RAS*) and aberrations (11q23, -5/5q-, -7/7q-, abn3q, and t(9;22)) was not possible with the use of GEP. Nevertheless, for abn3q, the most discriminative gene was *MECOM*, encoding an oncogenic transcription factor often involved in 3q26 abnormalities, and in 7(q) almost all differentially decreased genes were located on chromosome 7.

One of the most challenging tasks was to find unique features characterizing cytogenetically normal AML (CN-AML or NK-AML from normal karyotype AML) which accounts for 40-50% of all AML cases. Debernardi et al. [57] were the first who attempted to do that. Although the sample size was not large (28 adult AML samples, including 10 NK-AML) and NK-AML revealed higher variability than AML with translocations, the authors found NK-AML could be separated from AML samples with chromosomal rearrangements based on the expression levels of certain members of the class I homeobox A and B gene families, which were low or undetectable in AML with (t(8;21), t(15;17), and inv(16)). In NK-AML, expression level of 10 genes was extremely increased: *HOXA4, HOXA5, HOXA9, HOXB2, HOXB3, HOXB5, HOXB6,* and *HOXB7*, and two members of TALE family, *MEIS1* and *PBX3*. While overexpression of *HOXB* genes was unique for

NK-AML, the upregulation of the remaining 5 genes was shared with 11q23 AML where *MLL* (mixed lineage leukemia) gene, now renamed to *KMT2A* (Lysine Methyltransferase 2A), was fused with different partners. High expression of some homeobox genes (e.g., *HOXA3* and *HOXB6*) was later found as typical of hematopoietic stem cells (HSCs) [59].

In 2004, two remarkable papers, identifying not only known but also new molecular AML subtypes through global GEP, were published in the same issue of the New England Journal of Medicine [60, 61]. Bullinger et al. (2004) [60] who analyzed 116 adult AML samples with the use of cDNA microarrays, found that hierarchical clustering with over 6 thousands of the most varied genes divided all AML samples into two main clusters. Out of the cytogenetic groups, only t(15;17) (APL) generated one condense subcluster. To enable biological insight into AML pathogenesis, group-specific gene expression signatures were established and functionally characterized. Signature specific for APL included genes related to hemostasis (*PLAU, SERPING1, ANXA8,* and *PLAUR*), resistance to apoptosis (*TNFRSF4, AVEN,* and *BIRC5*), impairment of retinoic acid-stimulated cell differentiation (*TBL1X, CALR,* and *RARRES3*) and resistance to chemotherapy (*CYP2E1, EPHX1,* and a group of metallothionein (*MT*) genes). *MLLT4* (present *AFDN*, Afadin), one of *KMT2A* fusion partners, was among the genes with unique expression profile in t(8;21), which suggested similar mechanism of pathogenesis with t(6;11). High expression of *NT5E*, observed in inv(16), was correlated with resistance to cytarabine. Interestingly, high expression of homeobox genes (*HOXA4, HOXA9, HOXA10, PBX3,* and *MEIS1*) was detected in AML specimens with not only normal but also complex karyotypes. Within NK-AML, Bullinger et al. [60] distinguished two distinct groups: one, where *FLT3* aberrations and FAB subtypes M1 and M2 prevailed, and the second one, where FAB M4 and M5 subtypes were more common. Of note, patients classified to those groups had different outcomes. For AML with complex karyotype, AML with *KMT2A* partial tandem duplications, and AML +8, it was impossible to find statistically significant unique gene expression signatures. Valk et al. [61] who analyzed 285 AML patients using Affymetrix GeneChips, identified 16 AML groups with distinct gene expression profiles. Some of them were composed of AML samples with known cytogenetic aberrations: t(18;21), t(15;17), and inv(16). *RUNX1T1* gene, which is a *RUNX1* fusion partner, was the most discriminative gene for AML with t(8;21). Overexpression of *MYH11* was the most discriminative feature of inv(16), which produces *CBFB-MYH11* fusion gene. Simultaneous downregulation of *CBFB* observed in this subtype could be explained by e.g., negative regulation of a wild-type *(wt) CBFB* allele by the fusion transcript. For APL, growth factor-coding genes were the most discriminative (hepatocyte growth factor (*HGF*), macrophage-stimulating 1 growth factor (*MST1*), and fibroblast growth factor 13 (*FGF13*)). However, AMLs with 11q23 were segregated into two separate clusters and partially scattered among all samples studied. Also NK-AML samples were divided into several clusters. The observed heterogeneity could be at least partially explained by the presence of particular mutations and different outcomes.

Further AML transcriptome studies, performed on independent patient cohorts, usually confirmed earlier research, reporting partially overlapping gene expression signatures. However, each study delivered a portion of new information, which deepened our knowledge about AML pathogenesis. Gutierrez et al. [62] performed hierarchical clustering of BM samples from 43 adult AML patients, based on the expression of over 5 thousand genes. Four distinct clusters they obtained corresponded to AML with inv(16), monocytic AML, APL and other AML samples which included NK-AML. The authors developed a minimal 21-gene predictor which classified each sample to appropriate group with 100% accuracy. Its efficiency was then confirmed with an independent AML sample set. APL samples, which formed the most condense group among all samples studied, revealed high expression of several growth factors and other signaling proteins, e.g., *HGF, FGF13, MST1, VEGFA, IGFBP2,* and *FGFR1*. Contrary, overexpression of *HOX* family members (*A5, A6, A7, A9, A10, B2, B5,* and *B7*), including genes encoding TALE proteins (*MEIS1*; *PBX3*), and histone proteins was shared by all non-APL leukemias. Increased level of *MYH11* expression and downregulation of *CBFB* and *RUNX3* genes were noted specifically for AML with inv(16). In monocytic leukemia, *CSPG2*, other adhesion molecules such as the lectins *CLECSF6*, *CLECSF12, SIGLEC7* and *FCN1* were upregulated compared to remaining AMLs. The remaining AML samples presented more heterogeneity, which was reflected by the existence of two subclusters, one with overexpression of genes encoding hematopoietic serine proteases, present in azurophil granules of neutrophilic polymorphonuclear leukocytes (*AZU1*, azurocidin 1, *ELANE* (previous *ELA2*), elastase, *PRTN3*, proteinase 3, and *CTSG*, cathepsin G), second with upregulation of *CD34* antigen, reflecting an early maturation arrest and lack of granulocytic differentiation.

AML M3 was extensively studied by Payton et al. [63] who compared the malignant promyelocytes from APL patients to leukemic cells collected from other AML subtypes and to promyelocytes, neutrophils and *CD34+* cells extracted from healthy bone marrow donors. The identified "M3-specific dysregulome" was composed of 510 genes and many of them exhibited dramatic differences in expression level comparing to other AML subtypes or normal promyelocytes. For example, *GABRE, FGF13, HGF, ANXA8*, and *PGBD5* were the most overexpressed genes whereas *VNN1, MS4A6A, P2RY13, HK3*, and *S100A9* the most underexpressed genes in M3 vs. other AMLs. 33 genes selected from the identified signature were validated by another high-throughput digital technology (nCounter; NanoString), capable of detecting as little as 0.5 fM of a specific mRNA and measuring up to 500 genes in a multiplex reaction. The authors demonstrated nCounter reproducibility and applicability as a tool for biomarker analysis when limited amounts of clinical material are available. 33 genes validated by NanoString assay were also enriched in an independent AML dataset of 325 samples, and APL mouse model, but, notably, not in a cell line expressing *PML-RARA* fusion gene.

One of the most impressive microarray-based studies was that of Haferlach et al. [53] who analyzed almost 900 patients with leukemia, including 620 with AML, with the use of Affymetrix GeneChips and support vector machine (SVM) model. The authors identified 13 separate leukemia types, including 6 within AML. Some of them, e.g., AML with t(8;21) and with t(15:17), could be classified with 100% specificity and 100% sensitivity based on the expression profile of 100 genes per group. The overall prediction accuracy of 95.1% was achieved. The misclassification occurred mainly in subgroups with a low sample number or high intragroup heterogeneity. The largest and the most heterogeneous subgroup was AML with normal karyotype, which cosegregated with AML with less common cytogenetic aberrations classified as "other". Here, AML samples with different fusion partners of *KMT2A* gene were included. Haferlach et al. [64] showed that APL was not only distinct from other AML subtypes in the matter of gene expression, but two M3 phenotypes, one with heavy granulation and bundles of Auer rods and AML M3 variant (M3v) with non- or hypogranular cytoplasm and a bilobed nucleus, could be discriminated based on gene expression signatures.

The largest GEP study in hematology and oncology was conducted thanks to international collaboration within the European Leukemia Net. In 2010, Gene Expression Profiling Working Group directed by Torsten Haferlach published the results of analysis of 3,334 samples collected from leukemia (including 542 AMLs) and MDS patients by 11 laboratories across three continents [65]. Apart from European ones, laboratories from the United States, and one from Singapore joined the program. The main conclusion was GEP was a robust technology for the diagnosis of hematologic malignancies with high accuracy. According to the authors, GEP had invaluable application potential and was vulnerable to standardization, outperforming more subjective methods such as cytomorphology and metaphase cytogenetics. To enable better molecular understanding of leukemias, the authors deposited the collected data into a publicly available domain.

In 2012, de la Blétière et al. [66] proved that AML cytogenetic subtypes could be successfully determined with the use of GEP, even in samples with low leukemic blast content or poor quality. With the use of Illumina Expression BeadChips, the authors first classified 71 good quality samples from a training set, representing APL, t(8;21)-AML, inv(16)-AML, or NK-AML with at least 60 percent of leukemic blasts. The optimal 40-marker gene classifier (10 markers per class, including previously described as well as newly discovered genes) was applied to 111 suboptimal AML samples with low leukemic blast load (from 2 to 59%) and/or poor quality control criteria. The overall error rate was 3.6%. All APL and t(8;21) samples were correctly classified, even those containing as low as 2 percent blasts. The worst result was achieved for inv(16). Surprisingly, poor sample quality did not affect classification. By the way, de la Blétière et al. [66] demonstrated reliability, robustness, and sensitivity of Illumina bead-based technology which seemed to be not worse than other, commercially or academically developed, microarray platforms used before.

5. Between AML and ALL: Acute Leukemia with *KMT2A* Rearrangements

In 2002, Armstrong et al. [67] showed that ALL with translocations involving the *KMT2A* gene (previously known as *MLL*) presented a unique gene expression profile, different from ALL and AML without *KMT2A* abnormalities. The core of this unique gene expression signature consisted of multilineage markers of early hematopoietic progenitors and *HOX* genes, which corresponds with the fact that *KMT2A* gene encodes histone lysine methyltransferase, a transcriptional coactivator regulating expression of genes (including *HOX*) during early development and hematopoiesis. Therefore, the authors proposed to distinguish a distinct leukemia entity termed "MLL". Then, a common gene expression signature, enriched in homeobox genes (*MEIS1, HOXA4, HOXA5, HOXA7, HOXA9,* and *HIOXA10*), was determined for all acute leukemias with *KMT2A* fusion, irrespectively of their lineage (myeloid or lymphoid), by Ross et al. [68]. Similarly, Andersson et al. [69] associated childhood acute leukemias with *KMT2A* rearrangements with upregulation of homeobox genes (*HOXA10, HOXA4, MEIS1* and *PBX3*). In their study, *KMT2A*-positive AMLs were also enriched in genes involved in cell communication and adhesion, whereas some antiapoptotic genes (e.g., a tumor necrosis factor receptor, *TNFRSF21*) and tumor suppressor genes (*BRCA1; DLC1*) were downregulated in this AML subtype. Hierarchical clustering with a subset of genes encoding transcription factors showed that leukemic samples with *KMT2A* rearrangements grouped together, independently on lineage. Although *KMT2A* translocations are prevalent in infant and treatment-related leukemias, they also occur in adult leukemias that were studied by Kohlmann et al. (2005) [70] who wondered how the differing *KMT2A* partner genes influenced the global gene expression signature and whether pathways could be identified to explain the molecular determination of *KMT2A* leukemias of both lineages. The data analysis in both types of acute leukemias revealed t(11q23)/*KMT2A*-positive samples that were evidently distinct from other subtypes of the same lineage. As in the case of childhood leukemia, adult *KMT2A*-AML and *KMT2A*-ALL, despite a shared common gene profile, revealed also lineage-specific expression markers sufficient to segregate them according to their lineages, with no respect to the *KMT2A* fusion partner. The commonly overexpressed genes were obviously the homeobox genes and their regulators (*HOXA9, MEIS1, HOXA10, PBX3, HOXA3, HOXA4, HOXA5, HOXA7*), *NICAL* gene (present *MICAL*, encoding Microtubule Associated Monooxygenase), *RUNX2* transcription factor and *FLT3* gene. The common downregulated genes included TNF-receptor superfamily members (*TNFRSF10A* and *TNFRSF10D*), transcription factor *POU4F1*, tumor suppressor *ST18* or *MADH1* (present *SMAD1*), encoding a signal transducer and transcriptional modulator. Comparing to *KMT2A*-ALL, overexpression of *CEBPB*, *CEBPA*, *KIT*, *MADH2*, *MITF*, *FES* and *SPI1* (former *PU.1*) oncogenes, and *MNDA*, encoding the myeloid cell nuclear differentiation, was noted in *KMT2A*-AML. Summarizing their results, the authors concluded AML with

t(11q23)/*KMT2A* and ALL with t(11q23)/*KMT2A* are rather distinct entities.

6. AML Risk Classification and Outcome Prediction

AML chemotherapy does not always lead to complete remission (CR). 20-50% AML patients are primarily resistant to induction therapy. Having this information at the time of diagnosis would facilitate treatment decision making. Taking into account the success of GEP in AML diagnosis and classification to particular disease subtypes, its application in prognosis prediction was only a matter of time. Correlation of *HOXA9* upregulation with poor AML outcome was reported by Golub et al. in their first microarray paper, devoted to ALL and AML classification [20]. Later, Andreeff et al. [71] demonstrated that many AML cases with intermediate and adverse prognosis, presented *HOX* expression levels similar to the levels observed in normal *CD34+*. Interestingly, *HOXA* genes could distinguish favorable vs. unfavorable cases, but only *HOXB* genes effectively distinguished intermediate from unfavorable AMLs. Despite the high coordination in *HOX* gene family expression, *HOXA9* seemed to be the best single predictor of overall survival (OS), disease-free survival (DFS) and response to therapy, confirming earlier results of Golub et al. [20].

AML outcome prediction was often matched with AML classification. Analysis of AML GEP-based clusters defined by Valk et al. [61] in the context of prognosis showed that three clusters, overlapping with inv(16), t(15;17), and t(18;21), were associated with good outcome. Patients classified to the cluster that common feature was *MECOM* overexpression had clearly worse outcome.

Two prognostically different NK-AML subgroups were also identified by Bullinger et al. [60]. A group with predominance of *FLT3* aberrations and FAB subtypes M1 and M2 presented shorter OS. High expression of *GATA2, NOTCH1, DNMT3A* and *DNMT3B* in this group suggested pathological impact of aberrant methylation. Genes deregulated in the second group, where FAB M4 and M5 subtypes were more common, were associated with granulocytic and monocytic differentiation, immune response and hematopoietic stem-cell survival (*VEGF*). Analysis of prognostically relevant genes led to the identification of 133-gene based prognostic signature. *FOXO1A*, encoding transcription factor involved in cell cycle arrest and apoptosis regulation, was one of the genes correlated with favorable outcome. Poor outcome was determined by overexpression of *HOX* genes and *FLT3* gene. Prognostic gene expression signature proposed by Bullinger et al. [60] was 2 years later applied to an independent cohort of 64 NK-AML patients below the age of 60 by Radmacher et al. (2006) [72]. GEP of the new sample set, performed with Affymertix GeneChips, different array technology than one that was used to establish the prognostic signature, allowed segregation of patients into 2 clusters with significantly different OS and DFS. Strong association between the outcome classification and *FLT3*-ITD status was observed: 67% patients with poor outcome were *FLT3*-ITD-positive. However, *FLT3*-ITD was present in almost 20% of patients from the good-outcome class, which indicated contribution of other prognostic determinants. Nevertheless, Radmacher et al. [72] not only validated the previous prognostic signature, but also developed a well-defined classifier, which might be applied to individual patients, with best accuracy to patients with normal cytogenetics and wt *FLT3*.

Application of gene expression microarrays for prediction of patient sensitivity to therapy was also demonstrated by Heuser et al. [73] and Tagliafico et al. [74]. Heuser et al. [73] identified gene expression profile that distinguished AML M0-M5 (excluding M3) patients with good or poor responses. Hierarchical clustering performed on a training set of 33 AML samples divided good responders into two clusters, which suggested the effect of determinants other than treatment. Interestingly, samples with the lowest level of myeloid cell maturation, corresponding to FAB subtypes M0 and M1, were equally distributed between clusters representing good and poor response. Over 30% of poor-response-associated genes, e.g., *MN1, FHL1, CD34, RBPMS, LPAR6*, and *FLJ14054* gene (currently known as *NPR3*), were earlier described as overexpressed in hematopoietic stem or progenitor cells, particularly in the populations with the highest self-renewing capacity. Application of the identified gene expression signature to the test set of independent 104 AML samples enabled dividing them into two prognostic subgroups which correlated with the different response to induction chemotherapy. The accuracy of prediction was 80%. Tagliafico et al. [74] conducted a similar analysis, but their training set included 10 blast cell populations collected form AML patients and 6 AML cell lines with determined sensitivity to differentiation therapy. The identified prediction set, containing such genes as *MEIS1* and *MS4A3*, was then tested on the GEP datasets published by Valk et al. [61] and Bullinger et al. [60]. Despite a significant overlap in prognosis prediction, Tagliafico et al. [23] distinguished within the poor outcome groups described in original papers, a subgroup of patients (20-40%) which revealed sensitivity to maturation induction. From the practical point of view, it suggested that these patients could benefit from a differentiation therapy even though the initial prognosis was unfavorable.

Gene expression analysis of samples from 170 older AML patients (median age 65 years, all FAB subtypes except for M3), presented by Wilson et al. [75], showed the problem of response to therapy as even more complex. Hierarchical clustering divided patients into 6 groups with different rates of resistant disease, complete response, and DFS. Distribution of FAB subtypes and *NPM1* (but not *FLT3*-ITD) mutation differed significantly between clusters, but in only two clusters particular subtypes prevailed, e.g., one cluster almost exclusively consisted of monocytic leukemias (M4 and M5). Poor-risk clusters had lower WBC and blast counts whereas cluster with the best DFS and OS contained 75% of NK-AML and 78% samples with *NPM1* mutations. Each cluster was defined by a specific expression profile of the 50 most discriminating genes. For example, in a cluster with the poorest outcome, the authors observed upregulation of multidrug resistance genes (*ABCG2* and

ABCB1, former *MDR1*), homeobox gene *PBX1*, which prevents myeloid differentiation, and *STK17* gene, encoding apoptosis regulator. Another poor outcome cluster revealed overexpression of genes connected with immunity (*IRF4, IL10RA,* and *MALT1*). The most favorable outcome cluster was characterized by overexpression of genes encoding proteins implicated in cell signaling (*IL12A*), promoting apoptosis (*CASP3* and *LTBP1*), and leukemic transformation (*MEIS1, WT1,* and *FOXC1*), and downregulation of genes encoding major histocompatibility complex (MHC) proteins of class II.

Based on gene expression data from a training cohort of 163 AML patients collected by the German AML Coopertive Group, Metzeler et al. [76] elaborated 66 gene expression signatures to predict OS in CN-AML. Then, the signature was validated in two independent cohorts of 79 and 64 CN-AML patients from Europe and the United States, respectively. In all three cohorts, patients with low gene expression risk score had better outcome. Moreover, in multivariate analyses of validation cohort, the gene expression score proved to be a stronger prognostic factor than age, presence of *FLT3*-ITD, and *NPM1* mutation. The genes from the identified signature partially overlapped with the results of previous studies, e.g., *TCF4, FHL1, CD109*, and *SPARC* genes, had been earlier associated with poor response to chemotherapy [73].

7. Looking for New Therapeutics

Transcriptome, as well as proteome, reflects the current cell status that dynamically evolves under the influence of various stimuli, e.g., therapeutic agents. GEP is a sensitive tool to detect changes in genome activity; therefore it can be applied to monitor minimal residual disease (MRD) and cancer cell reaction to novel compounds. AML treatment is challenging because resistance to therapy is quite common and even those patients who achieve CR are prone to relapse. GEP was widely applied for analysis of resistance mechanisms and efficiency of potential drugs. Kinase inhibitors in the treatment of AML with *FLT3*-ITD, correlated with negative prognosis, have been studied for a long time. In April 2017, staurosporine derivative PKC412 (midostaurin), a multikinase inhibitor, was approved by the US FDA for the treatment of newly diagnosed *FLT3*-mutant AML in combination with chemotherapy [77]. Activity of this compound was analyzed, inter alia, in human myelomonoblastic cell line MV4-11 carrying *FLT3*-ITD by Stölzel et al. [78] with the use of gene expression microarrays. Two versions of MV4-11 cells, sensitive and resistant, were compared prior to and after treatment. Significant downregulation of *TP53* and upregulation of *JAG1* was observed in resistant cells before and after treatment. *MCL1* and *KIT* genes were upregulated in resistant MV4-11 cells after incubation with PKC412. The authors concluded that resistance against PKC412 was mediated by antiapoptotic gene activation and proapoptotic signal decrease, with contribution of deregulation of genes involved in normal and malignant hematopoiesis.

Tavor et al. [79] studied gene expression response of the AML cell line U937 under treatment with the CXCR4-antagonist, AMD3100. CXCR4, a receptor for SDF-1 chemokine secreted by stromal cells, participates in the interactions of leukemic stem cells with the BM microenvironment, necessary for cell migration and disease progression. In addition the role of elastase, neutrophil serine protease synthesized during the transition of myeloblast to promyelocyte, was investigated. The authors did not observe changes in gene expression after treatment with anti-CXCR4 antibody or elastase inhibitor, but found AMD3100-induced suppression of the SDF-1/CXCR4 axis or elastase inhibited leukemic cell proliferation as well as activated genes involved in myeloid differentiation.

Other candidates for target therapeutics in AML treatment are in clinical trials. One example is pinometostat (EPZ-5676), a small-molecule inhibitor of DOT1L (histone methyltransferase disrupter of telomeric silencing 1-like). Pinometostat, considered for combination therapies of acute leukemias with *KMT2A* gene rearrangements, was proved to target DOT1L and reduce H3K79 methylation in adult AML patients with 11q23 translocations [80]. Another promising therapeutic strategy is directed against members of Hedgehog (HH) signaling pathway, which plays a role in embryonic cell development as well as in proliferation and maintenance of adult stem cells, including cancer stem cells [81, 82]. Comparing chemotherapy-sensitive and resistant cell lines, Queiroz et al. [83] indicated HH pathway as an essential component of myeloid leukemia MRD. Overexpression of HH pathway effectors, *GLI1* and *PTCH1,* followed by constitutive activation of HH signaling, was correlated with chemoresistant phenotype. The efficacy of a HH pathway inhibitor, glasdegib, which targets a smoothened protein (SMO), a G protein-coupled receptor interacting with PTCH1, was evaluated by Cortes et al. in AML and high-risk MDS patients who were not eligible for intensive chemotherapy [81]. At the end of 2018 glasdegib has been approved in the USA, under the name DAURISMO™, for use in combination with low-dose cytarabine for the treatment of newly diagnosed AML patients excluded from intensive induction chemotherapy due to age or comorbidities [84]. Another compound, GANT61, the inhibitor of GLI family proteins, was shown to specifically target the *CBFA2T3-GLIS2* fusion gene in pediatric AML [82]. The authors demonstrated that GANT61 treatment significantly reduced the expression level of *GLIS2* and a gene encoding bone morphogenic protein (*BMP2*). Posttreatment microarray-based gene expression analysis revealed downregulation of *CBFA2T3-GLIS2* target genes as well as genes required for cell cycle progression, cell proliferation, and epigenetic regulation. New AML therapies are still being elaborated. Currently, the US National Cancer Institute (NCI) supports 75 clinical trials for adult AML treatment (https://www.cancer.gov/about-cancer/treatment/clinical-trials/disease/adult-aml/treatment). It is impossible to provide even a brief summary of all of them in this work.

8. Pediatric AML: Distinct but Similar

AML in children is less frequent than in adults but reveals similar level of heterogeneity. In both age groups, similar chromosomal aberrations and mutations occur, though with different proportions. In children, CN-AML concerns only about 20% of all AMLs and the frequency of mutations is generally lower [85]. The power of GEP demonstrated on adult AML samples triggered the research of childhood AML. First, the 35-gene expression signature was shown to predict prognosis in pediatric AML [86]. Genes encoding cyclins and cyclin-dependent kinases required for cell cycle progression (*CDK6, CCND1,* and *CDC25A*), and *TRAF2* gene encoding a signal transducer activating *NFKB1*, showed higher expression in patients with poor outcome. The levels of *NFKBIA*, encoding *NFKB1* inhibitor, and *STK17B*, encoding serine/threonine protein kinase inducing apoptosis, were lower in patients with poor outcome. *STK17B* downregulation and *NFKB1* enhancement might explain why patients with adverse prognosis escaped from chemotherapy-induced apoptosis. An additional reason for the poor outcome could be increased cell cycle progression. Comparing pediatric AML patients with different FAB subtypes, the authors selected 213 probe set representing genes, whose expression correlated with FAB subtype. Both signatures, prognostic and diagnostic, shared only three genes (*TYMP, STK17B,* and *ATP6V0B*).

Ross et al. [68] compared gene expression in 130 pediatric and 20 adult AML samples with Affymetrix GeneChips. Some AML groups, namely t(15;17), t(8;21) and FAB M7, more frequent in children than adults, were clearly distinguished whereas AML with *CBFB/MYH11* fusion gene (inv(16)) and *KMT2A* chimeric fusion genes revealed more heterogeneity, indicating the existence of additional subgroups. Biology of the disease seemed to be similar, independently on age, as only minimal differences were observed in gene expression profiles between pediatric and adult AML samples containing the same lesions. The authors identified a set of class discriminating genes, which included genes specifically overexpressed in particular AML FAB types, e.g., AML M2, was characterized by increased expression of genes coding for cell surface antigens (*CD34*; *CD19*), proteins regulating developmental processes (*ROBO1, TWSG1,* and *PELI2*) and transcription factor *POU4F1*. Genes upregulated in AML M3, M4Eo and M7 encoded proteins reflecting particular stages of myeloid differentiation or lineage, for example, *HGF*, *MPO* and *CPA3* in M3, *CD52* and *CHI3L1* in M4Eo, *GP1BB* and *ITGA2B* in M7. The results concerning AML with *KMT2A* rearrangements were described above. What is interesting, Ross et al. [68] tested on their dataset the 35-gene prognostic signature described by Yagi et al. [86] and did not confirm its correlation with patient outcome. Instead, Ross et al. [68] selected another, small set of genes whose high expression correlated with poor outcome. This shows gene expression profile dependence on sample set, sample size, protocols and laboratory. However, another study of childhood leukemia, published by Andersson et al. [69], confirmed the results obtained by Ross et al. [68], presenting 77–86% overlap between the differentially expressed genes (DEGs).

Analysis of 237 pediatric AML cases with gene expression microarrays and double loop cross-validation method allowed for the selection of 75 probe sets, representing 59 unique genes, able to classify AML with the five most prevalent cytogenetic subtypes, constituting about 40% of pediatric leukemia [85]. Among the most discriminative genes were *WHAMMP3* and *ITM2A* (encoding membrane associated proteins) for *KMT2A*-rearranged; *RUNX1T1*, *IL5RA* and *POU4F1* for t(8;21); *MYH11*, *LPAR1* and *NT5E* for inv(16); *HGF*, *STAB1* and *FAM19A5* for t(15;17); *TP53BP2* (coding for p53-binding protein), and *DNAAF4* (encoding protein interacting with the estrogen receptors and the heat shock proteins) for t(7;12). The accuracy of the classifier, validated on two independent cohorts of patients, was equal to 92% and 99%. However, GEP had limited predictive value for AML cases with *NPM1*, *CEBPA*, *KMT2A*(-PTD), *FLT3*(-ITD), *KIT*, *PTPN11*, and *N/K-RAS* mutations, perhaps because of generally lower frequency of mutations in children than in adults.

9. AML in the Elderly

AML is a disease of older adults. Within age, not only the incidence of illness increases; elderly AML patients usually present worse outcome and weaker response to therapy. Rao et al. [87] reanalyzed clinically annotated GEP data from 425 de novo AML patients in the context of age. From this dataset, two age-related cohorts were selected: 175 young (<or= 45 years) patients and 144 elderly (>or= 55 years) patients. Indeed, both cohorts significantly differed in OS and DFS. This difference could be explained by unique pattern of deregulated signaling pathway found for older AML patients, who had a lower probability of E2F and PI3-kinase pathway activation but a higher probability of RAS, TNF, SRC, and EPI pathway activation. Thus, the authors concluded AML in the elderly represents a distinct biologic entity. The same conclusion was made by de Jonge et al. [88] who discovered the downregulation of the tumor suppressor gene *CDKN2A* in older AML patients with intermediate- and unfavorable prognosis. *CDKN2A* gene encodes a cyclin-dependent kinase inhibitor known as p16(INK4A) protein whose amount increases with physiologic ageing. The authors showed that p16-INK4A besides cytogenetic risk groups, was an independent OS prognostic parameter in older patients. The conclusion was that in the elderly, oncogenesis might be facilitated by a suppression of defense mechanisms, which usually protect older cells against cell and DNA damage [89].

10. Between MDS and AML

Myelodysplastic syndromes (MDS) are a group of clonal heterogenous hematologic malignancies frequent in the elderly, characterized by progenitor cell dysplasia, ineffective hematopoiesis and a high rate of transformation to AML [90]. Due to the not clearly defined boundaries between MDS and other myeloid disorders, establishing MDS diagnosis with conventional method is often problematic. Looking for a novel diagnostic strategy, Miyazato et al. [91] compared the transcriptomes of MDS with de novo AML and other

bone marrow diseases, with the use of custom-made oligonucleotide microarrays. The hematopoietic stem-cell fractions were purified based on the expression of the surface marker *PROM1*, previously named *AC133* or *CD133*. The authors identified a small set of genes preferentially expressed in MDS (e.g., *DLK1, TEC,* and *ITPR1*) or AML (e.g., genes encoding solute carrier (*SLC*) family members, opioid receptor delta 1 (*OPRD1*) and leptin receptor (*LEPR*)).

AML with dysplasia, which has a poor outcome with conventional chemotherapy, was studied by Tsustumi et al. [92]. The authors analyzed three AML subcategories with dysplastic morphology, AML with multilineage dysplasia (AML-MLD), MDS-related AML (MDS-AML), and therapy-related leukemia (TRL), and compared them with de novo AML without dysplasia. As in the study of Miazato et al., fractions of BM hematopoietic stem cells presenting CD133 antigen were selected for microarray-based transcriptome analysis. 56 genes displayed different expression levels between AML-MLD and MDS-AML. The genes preferentially expressed in AML-MLD comprise many genes encoding nuclear proteins, ubiquitination-related proteins, and *PF4* gene encoding platelet factor 4, a chemokine secreted by platelets and influencing BM environment. The same gene was overexpressed in AML-MLD also when compared to AML without dysplasia, suggesting the correlation of PF4 expression with AML-MLD. Distinction between MDS-AML and AML without dysplasia was possible with the use of 28 genes, including 9 shared within the 56-gene signature differentiating AML-MLD and MDS-AML. One of them, *LAPTM5* gene, encoding lysosomal transmembrane protein, was clearly upregulated in MDS-AML, being a candidate for novel marker for MDS-related leukemia. However, the gene signatures determined by Tsustumi et al. [92] were not perfect, which showed global gene expression analysis may be not adequate for AML subgroups with high intragroup heterogeneity and subtle intergroup differences.

11. Bone Marrow Microenvironment

The main attention of AML investigators was focused on leukemic blasts. However, it is well known that other factors, such as tumor microenvironment, contribute to disease progression. In hematological malignancies, the interplay of cancer cells and surrounding stroma is particularly important. BM microenvironment consists of a heterogeneous population of cells directly involved in hematopoiesis or supporting hematopoietic cell function, migration, adhesion, metabolism, and differentiation, e.g., by production of ligands and cell adhesion molecules [93]. The role of BM niche in AML has not been fully elucidated and is currently intensely studied [4, 93]. Experiments with the mouse models indicated that the BM microenvironment not only facilitates the leukemic cell growth but can even initiate leukemogenesis in healthy cells [94]. The expansion of a single dominant hematopoietic progenitor clone is favored in the aged BM microenvironment, which causes monoclonality and may contribute to higher rates of leukemia incidence with age [95]. Moreover, BM niche protects quiescent LSCs, being responsible for MRD and relapse. On the other hand, BM stromal cells reveal high level of plasticity and can also be affected by malignant cells [93, 96]. Therefore, the disease progression depends on the leukemia-microenvironment crosstalk. One of the best recognized interactions between leukemic blasts and stroma is directed by a transmembrane chemokine receptor CXCR4, highly expressed by leukemic cells, and CXCL12 protein secreted by BM stromal cells. CXCR4-CXCL12 binding promotes the homing, residence, and survival of leukemic cells in the BM [4]. Another interaction, between the integrin VLA-4, expressed by leukemic cells, fibronectin present in the extracellular matrix, and VCAM-1 on BM stroma, contributes to chemoresistance [4].

In 2018, Kumar et al. described how AML blasts transform the BM niche into a leukemia-promoting and normal hematopoiesis-suppressive microenvironment through a secretion of exosomes, small vesicles mediating cell-to-cell communication [96]. The authors demonstrated that AML-derived exosomes target stromal and endothelial cells in the BM niche. Using human-to-mouse AML graft models, they proved AML-derived exosomes caused changes in mice, similar to those induced by AML cells, i.e., reshaped the BM niche cell composition and modulated gene expression in stromal cells. Genes required for normal hematopoiesis and bone development, e.g., *CXCL12*, *KITL* and *IGF1*, were downregulated whereas a hematopoiesis and osteogenesis suppressor, *DKK1*, was upregulated. Reduction of exosome secretion in AML cells delayed the disease progression.

One of the recent studies used a unique *ex vivo* model of growing leukemic cells on patients' own stroma (POS) derived in diagnosis (Dx), remission (Rm) and relapse (Rx) [97]. Compared to healthy mesenchymal stromal cells (MSCs), POS presented different morphology, larger cell size, reduced proliferation rate, slower expansion, and poor cell-cell contact. Coculture cross experiments revealed that POS preferentially supported proliferation of the same patient's AML cells, irrespective of the disease state POS was obtained in. The unique crosstalk between POS and AML cells was mediated by cytokines and chemokines, angiopoietin 1, secreted phosphoprotein 1, and SDF-1, encoded by *ANGPT1* (former Ang-1), *SPP1* (former *OPN*), and *CXCL12* genes, respectively. Compared to healthy MSCs, *SPP1* expression was higher in Dx/Rx and Rm POS whereas *ANGPT1* expression was upregulated in Dx/Rx POS and increased in the presence of AML cell. In contrast, *CXCL12* was decreased in Dx/Rx and Rm POS, which was associated by the authors with interruption in the CXCL12-CXCR4 signaling, and a consequent loss of hematopoietic progenitor quiescence and induced proliferation. Interestingly, POS demonstrated similar features in remission as in the active disease, which indicates the critical role of BM niche in relapse and treatment failure.

BM microenvironment-mediated protection of *FLT3*-ITD AML from tyrosine kinase inhibitors (TKIs) was recently reported by Chang et al. [98]. Drug resistance was a result of elevated expression of genes encoding cytochrome P450 enzymes, in particular *CYP3A4*, by BM stromal cells. Because CYP3A4 inhibitor reversed the protective effects of BM niche, the authors proposed a combination of *FLT3* TKIs with

CYP3A4 inhibitors as a novel strategy to treat *FLT3*-ITD AML.

Passaro et al. [99], who studied the BM vasculature in AML using intravital two-photon microscopy, associated increased vascular permeability in the BM microenvironment with disease progression and poor treatment response. Transcriptome analysis of BM-derived endothelial cells via RNA-seq identified deregulation of genes involved in vasculature development, angiogenesis, and response to hypoxia. *Nox4* gene, encoding NAPDH oxidase, responsible for production of reactive oxygen species (ROS), activation of nitric oxide synthase 3 (NOS3), and release of nitric oxide (NO), was particularly upregulated. Increased NO level contributed to the vascular leakiness in AML-engrafted mice and was associated with poor prognosis in AML patients. Application of NO synthase inhibitors combined with standard chemotherapy restored normal vasculature and improved the treatment response, demonstrating the efficacy of combined leukemia-niche therapies.

The role of lymphocytes and other blood cells has long been neglected in the studies of AML. However, Le Dieu et al. found that the absolute number of T-cells circulating in PB of de novo AML patients, not belonging to malignant clones, was increased compared to healthy controls [100]. Activation of T-cells might reflect a response to growth signals present in a local microenvironment. GEP of CD4+ and CD8+ T-cells from AML patients and healthy volunteers revealed global differences in transcription pattern, with little similarities to T-cells of CLL patients. Particularly, genes associated with the actin cytoskeleton and cellular polarization were deregulated in AML T-cells. According to the authors, T-cell aberrant activation leads to their dysfunction and impaired immune response, which is not a sufficient weapon against the leukemic blasts. Here, a rationale to apply immunomodulatory drugs appears.

12. Discovering the Power of Small Molecules: miRNA Expression Profiling

The discovery of regulatory role of RNA in cell and organism development completely changed our understanding of biology and at least partially explained the paradox that is the large size of mammalian genomes of which only a small percentage are the protein-coding genes [101–103]. Among small regulatory RNAs, microRNAs (miRNAs), termed due to their small size (18-23 nt), are best recognized [104, 105]. The function of miRNAs in gene expression regulation (usually repression), controlling cell fate and normal developmental processes as well as oncogenesis, is well-established [106–109]. As one miRNA targets multiple transcripts [110], dysfunction of miRNA may result in a wide-scale deregulation of gene expression, often triggering a cascade of events leading to pathogenesis. In 2002, Calin et al. demonstrated *miR-15* and *miR-16* are located at chromosome 13q14 region frequently deleted in B-cell chronic lymphocytic leukemias (B-CLL) [111]. Then, more than half miRNA genes were linked with cancer-associated genomic regions or fragile sites, and their amplification or deletion in human cancers supported miRNA role in malignant transformation [112]. Since 2005, when Lu et al. classified multiple human cancers, including AML, based on miRNA expression profiles exclusively, and proved general downregulation of miRNAs in tumors compared to normal tissues [51], miRNAs started to be widely investigated in cancers and other diseases.

MicroRNAs were also described as regulators of mammalian hematopoiesis [113, 114]. In 2004, three microRNAs, which modulate mouse hematopoietic lineage differentiation, were found by Chen et al. [113]: *miR-181*, a positive regulator of B-lymphoid cell differentiation, *miR-223*, nearly exclusively expressed in BM and myeloid cells, and *miR-142*, found at highest levels in B-lymphoid and myeloid lineages. Georgantas et al. identified 33 miRNAs specifically expressed in CD34+ hematopoietic stem-progenitor cells (HSPCs) [115]. The identified miRNA signature included *miRNA-17, -24, -146, -155, -128*, and *-181*, holding early hematopoietic cells at a stem-progenitor stage and blocking their maturation, *miRNA-16, -103*, and *-107* responsible for block differentiation of later progenitor cells, and *miRNA-221*, *-222*, and *-223* controlling terminal stages of hematopoietic differentiation. Some miRNAs indeed presented lineage-specific expression, which suggested the limitation of function to e.g., lymphoid (*miRNA-146*), erythroid (*miRNA-221* and *-222*), or granulocytic (*miRNA-223*) development. Inhibition of erythropoiesis and erythroleukemic cell growth through *KIT* gene suppression by *miR-221* and *-222* was also reported by Felli et al. [116] whereas granulopoiesis regulation by a minicircuitry involving *miR-223*, *NFIA* and *CEBPA*, by Fazi et al. [117]. Other miRNAs were able to control different processes, e.g., myelopoiesis and erythropoiesis, as *miRNA-155* [115]. Contrary to the results of Chen et al. [113] who studied hematopoiesis on murine model, *miR-142* expression was not detected in human hematopoietic cells by Georgantas et al. [115]. Later, Bissels et al. deepened the knowledge about miRNA-regulated hematopoiesis by combining analyses of microRNA and mRNA profiles in CD133+ and CD34+ hematopoietic stem and progenitor cells [118]. In both types of cells, 25 highest expressed miRNAs accounted for 73-74% of the total miRNA pool. However, the most abundant miRNAs were rather common for both progenitor cell types, except for *miR-142-3p*, which was upregulated in CD34+ cells, to the level of up to 5,000 copies per cell. Remarkably, one of *miR-142-3p* targets seemed to be *CD133* gene. The authors found 18 miRNAs expressed differentially between the CD133+ (ancestral) and CD34+/CD133- (later progenitor) cells. *miR-10a, -99a, -125a* and *b*, and *miR-146a* and *b*, expressed at highest level in CD133+ cells, were postulated to maintain the stem-cell character, whereas *miR-484* and other miRNAs upregulated in CD34+ cells, probably blocked cell differentiation at a later stage. Generally, differentially expressed miRNAs were involved in inhibition of differentiation, prevention of apoptosis, and cytoskeletal remodeling.

In 2007, Mi et al. [54] showed that discrimination of AML from ALL is possible through miRNA expression profiling. Among 27 miRNAs differentially expressed between AML and ALL, four were sufficient to distinguish these two types of acute leukemia: *let-7b* and *miR-223* were significantly upregulated in AML whereas *miR-128a* and *miR-128b* were

downregulated in AML comparing to ALL [54]. In 2010, Wang et al. [119] conducted similar research on Chinese cohort of 85 patients and found 16 miRNAs differentially expressed between AML and ALL. Half of them were previously reported by Mi et al. [54] (*miR-23a, miR-27a/b, miR-128a, miR-128b, miR-221, miR-222, miR-223*, and *let-7b*), but eight have not identified previously in this context (*miR-17, miR-20a, miR-29a/c, miR-29b, miR-146a, miR-150, miR-155*, and *miR-196b*). In addition, prognostically relevant signatures were determined for ALL, AML and non-M3-AML. One miRNA, *miR-146a*, was strongly inversely correlated with OS of both acute leukemias in two independent patient cohorts.

Garzon et al. studied miRNA expression in APL cells treated with all-trans-retinoic acid (ATRA) [120], and found upregulation of *miR-15a, -15b, -16-1, let-7a-3, let-7c, let-7d, miR-223, -342*, and *-107*, and downregulation of *miR-181b*. The observed ATRA modulation of *NFIA, RAS* and *BCL2* gene expression corresponded with the fact the mentioned genes were targets of *miR-107, let-7a* and *miR-15a/miR-16-1*, respectively. Then, Garzon et al. [121] evaluated the miRNA expression in 122 newly diagnosed AML cases comparing to *CD34+* cells from 10 healthy donors, and found 26 differentially expressed miRNAs, all downregulated in AML, e.g., *miR-126, -130a, -135, -93, -146, -106b*, and *-125a*. Expression level of some miRNAs was variable within AML and correlated with AML cytogenetics, prognosis and clinical features. For example, *miR-181* was downregulated particularly in AML with multilineage dysplasia whereas *miR-155* and *miR-181b* positively correlated with WBC (white blood cell count). In AML with balanced 11q23 translocations, many tumor suppressor miRNAs, targeting known oncogenes, were downregulated, e.g., *miR-34b* (targeting *CDK4* and *CCNE2*), *miR-15a* (targeting *BCL2*), *let-7* family (targeting *RAS* genes), and *miR-196* (targeting *HOX* genes). In trisomy 8, only upregulated miRNAs were identified, including those located at chromosome 8, e.g., *miR-124a* whose known target is *CEBPA*. NK-AML was the most heterogeneous; therefore the identified miRNA signature was not predictive of NK-AML. However, five miRNAs overexpressed in AML (*miR-199a and b, miR-191, miR-25*, and *miR-20a*) were associated with adverse patient outcome.

Debernardi et al. [122] showed strong correlation of *miR-181a* expression with the AML FAB subtypes (elevated in M1 and M2), and with the expression of its predicted targets. Half of them, e.g., *BCL2L11, KLF3, MAP2K1*, were negatively correlated with *miR-181a* expression. Havelange et al. [123] observed two other mRNA-miRNA interactions: negative correlation between *miR-181a* and *miR-181b*, *miR-155*, and *miR-146* expression with that of genes involved in immunity and inflammation (*IRF7* and *TLR4*), and positive correlation between *miR-23a, miR-26a, miR-128a*, and *miR-145* expression level with that of proapoptotic genes (*BIM* and *PTEN*). Association of the last three miRNA with apoptosis was experimentally validated. Lineage-associations were showed for *miR-23a* and *miR-196a* (positive correlation with myeloid differentiation), *miR-191*, *miR-222* and *miR-17* (negative correlation with erythroid differentiation). Interaction analysis induced the authors to conclude that a small group of miRNAs coordinately regulates protein-coding transcriptome influencing the same group of genes (presumably the key players) within the pathway.

In 2008, distinctive patterns of miRNA expression associated with cytogenetic and genetic AML subtypes were determined by Dixon-McIver et al.[124], Jongen-Lavrencic et al. [125], and Li et al. [126]. Dixon-McIver et al. [124] measured the expression level of 157 miRNAs in 100 AML patients and two AML cell lines, with the use of bead-based flow cytometric miRNA expression assay, and found 33 miRNAs with differential expression level between AML and normal BM, 17 upregulated (*let-7e, miR-27a, -30d, -142-5p, -155, -181a, -181b, -181c, -195, -221, -222, -324-5p, -326, -328, -331, -340, -374*), and 16 downregulated (*miR-9**, *-15b, -26a, -30a-3p, -34c, -103, -147, -151, -182, -184, -199a, -302b**, *-302d, -325, -367, -372*). Moreover, they associated t(15;17) translocation with upregulation of miRNAs located in the 14q32 imprinted domain, e.g., *miR-127, miR-154, miR-154**, *miR-299, miR-323, miR-368*, and *miR-370*. In AML with inv(16), high level of *miR-99a, miR-100*, and *miR-224* expression, was observed whereas t(8;21) AML presented high expression of *miR-146a* and a decrease of *miR-133a*. High degree of variability across samples was noted for *miR-10a* and *miR-125b*. Jongen-Lavrencic et al. [125] found a set of strongly upregulated microRNAs (*miR-382, -134, -376a, -127, -299–5p*, and *-323*) in t(15;17), partially overlapping with the APL signature defined by Dixon-McIver et al. [124]. Clustering of AML cases with miRNA expression revealed that inv(16) were sometimes mixed with t(8;21) and shared a part of miRNA signature, which is not unexpected as these both AML subtypes belong to CBF AMLs. Predictors of most AML subclasses, containing from a few to several dozen miRNAs, were built for AML with *NPM1* mutation, and even for AML with *FLT3*-ITD or *FLT3*-TKD mutations which were not separated from other AMLs as a result of global miRNA expression-based clustering.

Li et al. [126] found miRNA signatures composed of 2-24 miRNAs able to distinguish AML with *KMT2A* rearrangement, t(15;17), t(8;21) plus inv(16), t(8;21), inv(16), and normal controls. They noted that *miR-126/126** were specifically overexpressed in both t(8;21) and inv(16) AMLs, while *miR-224, miR-368*, and *miR-382* in t(15;17). In *KMT2A*-AML, significant overexpression of miRNAs from polycistronic miRNA cluster, *mir-17-92*, was observed. A minimal class-predictor contained only seven miRNAs: *miR-126, -126**, *-224, -368, -382, 17-5p*, and *-20a*. Interestingly, differential expression of *miR-126/126** was not associated with DNA duplication nor mutation, but probably resulted from epigenetic regulation. Gain- and loss-of-function experiments revealed that high expression of *miR-126* inhibits apoptosis and increases cell viability and proliferation, synergistically with the fusion gene *RUNX1-RUNX1T1*. From 674 predicted *miR-126* targets, the authors empirically tested 12 genes and confirmed that only one of them, *PLK2*, was indeed regulated by *miR-126*.

MicroRNA expression pattern correspondence with FAB classification was shown by Wang et al. [127] who noted that M1, M2, M3 and M4 tended to depart from each other more effectively than M5. Apart from miRNAs reported previously,

the authors identified a spectrum of new miRNAs whose expression strongly correlated with particular AML FAB types, e.g., *miR-1300, miR-1180, miR-297, miR-610* and *miR-650* overexpressed exclusively in AML M1. High expression of some miRNAs was common in a few FAB types, e.g., *miR-181a-d, miR-221* and *miR-222* by M1, M2 and M3. The most distinct miRNA expression pattern was shown in AML M3, with 36 miRNAs strongly and exclusively upregulated, e.g., *miR-370, miR-224, miR-382, miR-154* described earlier, and *miR-100, miR-195, miR-452, miR-654-3p* not reported previously. Comparing to normal PBMCs, all AML samples displayed downregulation of *miR-29a* and *miR-142-3p* which were proposed by the authors as AML diagnostic biomarkers.

Analysis of miRNA expression data in CN-AML, performed by Marcucci et al. [128] allowed identification of miRNA signature of prognostic relevance. Upregulation of *miR-181a* and *b* was associated with low risk whereas overexpression of *miR-124, -128-1, -194, 219-5p, 220a, and -320* with increased risk of failure to achieve CR, relapse or death. Increased miRNA levels were correlated with increased expression of genes involved in innate immunity, encoding toll-like receptors, interleukins and caspases. Some of them were putative targets of *miR-181*.

13. Mutation-Defined AML Subtypes

Progressive accumulation of transcriptomic data regarding both mRNA and miRNA expression allowed more precisely characterize AMLs with the recurrent mutations.

13.1. AML with Mutated CEBPA. Valk et al. [61] first tried to determine gene expression profiles specific for AML with particular mutations. Having a set of nearly 300 samples, the authors were able to distinguish a unique gene expression signature for AML with *CEBPA* mutations. *CEBPA* gene, mutated in 5% to 15% of all AML cases encodes a critical regulator of hematopoietic stem-cell maintenance and myeloid differentiation, therefore unique gene expression pattern was not unexpected for samples with the loss-of-function *CEBPA* mutation. Valk et al. [61] showed that the most prominent features of *CEBPA*-mutated AML were *CD7* overexpression and downregulation of *CTNNA1, TUBB* and *NDFIP1*. Interestingly, hierarchical clustering of all AML samples segregated AMLs with *CEBPA* mutations into 2 different clusters, and one of them included also samples without any known mutations or chromosomal aberrations. In their subsequent paper, the authors, using bisulfite genomic sequencing, revealed that this previously unidentified subset of AML was represented by samples where *CEBPA* gene promoter was hypermethylated [129]. In fact, within this mysterious AML cluster identified by Valk et al. [61], *CEBPA* levels were very high in AML cases with *CEBPA* mutations whereas in AML with wt variant, *CEBPA* expression was minimal or undetectable, due to its epigenetic silencing [129]. Detailed characteristics of *CEBPA*-silenced AML samples showed that they expressed both, myeloid markers (*CD13, CD33,* and *MPO*), and T-lymphoid markers (e.g., *CD7* mentioned above). In addition, high expression of the myeloid oncogene *TRIB2* and *NOTCH1* gene, encoding a membrane receptor and transcriptional regulator of T-cell development, was noted. In a part of those AML patients, activating *NOTCH1* mutation was identified. Moreover, *TRIB2* was determined to be a direct target of *NOTCH1* signaling. Later, the authors found that *CEBPA* methylation was accompanied by aberrant hypermethylation of many genes compared to *CEBPA*-mutated AMLs or with normal *CD34*+ hematopoietic progenitor cells [130]. This could explain an observed *in vitro* decreased response of *CEBPA*-silenced AML to myeloid growth factors and makes this AML subtype susceptible to dynamically developing treatment with demethylating agents. Interestingly, comparison of genome-wide methylation pattern with GEP revealed only a minimal overlap (12 unique genes, including *CEBPA*) between the differentially expressed and differentially methylated genes. This suggested that gene expression and genome methylation are biologically independent processes.

13.2. AML with Mutated NPM1 and the Paradox of HOX Gene Expression. Since the time a 4-nucleotide insertion in *NPM1* gene and its significance in AML was discovered by Falini et al. in 2005 [52], much attention was paid to unveiling the mechanism of AML triggered by *NPM1* mutation. *NPM1* encodes a multifunctional protein, involved in ribosome biosynthesis and transport, DNA replication and repair, chromatin remodeling, protein chaperoning, regulation of cell cycle, embryogenesis and oncogenesis [131, 132]. Although *NPM1* protein localizes mainly in the nucleolus, it constantly shuttles between nucleus and cytoplasm. The 4-nucleotide insertion in the last exon of *NPM1* gene results in aberrant cytoplasmic accumulation of a protein and, consequently, affects its functions. *NPM1* gene is mutated in about 30% of AML and in 50% to 60% cases of adult NK-AML. Given that NPM-cytoplasmic positive (NPMc+) AML reveals unique molecular and clinical features [133], it was introduced into WHO classification as a separate entity [13]. An invaluable contribution to cognition of AML promotion by *NPM1* mutation was made by GEP.

Alcalay et al. [134] first claimed NPMc+ AML represented a distinct entity, which can be easily distinguished from other AML samples, regardless of the karyotype. They compared global gene expression of 58 AML NPMc+ samples with prevalence of NK-AML and frequent occurrence of *FLT3* mutations, to the group of 20 NK-AMLs without *NPM1* mutations and lower occurrence of *FLT3* mutations. Unsupervised approach showed *NPM1* mutation status was the strongest clustering parameter. A selected 369-gene-predictor efficiently segregated NPMc+ from NPMc- patients. Interestingly, *NPM1* transcript level did not differ between these two groups, indicating the lack of *NPM1* mutation influence on *NPM1* gene expression. I confirmed this observation by analysis of *NPM1* alternative transcripts with droplet digital PCR (ddPCR) [135]. In the NPMc+ patients, *CD34* and *CD133* antigens, as well as *POU4F1* and *CDKN2C*, were suppressed whereas a number of homeodomain-containing transcription factors, including *HOX* and *TALE* genes, were activated [134]. Because several *HOX* genes are highly expressed in

HSCs and their expression decreases within cell differentiation, the authors concluded *HOX* gene activation is a mechanism of stem-cell phenotype maintenance utilized by AML blasts. The obtained results explained the overexpression of *HOX* genes described earlier in an uncharacterized subgroup of NK-AML samples [57], which probably in significant proportion carried *NPM1* mutation.

A miRNA-based expression signature of AML with mutated *NPM1* was later published by Garzon et al. [136]. The signature consisted of upregulated *miR-10a* and *b*, members of *let-7* and *miR-29* families, and *miR-15a-16-1* and *miR-17-18a-19a-20a* clusters. Contrary, *miR-204* and *miR-128a*, predicted to target *HOX* genes, were downregulated in *NPM1*-mutated AML, what is consistent with the observed upregulation of *HOX* genes in this AML subtype. The authors proved positive correlation between *miR-10a* and *HOXB4* expression, and confirmed that *miR-204* targets *HOXA10* and *MEIS1* genes. Similarly, other authors showed three miRNAs located in intergenic regions in the *HOX* clusters, *miR-10a, miR-10b*, and *miR-196a-1*, were highly positively correlated with *HOX* gene expression [122, 123]. Jongen-Lavrencic et al. [125] observed in AML with *NPM1* mutation not only overexpression of *miR-10a* and *miR-10b*, but also overexpression of *miR-196a* and *miR-196b*.

From the other side, Verhaak et al. [137] found that *HOX*-gene-based discriminative signature was not limited to AML with mutated *NPM1*. They showed *HOX*-based classification produced high percentage of false positive results, including AML cases with 11q23 abnormalities and *KMT2A* gene rearrangements, which corresponded with the results described above. One of possible explanations is the fact that *NPM1* mutation in AML is not exclusive. More insight into *HOX* gene phenomenon was given by Andreeff et al. [71], who measured, using real-time RT-PCR technique, expression of 39 *HOX* genes in 115 de novo AMLs representing various cytogenetic types. While in normal CD34+ cells homogeneous expression of *HOX* genes was observed, AML samples were very heterogeneous in the matter of *HOX* expression. As previously reported, low levels of *HOXA* and *HOXB* expression was noted in favorable cytogenetic AMLs. Overexpression of *HOX* genes was detected in AMLs with intermediate cytogenetics and in AMLs with *NPM1* mutation, usually associated with favorable prognosis. Considering impact of *FLT3*-ITD, the authors observed higher *HOX* expression in AML samples with both mutations, *NPM1* and *FLT3*-ITD, than in AML with exclusive *FLT3*-ITD.

Biological significance of *NPM1* mutation with concomitant *FLT3*-ITD, and *NPM1* mutation with concomitant NRAS mutation, was also verified with the use of mouse knock-in models [138]. Overexpression of HOX genes, enhanced self-renewal, expansion of hematopoietic progenitors, and myeloid differentiation bias, were common for both combinations, which indicated the persistence of transcriptional signature specific for *NPM1* mutation in hematopoietic progenitors of both double-mutants. Comparing to wt mice, dramatically altered gene expression profile was only observed in *NPM1-FLT3*-ITD mutants which also had higher leukocyte counts, early depletion of common lymphoid progenitors, and a monocytic bias, presenting more acute course of the disease. *NPM1* and Nras-mutants, characterized by granulocytic bias, developed AML with a longer latency and a more mature phenotype. Moreover, additional somatic mutations were required for AML progression. The molecular-level results, including GEP, underpinned the higher frequency and significantly worse prognosis of AML with simultaneous *NPM1* and *FLT3*-ITD mutations.

Kühn et al. [139] explained the phenomenon of *HOX* and *FLT3* gene upregulation in *NPM1*-mutated AML as a result of the activity of chromatin regulators, *KMT2A* and *DOT1L*. Earlier, KMT2A and DOT1L methyltransferases were known to positively regulate *HOX* gene expression in normal hematopoiesis and AML with *KMT2A* rearrangements [140]. Many *KMT2A* fusion partners interact with DOT1L. With the use of CRISPR-Cas9 genome editing, small-molecule inhibition and RNA-seq, the authors proved that KMT2A and DOT1L control the expression of *HOX*, *MEIS1* and *FLT3* (which is a downstream target of MEIS1), and also cell differentiation in *NPM1*-mutated leukemia, despite the lack of *KMT2A* rearrangement [139]. Interaction of wt KMT2A with another protein, called menin, and their association with *HOX* and *MEIS1* promoters, were required for *HOX*, *MEIS1*, and *FLT3* upregulation. DOT1L showed a synergistic effect. Combinatorial inhibition of the menin-KMT2A interaction and DOT1L more profoundly suppressed *HOX*, *MEIS1*, and *FLT3* expression, and induced differentiation of *NPM1*-mutated AML. Therefore, novel and possibly less toxic therapeutic strategies emerged for the acute leukemias with *NPM1* mutation and concomitant *FLT3*-ITD.

13.3. AML with FLT3-ITD. *FLT3* gene, encoding FMS-like tyrosine kinase 3, is mutated in one-third of AML patients. Usually, the consequence is constitutive activation of the kinase receptor what impairs hematopoietic cell signaling and disturbs hematopoiesis. The presence of *FLT3*-ITD without concomitant *NPM1* mutation is well-established marker of poor AML prognosis. In contrast to AML with mutated *NPM1* or *CEBPA*, gene expression signature specific for AML with *FLT3*-ITD was not found for a long time, probably due to the cooccurrence of other mutations. For example, in the study of Valk et al. [61], samples with *FLT3*-ITD were segregated into three clusters. Considering miRNA expression, Garzon et al. [113] reported *miR-155, miR-10a*, and *-10b* were upregulated in AML with *FLT3*-ITD.

Two classifiers, based on the expression of 10 or 34 genes predicting *FLT3*-ITD in *NPM1*-mutated CN-AML were determined by Huang et al. [141] by analysis of two independent AML patient cohorts, each with over 100 CN-AML patients. Among the 6 genes common for both classifiers, one was downregulated (*MIR155HG*, *miR-155* host gene, and noncoding oncogene) and 5 were upregulated, encoding membrane proteins (*TMEM273* and *STON2*), ectonucleotide pyrophosphatase/phosphodiesterase (*ENPP2*), matrix metallopeptidase (*MMP2*), and cytokine signaling suppressor (*SOCS2*).

In 2017, Zhu et al. [142] analyzed four microarray datasets and identified 22 DEGs between *FLT3*-ITD-positive and

negative AMLs shared by all four datasets. Reactome pathway analysis revealed correlation of the identified genes with hemopoiesis, hemoglobin metabolic process, hematopoietic or lymphoid organ development, immune system development, and myeloid cell differentiation. Expression levels of *AHSP, EPB42, GYPC* and *HEMGN* genes were negatively correlated with *FLT3* expression. High expression of these four genes in NK-AML with *FLT3*-ITD was associated with better prognosis. In concordance with the fact that *HEMGN* is a direct transcriptional target of *HOXB4*, a negative correlation between *HEMGN* and *HOXB4* expression was found. Based on the data collected, the authors concluded that *FLT3*-ITD might influence AML prognosis by decreasing the expression of *AHSP, EPB42*, *GYPC*, and *HEMGN* genes.

Wellbrock et al. [143] associated *FLT3* mutation with the expression of HH pathway downstream effector, GLI2. Just as the presence of *FLT3* mutation, *GLI2* expression significantly decreased event-free survival (EFS), relapse-free survival (RFS), and OS. Because *GLI2* was coexpressed with *SMO* (Smoothened) and *GLI1*, one can conclude *FLT3* mutation is generally associated with HH pathway activation. In fact, the analysis of an independent patient cohort revealed the expression of *GLI2* and *GLI1*, and *FLT3* mutation could serve as independent risk factors for the survival of AML patients. Interestingly, the expression of three HH pathway ligands, Sonic Hedgehog (*SHH*), Desert Hedgehog (*DHH*), and Indian Hedgehog (*IHH*), undetectable in AML blasts, was detected in primary BM stromal cells. Thus, BM microenvironment seemed to sustain activation of HH pathway, supporting leukemia progression and mediating AML resistance to conventional chemotherapy [143]. Targeting HH pathway emerges as an alternative or complimentary therapeutic strategy against *FLT3*-mutated AML.

13.4. AML with IDH Mutations. Mutations in *IDH1* and *IDH2* genes, encoding two isoforms of the nicotinamide adenine dinucleotide phosphate (NADP)-dependent isocitrate dehydrogenases, cytosolic and mitochondrial, respectively, occur in 33% of CN-AML patients and confer unfavorable prognosis [144]. Marcucci et al. [144] identified a novel subset of CN-AML with R172 IDH2 mutation, which was mutually exclusive with other known prognostic mutations, associated with lower CR rates and presented distinctive gene and miRNA expression profiles. Comparing to *IDH1/IDH2*-wt patients, AML with R172 IDH2 mutation revealed higher expression of *APP, CXCL12, PAWR, CDC42BPA,* and *SPARC* genes, and decreased expression of *KYNU, SUCLG2, CD93, LY86, LIST1* and *PTHR2*. As far as the above-mentioned overexpressed genes were more or less directly related to AML and cancer, none of the downregulated genes had previously been associated with AML. In the miRNA expression signature specific for R172 *IDH2*-AML, members of *miR-125* family (including *miR-125b* which targets the tumor suppressor gene *TP53* and inhibits myeloid differentiation), and two microRNAs not associated with cancer but involved in embryonal stem-cell differentiation, *miR-1* and *miR-133*, were upregulated. None of the downregulated miRNAs (e.g., *mir-194-1, miR-526, miR-520a-3p*, and *mir-548b*) had been associated with normal hematopoiesis or AML.

13.5. AML with RUNX1 Mutations. Apart from translocations and fusion transcripts, small mutations were also found in runt-related transcription factor 1 (*RUNX1*) gene in 6% [145] to more than 30% of AML patients [146]. In older AML patients, the frequency of *RUNX1* mutation was twice as high as in younger patients [147]. Presence of *RUNX1* mutation was also associated with the resistance to induction chemotherapy [145].

Gaidzik et al. [145] found 148 genes differentially expressed between *RUNX1*-mutated AML and AML with wt *RUNX1*. However, the identified gene expression signature was not exclusive for *RUNX1* mutation but shared with AML with monosomy 7 and MECOM rearrangements, and AML with complex karyotypes, both deprived of *RUNX1* mutation. A key feature of *RUNX1*-mutated AML was deregulation of apoptotic pathway, supported by an increased expression of *BCL2*-like gene, *BCL2L1*.

Association of *RUNX1* mutations with CN-AML poor outcome and distinct gene and miRNA expression was confirmed by Mendler et al. [147]. In older (> 60 years) CN-AML patients with *RUNX1* mutation and wt *NPM1*, genes normally expressed in primitive hematopoietic cells (e.g., *BAALC, CD109, GNAI1, HGF,* and *FHL1*) and early lymphoid precursors, B-cell progenitors (e.g., *DNTT, BLNK, FOXO1,* and *FLT3*), were upregulated whereas myelopoiesis promoters, such as *CEBPA*, components of neutrophil granules (*AZU1, MPO*, and *CTSG*), were downregulated. Regarding miRNA profile, *miR-223* and two members of the *let-7* tumor suppressor family were decreased in AML with *RUNX1* mutations. Three other miRNAs, of unknown functions in leukemogenesis, *miR-211, miR-220*, and *miR-595*, were upregulated in *RUNX1*-mutated blasts.

The collected data indicated definitely different biology of *RUNX1*-mutated AML than, for example, *NPM1*-mutated AML, and contributed to the distinction of AML with mutated *RUNX1* as a provisional entity in the revised WHO classification of AML [13].

14. AML with Overexpression of Particular Protein-Coding Genes

Valk et al. [61] distinguished a compact cluster of AML samples with overexpression of *MECOM*, transcriptional regulator, and oncoprotein involved in hematopoiesis, apoptosis, development, cell differentiation, and proliferation. High expression of *BAALC* gene, postulated marker of early hematopoietic progenitor cells, was earlier established as an independent poor prognostic factor in CN-AML [42]. Langer et al. [148] proved younger (<60 years) CN-AML patients with *BAALC* overexpression presented distinct gene expression signature, with upregulation of genes earlier associated with poor outcome (e.g., *HGF, MN1, CD200*), genes involved in drug resistance (e.g., *ABCB1* alias *MDR1*) and hematopoietic stem-cell markers (*PROM1* alias *CD133*, *CD34*, *KIT*). *CD133* was the most upregulated gene in high

BAALC expressers. Interestingly, no differences were found in global microRNA expression, but an inverse correlation between the expression levels of *miR-148a* and *BAALC* was observed suggesting that *miR-148a* might act as a negative regulator for *BAALC*. Later, the same authors focused on the meningioma 1 (*MN1*) gene, encoding a member of gene transcription regulator complex with the nuclear receptor RAR-RXR or the vitamin D receptor [149]. They associated high *MN1* expression with the lack of *NPM1* mutation, increased *BAALC* expression, less extramedullary involvement, and worse outcome. Gene- and microRNA-expression patterns determined from high *MN1* expressers had common features with high *BAALC* expressers (upregulation of *PROM1, CD34, FZD6, CRYGD*, *CD200*, and *ABCB1* genes) and patients with wt *NPM1* (low levels of HOX genes). Positive correlation was also found between the expression of *MN1* gene and the *hsa-miR-126* family, contributing to proangiogenic activity of VEGF and formation of new blood vessels, and *hsa-miR-424*, which regulates monocyte and macrophage differentiation. Apoptosis-related *hsa-miR-16* and miRNAs involved in malignant transformation (e.g., *hsa-miR-19a* and *hsa-miR-20a* members of the *miR-17-92* polycistron), as well as *hsa-miR-100* and *hsa-miR-196a*, were downregulated in AML samples with higher *MN1* expression.

Metzeler et al. [150] who analyzed the expression levels of *ERG, BAALC* and *MN1* in over 200 CN-AML patients with the use of oligonucleotide microarrays, confirmed the association of high level of expression of the studied genes with inferior OS and a lower rate of CR. Indeed, the expression levels of all three genes were highly correlated. However, in multivariate analyses, high *ERG* expression, similarly as *FLT3*-ITD, seemed to be an independent and strongest predictor of negative prognosis in younger and older CN-AML patients. The results suggested the prognostic value of *ERG, BAALC*, and *MN1* genes might partially overlap, and high *ERG* expression, together with the presence of *FLT3*-ITD, might be a sufficient combination of factors for high-risk stratification in CN-AML.

15. Time for Meta-Analyses

After a significant amount of gene expression data had been collected, papers reporting meta-analysis started to appear. For example, in 2010, Miller et al. [151] systematically analyzed the results of 25 AML studies published between 1999 and 2008. In total, close to 16 thousand expression features, corresponding to 5 thousand unique genes, were available from 2,744 patient samples analyzed with 10 different microarray platforms. One-third of genes were reported in more than one study. Several genes, e.g., *VCAN* and *PGDS*, were identified only in AML cell lines. 25 genes, including 7 *HOX* family members, *POU4F1*, *TSPAN7*, *MYH11*, *RUNX1T1*, *RUNX3*, *CD34*, and *MN1*, were reported as AML-specific by at least 8 independent studies. *HOX/TALE* expression was increased in AML with normal cytogenetics, *NPM1* and *FLT3* mutations, and 11q23 abnormalities involving the *KMT2A* gene. Decreased expression of these genes was typical of CD34+ cells, AML with *CEBPA* mutations and AML with cytogenetic aberrations. Considering prognosis-relevant signatures, the authors found only a minority of genes (9.6%) were reported by at least two studies. Among these genes, *BCL11A, TBXAS1, HOXB5, HOXA10, CD34, MN1, NME1, FLT3*, were upregulated whereas genes such as *EML4, C3AR1, SMG1, FOXO1, AZU1*, were downregulated in AML samples with poor prognosis. In AML with *NPM1* mutations, increased expression of *SMC4* gene was reported by 5 different studies. Apart from the selection of genes and pathways shared by different AML studies, meta-analysis made by Miller et al. [151] enabled identifying novel marker genes and potential therapeutic targets which were skipped by single studies. The examples were two genes whose expression correlated with response to therapy, namely *TBXAS1* and *SEMA3F*, increased in AML samples with poor and good prognosis, respectively. This evidently shows that reanalysis of collected transcriptomic data and combining the results from different studies may be an underestimated source of new AML-relevant information.

16. Custom-Made Microarrays: An Alternative to Global GEP

Since the time microarray technology was established, multiple types and applications of microarrays were developed [45]. To benefit from this dynamically developing technology, guidelines for microarray gene expression analyses in leukemia were formulated by three European leukemia networks in 2006 [152]. Among all microarray platforms used for gene expression analysis of AML, commercially available GeneChips of Affymetrix™, predominated (see Supplementary Table 1). However, a few prominent AML papers were published based on cDNA microarrays developed at the Stanford University [60, 73] or Lund University [69]. All of the above microarray platforms were generated to study global gene expression. Alternatively, small custom-made microarrays, dedicated to analysis of a selected subset of genes, were harnessed to AML studies. IntelliGene Human Cancer CHIP, cDNA microarray from Takara Biomedicals, as well as two kinds of custom oligonucleotide microarrays, covering 2,304 genes, mainly encoding transcription factors, membrane proteins, growth factors, and proteins involved in redox regulation, were used by Miyazato et al. [91] to identify MDS-specific genes. In-house microarray was applied by Park et al. [153] to study the expression of about 300 prognosis-related genes in 4 clinical AML samples. The genes were selected based on global GEP of AML cell lines and previously published data. Taking advantage of our own experience with custom microarrays, we also designed and generated a boutique microarray dedicated to gene expression analysis of AML [154]. Our AML-array was composed of about 900 oligonucleotide probes complementary to genes selected by the literature search: proven and postulated acute leukemia biomarkers, general oncogenes, genes specifically involved in leukemic transformation, genes related to immune response, and a set of positive (housekeeping human genes) and negative (plant and bacterial) control genes. AML-array was used to analyze gene expression in 33 AML patients without

or with minimal maturation (FAB M1 and M2 subtypes) and 15 healthy volunteers (HV). Based on 83-gene classifier, we were able to perfectly distinguish AML from HV samples. The genes overexpressed in AML included well-established AML markers such as *KIT, MYH11, MYC, CEBPA, MN1, MPO, SET*, and *HOXA10*, but also genes rarely discussed in the context of AML pathogenesis, e.g., *STMN1* (the most discriminative gene in our analysis), *CDK6, ANGPT1* or *ENO1*. The role of genes determined in our analysis as underexpressed in AML, e.g., *IFITM1, FCN1, S100A9, LTB, LYZ, FCER1G*, was even less clear and demand further research. We found that the upregulation of *CPA3* gene was specific for AML with mutated *NPM1* and *FLT3* genes. Although we observed some gene expression trends, we were not able to find any genes with statistically significant differences between AML subgroups divided according to FAB subtype, mutation status, or response to therapy. This may be due to too small sample size, high homogeneity level within the study group, limited to M1 and M2 FAB subtypes which are not dramatically different, too high technical bias or a preselection of genes which could skip more discriminative genes. Nevertheless, we showed applicability of a small custom array to AML gene expression analysis. Following optimization, it could serve, for example, as a first-line diagnostic tool. With the use of complementary quantitative RT-PCR methods, we identified three genes (*S100A9, ANXA3* and *WT1*) whose expression levels can be used to distinguish between M1 and M2 FAB subtypes. We showed relationship between *STMN1* and *ABL1* expression level, and *FLT3* and *NPM1* mutation status. We have also found correlation between positive response to treatment and high *CAT* expression and low *WT1* expression [154].

17. SAGE: Alternative to Microarrays before Massive Sequencing Era

Apart from microarrays, serial analysis of gene expression (SAGE) technique was also applied to AML gene expression profiling [59, 155, 156]. Although this method did not demand prior gene sequence knowledge, produced more quantitative results and was described as very sensitive, it was definitely less common than microarrays and finally was ousted by NGS.

With the use of SAGE, 22 AML samples with four most common translocations, t(8;21), t(15;17), inv(16), and t(9;11), were compared to normal myeloid progenitor cells [155]. Over 2.6 thousand transcripts were abnormally expressed. Altered expression of 56 genes was shared by all AML samples, e.g., *NUBPL, TRAM2, PTRF* (present *CAVIN1*) (upregulated), *FCN1, LCN2* and *FASN* (downregulated). Other genes were differentially expressed in one or some of the translocations studied. In all translocations except t(8;21), more than 2/3 DEGs were underexpressed. Of note, only a small part of the SAGE results corresponded with the results of published microarray-based experiments. For example, Lee et al. [155] did not observe *MYH11* overexpression in inv(16) nor *RUNX1* and *RUNX1T1* overexpression in t(8;21). In subsequent paper, the authors compared SAGE results obtained for three pooled primary AMLs with t(9;11)(p22;q23) with SAGE-based GEP of Mono Mac 6 (MM6) cell line, representing AML with this particular translocation. Despite generally similar gene expression profile, the authors identified 884 alternatively expressed transcripts corresponding to 83 known genes, mainly related to biosynthetic and metabolic processes. Interestingly, *HRAS* with well-established role in leukemogenesis, and three other genes from ERK1/ERK2 MAPK pathway, governing cell growth, proliferation, differentiation and survival, were overexpressed exclusively in MM6.

18. Next Generation Sequencing: Unlimited Perspectives

Microarray boom lasted about 15 years. Since 2006, when a first high-throughput automatic sequencer, Genome Analyzer was launched by Solexa, DNA microarrays were being gradually replaced by the NGS, termed also massive parallel sequencing (MPS). At the beginning, the costs of NGS outbalanced the costs of microarray experiment, but they were soon compensated. While microarrays are still applied for genotyping, due to their simplicity compared to the whole genome sequence analysis, in the field of transcriptome research, NGS is incomparably better. Transcriptome sequencing, called RNA-seq, is able to detect all types of transcripts present in a cell, including noncoding RNAs, products of gene fusions and alternative splicing. In addition, transcriptome sequencing is often combined with a whole genome, exome or targeted resequencing which allows completely characterizing the studied object. In 2013, such comprehensive study of AML was published by The Cancer Genome Atlas Research Network in the New England Journal of Medicine [23]. A total number of 200 cases represented different AML subtypes were sequenced (50 whole genomes and 150 exomes). For the same individuals, analyses of global mRNA and miRNA expression, and DNA methylation were performed. In several cases, RNA-seq revealed increased or exclusive expression of the mutant *DNMT3A*, *RUNX1*, *PHF6,* and *TP53* genes. Gene fusions, including 15 new fusion events with maintained open reading frame, were detected in almost half of AML patients. Hierarchical clustering of gene expression data enabled distinguishing seven AML groups based on mRNA expression and five groups based on miRNA expression. Similarly as in microarray data analysis, the identified groups were highly correlated with AML FAB subtypes, differentiation stage, presence of the recurrent mutations, and patient outcomes. Integration of gene expression and DNA methylation data led to the discovery of a small RNA set within an imprinted locus on chromosome 14. These small RNAs were specifically dysregulated in APL. Patients with *PML-RARA* fusions had generally very distinct mRNA and miRNA signatures that were strongly correlated with each other and with a specific DNA methylation signature. AML with *RUNX1-RUNX1T1*, AML with some *KMT2A* fusions, and AML with three mutations (in *NPM1*, *DNMT3A*, and *FLT3*) together were also associated with mRNA and miRNA expression signatures. In compliance with previous research, the most discriminatory miRNAs for the triple-mutant AML

were *miR-10a, miR-424, miR-196b, miR-130a*, and *let-7b*. Other transcription factor fusions were correlated with only mRNA expression signatures.

At the end of 2018, functional genomic landscape of acute myeloid leukemia was highlighted by Tyner et al. [25]. The study, within the frame of Beat AML program, included half thousand AML patients, for whom whole-exome sequencing and RNA sequencing data were integrated with the analyses of *ex vivo* drug sensitivity. Clustering of the 2,000 most variably expressed genes allowed to distinguish gene expression signatures associated with genetic and cytogenetic AML groups. Mutations in several genes, e.g., *TP53* and *ASXL1*, seemed to be responsible for a broad pattern of drug resistance. Specific gene expression signatures were also identified for 78 out of 119 testable drugs. For example, 17 gene expression-based signature predicting sensitivity to ibrutinib was determined. Multivariate modelling was harnessed to estimate contributions of both mutation and gene expression patterns in drug response prediction.

Apart from two most prominent studies mentioned above, a significant number of NGS-based AML papers were published within the last several years. Since referring to all of them is impossible in one review, a few selected examples are highlighted below.

19. Better Tools: Better Characterization

Like the microarrays, RNA-seq was used to better characterize particular AML subgroups and cell lines. For example, transcriptome sequencing of three basic myeloid leukemia cell lines, K562, HL-60, and THP1, representing chronic myeloid leukemia (CML), APL and acute monocytic leukemia, respectively, was conducted by Wang et al. [157]. They found ERK/MAPK and JAK-STAT signaling pathways were more highly activated in K562 than in HL-60 cells. Contrary, PI3K/PKB pathway, induced by oncogene *KIT* or *FLT3*, as well as *PML* and *RARA* genes, which are fusion partners in APL, were upregulated in HL-60. Genes related to cell cycle, cell division, and chemokine signaling pathway were also overexpressed in HL-60 cells. Genes upregulated in THP1 cells were enriched in immune defense, inflammatory response, and other processes connected with monocyte functions (e.g., *LYZ, MPO, HLA-B, IL8*, present *CXCL8, PRG2, SPI1*, former *PU.1*, and *TFRC*). Based on GEP, the authors concluded K562 cells are a good model to study erythroid differentiation, HL-60 cells, chemotaxis and phagocytosis, and THP1, inflammatory response. Gosse et al. [158] described a novel NK-AML cell line, termed CG-SH. A whole genome sequencing revealed the absence of recurrent mutations but novel small alterations were found in several genes, including *GATA2* and *EZH2*. Comparing genome and transcriptome data showed allele-specific expression of *GATA2* gene which resulted from epigenetic silencing. Although the mutation was heterozygous, only a mutated variant was transcribed. Interestingly, genes which are frequently mutated in AML, but not mutated in CG-SH (e.g., *NPM1*, *GATA2, IDH2*, *RUNX1*, and *TP53*), were upregulated in the studied cell line, however, their levels of expression remained within the ranges observed for 55 AML patients. Differential expression of genes implicated in proliferation, apoptosis and differentiation, was noted for CG-SH cells following cytokine treatment.

Two subtypes of pediatric CBF AML, t(8;21), and inv(16), were compared with the use of RNA-seq by Hsu et al. [159]. Although both CBF leukemias revealed many common features, the authors were able to discover two hundreds of DEGs. In t(8;21) samples, the most upregulated gene was *RUNX1T1*, fusion partner gene, whereas the most underexpressed was *RFX8*. Overexpression of matrix metallopeptidase gene *MMP14* and downregulation of collagen gene *COL23A1* was typical of inv(16). Compared to NK-AML samples, *HOX* gene family, including *MEIS1* and *NKX2-3* transcription factors, were downregulated in both CBF AMLs. Within NK-AML, two subgroups, with and without *FLT3*-ITD, were not able to distinguish based on GEP. In total, 287 fusion transcripts were identified; 16 of them were novel, including three involving NUP98 gene. In the whole cohort of 64 patients, alternative splicing events (ASEs) differentially expressed across all subtypes were also detected. The predominant alternative splicing events were skipped exon (SE), mutually exclusive exons (MXE) and retained intron (RI).

Singh et al. [160] compared genome-wide DNA binding sites and transcriptome data associated with *RUNX1-RUNX1T1*, *CBFB-MYH11*, and *PML-RARA* oncofusion protein expression and found many target genes, pathways, and acetylation patterns are shared between these three fusion transcription factors. In the case of *RUNX1-RUNX1T1* and *PML-RARA*, the percentage of common target genes reached 40%. Gene expression analysis revealed both common and unique signatures for each translocation. The unique DEGs included genes described earlier as specific for particular translocation, namely, *TRH, POU4F1, PRAME* and *RUNX1T1* genes for t(8;21), *VCAN, MN1* and *S100A12* for inv(16), and *CTSG* and *PTGDS* for t(15;17). However, even these unique genes were members of the similar pathways, in particular, linked to cell proliferation (e.g., TGFB signaling pathway) and apoptosis. Therefore, the authors hypothesized the three different AML subtypes, despite distinct molecular properties (binding sites; mechanisms of action) exploit common programs of malignant cell transformation.

Eisfeld et al. [161] studied AML with a sole monosomy of chromosome 7 (-7 AML), the most frequent autosomal monosomy associated with poor outcome. In over 30 cases analyzed, the authors not only identified the most frequent AML mutations but found different mRNA and miRNA expression profiles compared to AML with both copies of chromosome 7. Among DEGs, downregulated prevailed, with 94% genes mapping to chromosome 7, affirming dosage effect. The most overexpressed genes were *PTPRM*, encoding a protein tyrosine phosphatase receptor, a regulator of cell growth, differentiation and oncogenic transformation, ID1, a downstream target of oncogenic tyrosine kinases, and *MECOM*, coding for a transcriptional regulator and oncoprotein implicated in hematopoiesis, apoptosis, development, cell differentiation and proliferation. Out of 16 differentially expressed miRNAs, 6 were significantly downregulated,

including 5 from chromosome 7 and *miR-9-1* from chromosome 1. Upregulated miRNAs came from two clusters, located on chromosome X (*miR-20b, miR-363,* and *miR-106a*) and chromosome 19 (*miR-99b, miR-125a*, and *miR-let7e*).

RNA-seq data were also collected from 13 patients with deletions of the long arm of chromosome 9 [del(9q)], a rare aberration occurring in about 2% of all AML cases, as a sole abnormality or accompanied by t(8;21), t(15;17), or other cytogenetic aberration [162]. Transcriptome of del(9q), combined with the exome and target amplicon sequencing, was compared with the transcriptomes of 454 AML patients with normal karyotype or various cytogenetic aberrations. Characteristic features of del(9q) AML were mutations in *NPM1*, *DNMT3A,* and *WT1,* more frequent than in other AML subtypes, and downregulation of *TLE4* gene.

Mixed-phenotype acute leukemia (MPAL), a rare type of progenitor leukemia with ambiguous expression of myeloid and lymphoid lineage markers, was studied by Pallavajjala et al. (2018) [163]. RNA-seq combined with whole genomic sequencing (WGS) enabled identifying mutations in 70 genes, different translocations, residing mainly in the noncoding regions of the genome, and describing gene expression profiles in samples from four patients with T/Myeloid MPAL. For two patients with matched diagnostic and remission samples, enriched pathway analysis allowed for association of genes which were upregulated at diagnosis, with pathways involving nucleosome and chromatin assembly and organization.

20. Discovery of New Fusion Transcripts

Being a useful tool for fusion transcript detection, RNA-seq was applied to identify a complex 3-way translocation t(8;12;21)(q22;p11;q22) in an individual AML M2 patient [164]. In addition to *RUNX1–RUNX1T1* fusion, typical of t(8;21) AML, the patient harbored two additional translocations with the contribution of *VPS13B* gene, a causative gene of Cohen syndrome, encoding vacuolar protein sorting 13, forming *TM7SF3–VPS13B* and *VPS13B–RUNX1* fusion genes.

With the use of a whole transcriptome sequencing, 88 new fusion transcripts were discovered in AML by Wen et al. [165]. In total, 134 fusion transcripts were detected in 45 AML samples, including 29 NK-AMLs. The fusions were predominantly formed between the genes adjacent in the same chromosome, in different orientations, and distributed at different frequencies in the AML cases, regardless of the karyotype. While comparing to other tumors, the authors found only 5 common fusions, all shared with only one tumor type (prostate cancer). It underpins the AML-specificity of the discovered fusions. Out of 114 fusions identified in NK-AML, seven were unique for this AML subtype. Moreover, *CIITA-DEXI* fusion transcript, occurring in three isoforms, was found in 48% of NK-AML cases. Of note, the maximal number of fusion transcripts found in one NK-AML case was 57. Although some fusions were generated posttranscriptionally, these results suggest that genome-level changes are not so rare in AMLs with normal karyotypes. The significance of particular fusions remained to be elucidated.

Also in pediatric CN-AML, novel fusion transcripts were identified with RNA-seq, e.g., *NUP98-PHF23* [166] or *CBFA2T3-GLIS2* [167, 168], occurring with 2.6% and 4.3-8.4% frequency, respectively. More frequent *CBFA2T3-GLIS2* fusion resulted from a cryptic inversion of chromosome 16 and was correlated with high risk of relapse and poor outcome [167, 168]. Schuback et al. [167] demonstrated the fusion was most prevalent in the youngest patients (<5 years) and absent in adults (>20 years). In another work, Masetti et al. identified another fusion transcript in 40% of the *CBFA2T3-GLIS2*-positive patients [169]. The novel fusion derived from a member of Hedgehog signaling pathway, Desert Hedgehog (*DHH*), and Ras Homologue Enrich in Brain Like 1 (*RHEBL1*) gene, coding for a small GTPase of the Ras family. *DHH-RHEBL1*–positive patients exhibited a specific gene expression pattern, with upregulation of *FLT3*, *BEX1*, *MUC4* and *AFAP1L2* genes. The outcome of these patients was even worse than that of the patients with exclusive *CBFA2T3-GLIS2*-rearrangement. Notably, targeted treatments against AML with *CBFA2T3-GLIS2* are under evaluation. GANT61, the most potent inhibitor of GLI family proteins, which are the final effectors of Hedgehog pathway, seems to be efficient also against GLIS2 chimeric proteins [82].

21. Alternative Transcripts: Another Source of Transcriptome Variability

The role of alternative splicing (AS) in AML pathogenesis was first highlighted by Tanaka et al. (1995) [36] who analyzed two of three previously identified alternative isoforms of *AML1* gene, at present termed *RUNX1*. Both transcripts, *AML1a* and *AML1b*, shared a runt homology domain, responsible for DNA binding, whereas transcriptional activation domain was present only in *AML1b*. The authors found that the two AS products regulated hematopoietic myeloid cell differentiation in an antagonistic way, presumably via competing for the binding to *CBF2B* gene encoding transcriptional activator. While *AML1a* inhibited granulocytic differentiation and induced cell proliferation upon granulocyte colony-stimulating factor (G-CSF) treatment, concomitant *AML1b* overexpression recovered the granulocytic differentiation.

Development of NGS contributed to the progress in AS research. Now, the role of splicing abnormalities in AML progression and drug resistance is incontestable [170]. While overexpression of *SRSF1* was associated with solid tumor promotion, mutations in genes encoding splice factors, i.e., *SF3B1, SRSF2, U2AF1*, are considered as important drivers of hematological disorders such as MDS and AML [170]. Contribution to AML pathogenesis was assigned to splice variants of *FLT3*, aberrant splicing of *BCL2* gene, linked with drug resistance, and overexpression of *WT1* and *E2F1* genes, which encode transcription factors taking part in AS regulation [170]. We recently demonstrated that alternative transcripts of *NPM1* gene are upregulated in AML and ALL at diagnosis, decrease in CR and increase again at relapse [135]. High expression of two *NPM1* gene isoforms was significantly associated with shorter overall and disease-free survival. This suggested that not only mutation but also expression level of

NPM1 gene affects patient outcome. Aberrant proportions of particular *NPM1* splice variants could be linked to abnormal expression of genes encoding alternative splicing factors.

RNA-seq of two samples collected from an individual AML M2 patient at diagnosis and remission were explored in the context of AS by Li et al. (2014) [171] and Gao et al. (2014) [172]. In both studies, a few dozens of differentially splicing events were detected in differentially splicing genes, associated with RNA processing, cellular macromolecule catabolic process and DNA binding.

Shirai et al. (2015) investigated the consequences of the most common *U2AF1* mutation in a transgenic mouse model [173]. Whole transcriptome analysis of hematopoietic progenitor cells of *U2af1*-mutated mice revealed altered hematopoiesis and changes in premRNA splicing. Comparing the results of the analysis with human RNA-seq data from TCGA AML cohort displayed enrichment of *U2AF1*-induced splicing alterations in processing genes, ribosomal genes, and recurrently mutated MDS and AML-associated genes (e.g., *NPM1*, *BCOR,* and *KMT2D*). The authors concluded sequence-specific AS pattern triggered by mutant *U2AF1* was similar in mouse and human cells.

Li et al. (2018) integrated AS events derived from RNA-seq with H3K79me2 ChIP-seq data across 34 human normal and cancer cell types [174]. Clustering based on skipping exon-associated sites divided all cell types to 6 clusters. Two of them consisted predominately of cell lines derived from hematological malignancies. Moreover, four AML cell lines, mainly with *KMT2A* rearrangements, were found in one cluster, together with one CML cell line. Deregulated genes associated with this particular cluster were involved in mRNA splicing via spliceosome. The obtained results corroborated contribution of epigenetic-mediated splicing events to progression of *KMT2A*-AML and associated alternative splicing mediated by K79me methyltransferase, encoded by *DOT1L* gene, with leukemogenesis.

22. Chimeric RNAs: Newly Discovered Contribution to Transcriptome Complexity

Apart from well-established fusions and alternative transcripts, another class of transcripts has been recently discovered in tumor as well as in normal cells by RNA-seq [175, 176]. The new class of functional and potentially oncogenic RNAs, called chimeric RNAs (chRNAs), not only are transcribed from genome regions modified by translocation, inversion, or more complex chromosomal rearrangement, but can be generated as a result of posttranscriptional RNA processing, e.g., cis- or trans-splicing. By combining two or more gene loci, chRNAs also differ from conventional splicing variants. The existence of chRNAs in AML was proved by Ruffle et al. in 2017 [177]. In RNA-seq data from three AML patients, 17 chRNAs were identified, including new *PML-RARA* transcripts with exon junctions not described earlier in t(15;17), and expression changes with time and treatment. Other chRNAs originated from two adjacent genes (e.g., *VAMP8-VAMP5*), nonadjacent exons separated by thousands of base pairs (e.g., *SLC16A3-METRNL* and *UBR5-AZIN1*), two distant genes (e.g., *NONE-CTDP1*), or gene fragments corresponding to opposite chromosome strands. Extending their research to a larger AML patient cohort and normal CD34+ transcriptome, the authors identified four new types of tumor-specific chRNAs recurrently expressed in AML samples (*TRIM28-TRIM28, DHRS7B-TMEM11, PLXNB-BLRD1,* and *SLC16A3-METRNL*). On the basis of *PML-RAR* fusions, it was shown in one patient several isoforms of the same chimeric transcript can coexist and present various sensitivity to therapeutic agents. The resistance to treatment can be indicated by the appearance or increase of fusion transcripts. However, the oncogenic potential of chRNAs needs to be verified by further research.

23. Small RNAs from the NGS Perspective

Compared to microarrays, NGS enabled more comprehensive and quantitative analysis of miRNome. Ramsingh et al. [178] analyzed miRNome of one CN-AML patient, female diagnosed as FAB M1, to assess miRNA expression and mutations in miRNA or miRNA binding sites. Small RNA sequencing of leukemic myeloblasts and CD34+ cells pooled from 5 healthy donors revealed expression of 472 miRNAs, including 7 novel miRNAs. The most highly expressed miRNA in both AML and CD34+ cells was *miR-233*. In AML, it represented almost 50% of all miRNA reads. miRNAs, which displayed differential expression between AML and control CD34+ pool, included *miR-362-3p* and *miR-25*, overexpressed and underexpressed in AML, respectively. Comparison of NGS-based miRNA profiling with the array- and RT-PCR-based approaches, showed microarray and real-time analyses underestimated the expression of some miRNA, although the general correlation between platforms was significant. The authors did not find acquired mutations in miRNA genes but revealed several novel germline polymorphisms. Comparing the results of miRNA expression with the sequence of this particular AML patient genome, which was known earlier [21], they identified a single mutation in the putative tumor suppressor gene *TNFAIP2* and proved this mutation generated a new miRNA binding site. As this mutation resulted in a Dicer-dependent translational repression of a reporter gene, the consequence could be a translational repression of *TNFAIP2*, previously described as a target of *PML-RARA* or *PZLF-RARA* fusion genes and highly expressed in hematopoietic cells. *TNFAIP2* mutation was predicted to generate imperfect binding sites for *miR-223* and *miR-181b*, but the experiments conducted on AML cell lines did not confirm contribution of *miR-223* and *miR-181b* and any other known miRNA to the translational repression of mutant *TNFAIP2* 3′-UTR. The authors did not exclude possibility of regulation by a new, unknown yet, miRNA. Nevertheless, the *TNFAIP2* 3′-UTR mutation must be rare as it was not found in any other AML samples from 187 patients screened.

Integrating SNP and mRNA arrays with microRNA profiling of 16 myeloid cell lines, García-Ortí et al. [179] associated expression levels of 19 miRNAs with CNVs affecting their loci. One of these miRNAs, *miR-370*, often

upregulated in AML, was proven to target tumor suppressor *NF1*, downregulated in more than 30% AMLs. Because *NF1* suppression activates RAS similarly as RAS-activating mutation, AML patients with *miR-370* overexpression may potentially benefit from additional treatment with either RAS or mTOR inhibitors.

Starczynowski et al. [180] who globally analyzed miRNA localization and expression in human genome using cell line models, discovered that 77% miRNAs mapped to leukemia-associated copy-number alterations, and the expression of only 18% of them was detectable. Furthermore, they found the loss of two selected miRNA, *miR-145* and *miR-146a*, localized in a commonly deleted in AML region 5q, initiated leukemia in mice. Using small RNA sequencing they identified 28 novel miRNAs, 18 of which mapped to leukemia-associated copy-number alterations, and may play a role in leukemogenesis.

Using t(8;21) AML mRNA- and miRNA-sequencing data from TCGA project, Junge et al. [181] constructed a network of 605 transcripts, potential competitors of *RUNX1T1* in miRNA binding. The so called competing endogenous RNAs (ceRNAs) cross-regulate each other by competing for binding to shared miRNAs. The predicted set of ceRNAs contained multiple oncogenes and members of the integrin, cadherin, and Wnt signaling pathways. One-third of those genes were differentially expressed between t(8;21) AML and normal granulocyte-macrophage progenitor cells. Taking into account experimentally validated miRNA binding sites, the authors selected 21 top *RUNX1T1* ceRNAs, including 13 which shared miRNA binding sites with *RUNX1T1*, e.g., *PLAG1, TCF4, NFIB*, and *YWHAZ*. Therefore, the authors supported the hypothetical miRNA sponge function of *RUNX1T1* gene, particularly its 3'UTR, present in a leukemic *RUNX1-RUNX1T1* fusion transcript and overexpressed up to 1000 times in t(8;21) versus other and control samples.

24. piRNAs, Extracellular Vesicles, and Transposable Elements

From small RNAs, miRNAs are definitely best characterized and their association with normal and malignant development is well established. Recently, a little longer (25-33 nt) P-element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs), responsible for epigenetic silencing of transposable elements (TEs) in germline tissue, have been correlated with brain functioning and tumor transformation [104, 182]. Aberrant piRNA expression was detected in multiple myeloma and various solid tumors [104]. In cancer cells, piRNAs and PIWI proteins may contribute to tumorigenesis through aberrant DNA methylation leading to genomic silencing and promotion of a "stem-like" state, or, oppositely, through gene expression activation via regulation of histone acetylation and euchromatin formation. Comparative analysis of malignant and normal tissues from 11 organs showed that out of approximately 20,000 piRNA present in human genome, less than 300 are expressed in somatic tissues and more than 500 in corresponding tumors [183]. Although most piRNAs were commonly upregulated across tumors, some piRNAs were expressed in tumor-specific manner. A fraction of small RNAs, abundant in miRNAs and piRNAs, was detected in extracellular vesicles (EVs), secreted by bone marrow mesenchymal stem cells (BM-MSC), which are a component of hematopoietic microenvironment [184]. EVs treatment of hematopoietic stem cells extracted from umbilical cord blood (UCB-CD34+ cells) induced cell survival, suppressed apoptosis and decreased cell differentiation. However, piRNA role in AML remains to be elucidated. Up to date, the only evidence of direct relation between piRNAs and AML pathogenesis was presented by Shiva Bamezai in her Ph.D. thesis, devoted to the role of Argonaute protein PIWIL4 in hematopoiesis and AML [185]. The author showed upregulation of *PIWIL4* gene, encoding one of PIWI proteins, in AML samples, the highest in AML with *KMT2A-AF9* translocation conferring poor prognosis. Of note, prognostically favorable AML with *PML-RARA* and inv16 showed the lowest levels of *PIWIL4*. Overexpression of *PIWIL4* was correlated with high expression of genes involved in cell proliferation, such as *FLT3*, *CBL* and *NRAS*. Contrary, depletion of *PIWIL4* in AML with *KMT2A* rearrangements drastically reduced leukemic cell growth *in vitro* and *in vivo*, but did not affect normal cord blood *CD34+* cell growth. Out of 10 thousand unique piRNAs detected in wt THP-1 cells, over 1000 revealed changed expression following *PIWIL4* knockdown, including 80 mapped to genes indicated by RNA-seq as deregulated by *PIWIL4* depletion. Interestingly, most of these genes belonged to the actin cytoskeleton regulation pathway.

The role of TEs in AML and MDS was highlighted by Colombo et al. [186]. While highly expressed in embryogenesis, TEs are usually methylated and silenced by heterochromatin in the somatic cells. Activation of TEs is being observed in ageing tissues and cancers. In LSCs, which are the most therapy-resistant fraction of AML cells, low expression of TEs was noted, along with the suppression of genes involved in interferon pathway, inflammation, and immune response. Significant suppression of TE expression was also identified in high-risk MDS compared to low-risk MDS. Considering the suppression of TEs in in AML and MDS as a mechanism for immune escape, indicates the potential targets to activate cancer immunogenicity in these myeloproliferative malignancies.

25. snoRNA

Small nucleolar RNAs (snoRNAs) are basically involved in the posttranscriptional modification of ribosomal RNAs, in cooperation with protein partners [102, 187]. In recent years, new functions of snoRNAs, which can be submitted to an extensive processing, have been discovered, namely in alternative splicing, regulation of chromatin structure, metabolism, and neoplastic transformation [187]. NGS-based analysis of snoRNAs is more tricky as their size, ranging from 60 to 250 nucleotides [187], overlaps with a gap between conventional small RNA sequencing and RNA-seq, devoted to mRNAs and other RNA molecules longer than 200 nt. Developing their own sequencing approach, Warner et al. [188] showed that snoRNAs, e.g., orphan snoRNAs

contained in the imprinted *DLK-DIO3* and *SNURF/SNRPN* loci, are expressed in a lineage- and developmentally restricted manner in human hematopoiesis. Moreover, 120 snoRNAs, including *SNORA21*, *SNORA36C* and *SCARNA15*, displayed consistent differential expression in AML, and, what is even more interesting, all of them were decreased in AML samples compared to normal CD34+ cells. Of note, although the majority of snoRNAs were embedded in the introns of host genes, expression levels of snoRNAs did not correlated with expression or alternative splicing of host genes, which suggested cellular levels of mature snoRNAs were determined by other factors. No somatic mutations were detected in the snoRNA genes, either. By the way, the authors found a few novel snoRNAs and proved standard transcriptome sequencing cannot reliably distinguish unspliced primary host gene RNA from correctly processed snoRNA [188].

26. Long Noncoding RNAs

A novel class of functional RNAs, which do not encode proteins and are longer than 200 nt, are referred to as long noncoding RNAs (lncRNAs) [189]. Their role in imprinting and regulation of cell cycle, cell differentiation and apoptosis has been postulated [190]. In 2014, Garzon et al. [191] first reported significance of lncRNAs for AML pathogenesis and prognosis. Using a custom microarray platform, they evaluated lncRNA expression in 148 older CN-AML patients and validated the results in an independent cohort of 71 patients. Distinct lncRNA patterns were determined for AML with *FLT3*-ITD and mutations in such genes as *NPM1*, *CEBPA*, *IDH1*, *IDH2*, *ASXL1*, and *RUNX1*. For example, patients with mutated *NPM1* revealed upregulation of several antisense transcripts of *HOX* genes (*HOXB-AS3*; *MEIS1-AS2*), plasmacytoma variant translocation 1 (*PVT1*), and the coiled-coil domain containing 26 (*CCD26*) lncRNAs. Wilms tumor 1 antisense RNA (*WT1-AS*) lncRNA was found as typical of *FLT3*-ITD signature whereas downregulation of *HOXB-AS3* lncRNA was noted in *CEBPA*-mutated AML. *RUNX1* mutation was associated with the increase of lncRNAs located in the proximity of lymphoid marker genes (e.g., *BLNK*), the immunoglobulin heavy locus (*IGH*) complex, and vault RNA 1-1 (*VTRNA1-1*), which was linked with multidrug resistance. Of note, no specific lncRNA profiles were found for *DNMT3A* and *TET2* mutations which frequently occur in older CN-AML patients. Instead, prognostic signature composed of 48 lncRNAs was identified, indicating correlation of lncRNA expression with AML treatment response and survival.

In 2017, Schwarzer et al. [192] presented the comprehensive transcriptome landscape of the normal human hematopoietic stem cells and their differentiated progenies. Short and long noncoding RNAs (ncRNAs), together with mRNAs, were characterized with the use of three microarray platforms in 12 distinct cell populations purified with multicolor flow cytometry from blood of healthy donors. The observed cell-type-specific ncRNA expression indicated the tight regulation and coordinated function of this RNA class in human hematopoietic system. Functional analysis of the identified ncRNA fingerprints in the studied cells and in two independent datasets of more than 600 AML samples, revealed 80% overlap of associated gene sets. For example, *HOTAIRM1*, granulocyte-specific lncRNA, was associated with inflammatory and innate immune response pathways, and was strongly correlated with genes upregulated in AML with *NPM1* mutation. In addition, novel ncRNA regulators of granulopoiesis were predicted, e.g., *LINC00173* expressed specifically in mature granulocytes and negatively associated with the expression of genes related to stemness, cell cycle progression and cancer. The role of *LINC00173* in granulocyte proliferation and differentiation was then confirmed by its transcriptional repression with CRISPR-interference (CRISPRi) in NB4 leukemia cell line. Similarly, gain- and loss-of-function experiments validated the function of miRNAs and lncRNAs of the human *DLK1-DIO3* locus in the differentiation and maintenance of megakaryocytes. Transcriptome analysis of 46 pediatric AML samples allowed identification of prognostically relevant ncRNA signatures shared by normal HSCs and AML blasts of distinct cytogenetic and morphologic subgroups.

In the same year, a systematic analysis of lncRNAs in hematopoiesis and hematological malignancies was also conducted by Delás et al. [193] with a murine model and AML cell lines. First, a catalog of lncRNAs was made, in the 11 types of cells, representing different stages of hematopoietic differentiation and blood cancers. Remarkably, similar expression patterns were observed for protein-coding genes and lncRNAs through hematopoietic system and disease development, which indicated involvement of similar mechanisms of expression regulation. A loss-of-function screen revealed that 20 lncRNAs were required for leukemia progression *in vivo*. Some of them seemed to promote leukemia stem-cell signatures, e.g., *Pvt1* and *Lilam*, whose functions were correlated with the function of *MYC* oncogene. Another lncRNA, termed *Pilna*, was required for the myeloid lineage during bone marrow reconstitution.

Transcriptome analysis of an individual primary AML sample from TCGA dataset enabled discovering 194 unannotated small RNAs in the 17-35 nt size range, 258 unannotated small RNAs in the range of 36-100 nt, and 977 previously unannotated multiexon lncRNA transcripts [194]. A majority of them were also found in the sequencing data of other AML patients from TCGA collection. Integration of the collected data with RNA-seq data from 179 other AML cases led to identification of a subset of lncRNAs with enriched expression in AML M3 (e.g., *MEG3*) and other FAB subtypes comparing to normal CD34+ cells. Reanalysis of 200 transcriptomes from TCGA AML dataset was also used to construct prognosis-related lncRNA module pathway network [195]. First, lncRNA coexpression network, composed of 42 functional modules, was generated thanks to integration of data from small and mRNA sequencing. Then, survival analysis was performed for each of the identified lncRNA modules and 8 of them, significantly enriched in 70 pathways (including AML pathway, chemokine signaling pathway and pathway in cancer), appeared to be correlated with patient outcome.

Relation between lncRNA expression and AML prognosis was also studied by Mer et al. [196] who sequenced transcriptomes of 274 intensively treated AML patients from a Swedish cohort and found 33 individual lncRNAs significantly associated with OS. Based on lncRNA expression, the authors classified all AML patients to four distinct molecular subtypes. The reproducibility and prognostic significance of the identified lncRNA-based signatures were validated in an independent AML patient cohort (142 TCGA samples). Remarkably, neither of the lncRNA-driven AML subtypes was found to be highly concordant with any of the conventional clinical or genetic factors, although enrichment in *CEBPA*, *NPM1*, *FLT3*-ITD and *TP53* mutation was noted for particular subtypes. Despite some similarities between lncRNA and mRNA expression, both types of transcripts stratified AML patients in different ways. This suggested a limited overlap in information retrieved from various levels of transcriptome analysis and underpinned the rationale for further lncRNA studies.

In 2019, Bill et al. [197], while analyzing a large set of transcriptome data, collected from 450 CN-AML patients, identified a 111-lncRNA-based signature specific for LSCs. Interestingly, only four out of 111 lncRNAs were downregulated in samples with high stem cell like gene expression profile. The lncRNA signature was mainly composed of long intergenic noncoding RNAs (lincRNAs; 30%), antisense RNAs (19%) and sense intronic RNAs (13%). One of the upregulated lncRNAs in LSCs was *DANCR* (*Differentiation Antagonizing Nonprotein Coding RNA*), lincRNA highly conserved between mice and humans, overexpressed in hepatocellular carcinoma. *DANCR*, described as a regulator of the Wnt pathway, crucial for the biology of LSCs, was proved to play a role in LSC self-renewal and quiescence. Decreased expression of *MYC* and other genes from Wnt pathway in AML cell line after *DANCR* knockdown confirmed association of *DANCR* with Wnt pathway.

27. Circular RNA

Though most human and mammalian premRNAs are spliced into linear molecules, the existence of circular RNAs (circRNAs), generated by noncanonical splicing (also termed backsplicing), was sporadically reported within the last 30 years [198]. In leukemia, a spectrum of abnormal *KMT2A* transcripts, including circular isoforms, resulted from exon scrambling was shown by Caldas et al. in 1998 [199]. However, until deep transcriptome sequencing was developed, this phenomenon could not be studied on a large scale. At present, it is known that circRNAs arise from hundreds of human genes in normal and malignant cells [198]. The abundance, diversity, and enhanced stability of circular transcripts in human cells suggest that they not only are a result of splicing side effect, but can play a role in the regulation of important physiological and pathological processes.

You and Conrad who elaborated the acfs algorithm for identification and quantification of circRNAs from single- and paired-ended RNA-seq data [200], demonstrated its efficiency on published AML datasets. Comparing 5 APL and 5 CN-AML cases, the authors found 80 circRNAs with opposite pattern of expression, generated from the host genes crucial to the differentiation and proliferation of myeloid cells. For example, *circ_EMB*, generated from *EMB* gene encoding embigin, transmembrane protein from the immunoglobulin superfamily considered as a cancer biomarker, was highly abundant in APL. Upregulation in CN-AML was noted for *circ_SMARCA5*, originated from *SMARCA5* gene encoding a core component of chromatin remodeling and spacing factor RSF, which promotes cell proliferation. In compliance with the theory of circRNA functioning as miRNA sponges, the authors found *miR-10b* (a member of the *miR-99* family) binding site on *circ_SMARCA5*. As *SMARCA5* expression was shown to be affected by *miR-99*, binding of *miR-10b* by *circ_SMARCA5* could relieve linear *SMARCA5* transcripts from repression and contribute to the accumulation of undifferentiated myeloid cells. Analysis of data from a larger AML cohort revealed that the gene loci frequently mutated in AML produced significantly more circRNAs.

In 2017, circRNAs generated from *NPM1* gene have been extensively studied by Hirsch et al. [201]. In total, in six AML cell lines (including OCI-AML3 line with *NPM1* mutation), one CML cell line, and 3 samples derived from the PBMC fraction of healthy volunteers, 15 circular *NPM1* transcripts were identified, including those previously deposited in circBase and novel ones. Oxford Nanopore technology of long read sequencing was harnessed to verify the internal structure of identified transcripts. One of them (hsa_circ_0075001), which exhibited highly differential expression in the AML cell lines, was quantified in a cohort of 46 AML patients. Based on hsa_circ_0075001 expression, patients were divided into two groups that differed in the expression of more than 2000 other genes. AML patients with high hsa_circ_0075001 expression, presented upregulation of ribosomal protein genes, increase of total *NPM1* expression, and downregulation of genes involved in the Toll-like receptor (TLR) signaling pathway and genes targeted by *miR-181* (e.g., *CARD8, CASP1, MSR1, SLC11A1, TLR4*), which is deregulated in CN-AML. The expression of hsa_circ_0075001 correlated with total *NPM1* expression, but was not affected by the *NPM1* mutational status. Global analysis of circRNA expression in 10 AML samples and 10 sorted cell fractions form healthy hematopoietic controls evidenced the existence of circRNA transcripts for almost half of all highly expressed genes [201]. Despite a general tendency towards higher circRNA expression from genes with higher parental gene expression, there were some exceptions, e.g., *circFLT3* expression was not correlated with *FLT3* gene expression in AML samples (though it was in healthy samples). AML patients and healthy controls differed in the expression levels of circRNAs arisen from 27 genes, including *ANGPT1*, *UGCG* and *FLT3*. In addition, AML subgroup-specific circRNA signatures were identified, e.g., *NPM1*-mutated patients could be distinguished from *NPM1*-wt patients based on their global circRNA expression (but not based on *circNPM1* expression).

L'Abbate et al. [202] who analyzed the architecture and expression pattern of chromosome 8 region with MYC amplification in 23 cases of AML, detected a significant overexpression of *circPVT1*, a circular transcript of *PVT1*

gene in the studied AML cases compared to NK-AML. The expression of *circPVT1* was correlated with *PVT1* gene copy-number increase and high *PVT1* expression in AML patients with *MYC* amplification.

Contribution of circRNA to drug resistance was recently shown by Shang et al. with the use of doxorubicin-resistant THP-1 AML cell line (THP-1/ADM) [203]. The authors identified 49 circRNAs differentially expressed between THP-1/ADM and THP-1 cells. One of them, *circPAN3*, was overexpressed not only in THP-1/ADM cells but also in refractory and recurrent AML samples. Silencing of this circRNA restored sensitivity to doxorubicin in THP-1/ADM cells, which indicated its significant role in chemoresistance mediation.

28. Focus on Stem Cells

Leukemic blasts which accumulate in BM and PB of AML patients represent cells blocked at a particular stage of differentiation. However, in the majority of GEP studies performed within the first years of microarray technology development, bulk AML cells were compared to the whole pool of mononuclear cells from healthy control samples. Then, the cell sorting techniques were introduced to select particular cell fractions. The example of a study where AML cells were compared not only to unselected healthy BM and PB samples, but also to normal hematopoietic CD34+ cells extracted from BM and PB, was that of Stirewalt et al. [204]. They found 13 genes deregulated in AML compared to all subpopulations of normal hematopoietic cells, including 7 upregulated (*BIK, CCNA1, FUT4, IL3RA, HOMER3, JAG1, WT1*) and 6 downregulated (*ALDHA1A, PELO, PLXNC1, PRUNE, SERPINB9, TRIB2*). Moreover, the expression levels of *WT1, FUT4, CCNA1, HOMER3, JAG1, TRIB2*, and *SERPINB9* were strongly associated with FAB classification whereas *WT1, JAG1, ALDH1A1, TRIB2*, and *PLXNC1* with cytogenetics. For example, *WT1* was the most overexpressed in AML with inv(16) or t(15;17), CCNA1 in t(15;17), while *BIK* expression was absent or extremely low in t(8;21). 7 upregulated genes were also measured in pediatric AML, where *BIK, FUT4*, and *WT1* showed the most significant increase in expression.

After the discovery of the self-renewing leukemic stem cells (LSCs), the main focus was shifted to these early progenitor cells capable of initiating leukemogenesis. How they are different from normal human hematopoietic stem cells (HSCs) was first shown in 2009 by Majeti et al. [205] who identified over 3000 DEGs between normal HSCs and LSCs extracted from AML patients. The selected genes encoded mainly proteins with kinase activity, associated with nucleoplasm, Golgi apparatus, chromosomes, vacuoles and actin cytoskeleton. KEGG pathway analysis revealed the top dysregulated pathways in LSCs were those related to adherens junction, ribosome, regulation of actin cytoskeleton, tight junction, focal adhesion, apoptosis, MAPK signaling, T-cell receptor signaling, pathway, JAK-STAT signaling and Wnt signaling. Some of these pathways were already associated with stem-cell biology and cancer development, other were not studied in cancer stem cells yet. The obtained results emphasized the importance of the LSCs' interaction with their niche in leukemia initiation and progression.

Gentles et al. showed the correlation of high expression of leukemic stem-cell genes with adverse outcomes in AML [206]. Comparing subpopulations of cells extracted from 163 NK-AML samples, the authors identified 52 genes discriminating the LSC-enriched subpopulations (CD34+/CD38-) from the leukemic progenitor cell (LPC)-enriched subpopulations (CD34+/CD38+), among others genes involved in early hematopoiesis, e.g., *VNN1, RBPMS, SETBP1, GUCY1A3, MEF2C*, and *HOPX*. Genes associated with proliferation, cell cycle, and differentiation were systematically repressed in the LSCs. The identified LSC gene expression signature (LSC score), reflecting self-renewal ability, was validated on four independent datasets of 1047 patients in total, leading to similar conclusions. OS, EFS, and RFS were worse for patients with high LSC score [100].

Ng et al. [207] compared gene expression profiles between 138 LSC+ and 89 LSC- cell fractions from 78 AML patients. From the list of 104 DEGs, 17 most related to stemness were selected to generate a LSC score (LSC17), which could be calculated for each patient as the weighted sum of expression of the 17 genes. Strong association between high LSC17 scores and poor overall and event-free survival was observed, as in the case of higher bone marrow blast percentage at diagnosis, higher incidence of the *FLT3*-ITD and adverse cytogenetics. The LSC score was validated by xenotransplantation assays and by reanalysis of microarray and RNA-seq data from five independent cohorts of more than 900 AML patients with different subtypes. Comparing with other prognostic determinants such as age, WBC count, cytogenetic risk group, and mutational status, LSC17 score was the strongest and independent prognostic factor. In the end, the authors designed a custom NanoString assay to easily analyze expression of 17 genes from the LSC signature. The assay should allow for rapid risk assessment at diagnosis, application of more intensified investigational therapies in the case of high-score patients, and protection of low-score patients against unnecessary toxicity. To test its efficiency in childhood leukemia, Duployez et al. applied the LSC17 score to 228 de novo pediatric AML patients [208]. Indeed, children with low LSC17 score had significantly better outcome (OS and EFS) compared to children with high score. Then, the stemness signature was validated in 257 children from an independent AML cohort. However, prognosis of pediatric patients with low LSC17 was not significantly better than those with intermediate LSC17 score. The differences between adult and pediatric AML patients might result from the different proportion of CBF AML in both groups (twice as high in children). Nevertheless, the authors extended the LSC17 prognostic value to pediatric AML patients, at least with non-CBF AML [208]. Among all negative prognostic factors, including the high LSC17 score, high WBC count, cytogenetic group "other aberrations" and presence of WT1 and *RUNX1* mutations, high LSC17 score gained the best statistics.

Recently, GEP of LSCs, HSCs, and leukemic progenitors from the same AML bone marrow enabled identifying three

genes overlapping in the results of two pairwise comparisons (LSCs vs. HSCs and LSCs vs. leukemic progenitors): *S100A8, SOD2*, and *IGFBP7* [209]. Significantly lower expression of *IGFBP7*, encoding insulin-like growth factor-binding protein 7, in LSCs was correlated with reduced sensitivity to chemotherapy. Restoration of *IGFBP7* expression by a recombinant human gene resulted in differentiation, inhibition of LSC survival, and improved response to therapy, without affecting normal hematopoiesis and HSC survival. Therefore, *IGFBP7* gene was postulated to be a factor responsible for the persistence of LSCs.

29. Taking Advantage of Model Organisms

Deep insight into the biology of the disease is not possible by the analysis of cells extracted from patient BM or PB. *In vitro* experiments with cell line models are convenient, but have also some limitations. In fact, animal models are the best option to study particular gene function, consequences of early pathogenic events or treatment with potential therapeutics. Using retroviral insertion mutagenesis in mice, Erkeland et al. (2004) [210] identified a number of genes that could be involved in the pathogenesis of AML. In one of their later works, the authors studied the virus integration sites (VISs) and virus common integration sites (CISs) in the human GEP datasets of 285 adult AML samples [61] and 130 pediatric AML samples [68]. First, they noted VIS flanking genes were significantly more differentially expressed between AML clusters than random genes in both datasets. Then the authors identified five regulatory networks involving 110 VIS/CIS genes most differentially expressed in the adult dataset. Network associated with cell growth contained only these genes. Many of them, e.g., interleukin and *STAT* genes, were implicated in cytokine signaling. Another network, consisted of gene expression regulators, was able to discriminate between AML patients with favorable and unfavorable prognosis. In the unfavorable group, *HOXA9*, *MEIS1*, and *CCND3* genes were increased whereas *BCOR* and *GFI1* genes were decreased.

Glass et al. [211] used NGS platform to identify *MECOM* (previously *EVI1*) target genes by comparison in *MECOM*-overexpressing murine myeloid leukemia cell lines (DA-1, NFS-60) before and after shRNA-mediated *MECOM* knockdown. *MECOM*, oncogenic transcription factor associated with human myeloid malignancies of poor prognosis, is overexpressed in 8–10% of adult AML and up to 27% of pediatric leukemias with *KMT2A* rearrangements. *MECOM*-induced leukemic cells present impaired myeloid differentiation, resistance to apoptosis, and aberrant cell cycle regulation, which results in excessive proliferation. Combining RNA-seq expression profiling with ChIP-seq, the authors found MECOM directly bound to and downregulated *Cebpe* gene, encoding a master myeloid differentiation regulator, *Serpinb2* gene, encoding serine protease inhibitor involved in cell cycle regulation, and numerous genes from the Jak-Stat signaling pathway that drive cellular differentiation. In addition, several P2X purinoceptors, responsible for ATP mediated apoptosis in neutrophils and macrophages, appeared to be significantly downregulated in *MECOM* leukemic cells.

An interesting approach was applied by Wilhelm et al. (2011) [212] who generated two related murine leukemic clones through the retroviral overexpression of *Meis*1 and *Hoxa9* genes in the purified fetal liver (FL) cells. Both clones differed in the hematopoietic stem-cell frequency and gene expression profile. Considering microRNAs, only a few miRNAs were really highly expressed; more than 95% of all miRNA transcripts came from the top 15 miRNAs. Functional annotation analysis showed the most differentiating genes between Meis1- and Hoxa9-overexpressing clones were related to immune system development, hemopoietic or lymphoid organ development, hemopoiesis, and myeloid cell differentiation. Both, differential expression and/or differential splicing, were observed in the studied transcriptomes. Moreover, hundreds of single nucleotide variants (SNVs), shared by both cell clones or unique for one of them, were identified with respect to the public reference sequence. The study revealed also a number of unannotated transcribed elements.

30. Gene Expression Regulation: New Directions

McKeown et al. (2017) [213] matched the epigenomic circuitry with the transcriptional state of leukemic cells to identify potential new treatment strategies. The authors focused on the large (>20 kb), highly active chromatin regions called "super-enhancers" (SEs) described previously as key oncogenic drivers in tumor cells [214]. Although SEs constitute only about 5% of all enhancers, they are involved in the regulation of the crucial genes defining cell identity and phenotype. 66 AML patients were characterized in terms of enhancers, super-enhancers, gene expression, and mutational status of blasts [213]. On average, 807 SEs per sample with a median length of 22 kb were identified. Most of them were linked to multiple genes. In addition, SE maps from normal HSPCs and monocytes were used to define the signature of differentiation state. The two most pervasive enhancer signatures were found in the genome and named Myeloid Differentiation and HOX Factor Activation, the last correlated with enhancers associated with homeobox (*HOX*) genes, *PBX3*, and *MEIS1*. Based on overall SE distribution, patients were classified to 6 subgroups, including 4 enriched in *NPM1* and *FLT3* mutation, and one containing all AML samples with *KMT2A* translocation. The authors concluded *KMT2A* translocations might induce a unique epigenetic state affecting overall enhancer landscape, which is consistent with the association of *KMT2A* fusion proteins with *DOT1L* deregulation and, consequently, aberrant histone H3K79 methylation. Moreover, patients classified to different SE-clusters showed differences in OS. Discovery of *RARA*-associated SE which differentiated patient samples and correlated with significantly higher *RARA* expression in 25% patients, prompted the authors to test the sensitivity of AML cells presenting a strong SE at the *RARA* locus to RARα targeted compounds. The results of tests

conducted on AML cell lines, AML patient derived mouse xenograft models and AML cells *ex vivo*, demonstrated that RARα agonist SY-1425 (tamibarotene) selectively inhibited the proliferation of AML cells with high (but not low) *RARA* expression, stimulating the expression of genes linked to granulocytic differentiation (e.g., *CD38, ITGAX, ITGAM,* and *CD66*) and retinoic acid response (e.g., *DHRS3*). In the context of efficiency, comparison of SY-1425 to ATRA, used clinically for APL therapy, showed the prevalence of the tested compound. Transcriptional response of APL to retinoids was similar to the response of AML cell lines with high *RARA* expression to SY-1425. The authors presented the following interpretation of the observed results: while APL-specific PML-RARA fusion protein represses transcription of differentiation-related genes, in non-APL AML, *RARA*-associated SE induces overexpression of unliganded RARα which acts as a transcriptional repressor of genes regulating by RARα. The practical consequence of clinical significance is that some non-APL AML patient may benefit from the ATRA-like therapy, and increased levels of *RARA* mRNA could be used as a prerequisite for the treatment by SY-1425, which has a power to reset RARα transcriptional activity.

Not only genetic and epigenetic factors contribute to AML pathogenesis; initiation, progression and maturation of AML are also affected by posttranslational modification of proteins. The important role of SUMOylation of *sPRDM16* in AML progression was demonstrated by Dong & Chen on leukemic cell lines [215]. *PRDM16*, previously termed *MEL1*, encodes transcription factor acting as a H3K9me1 methyltransferase responsible for maintenance of heterochromatin integrity. However, only protein isoform deprived of the histone methyltransferase domain, encoded by a short transcript variant, *sPRDM16*, was associated with AML pathogenesis. The authors showed that SUMOylation of *sPRDM16* changed the expression of genes implicated in wound response, cell proliferation, chemotaxis, differentiation, and cell cycle progression, including 13 genes (e.g., *KLF10, BCL3, HDAC9, CCL5, IL6R, LIF,* and *NUMB*) involved in proliferation and differentiation of hematopoietic and leukemic cells.

31. Single-Cell Sequencing: The Future

Considering AML as a multiclonal cancer, we should be aware of the fact that bulk RNA sequencing reflects what is going on in a dominant clone that is not necessarily a clone of origin. Even application of cell sorting technique may not be sufficient to catch the whole AML heterogeneity as far as we sequence a pool of cells. A promising approach, which can overcome this problem is single-cell gene expression profiling. A pilot single-cell AML study was performed on twenty CD45-positive cells collected from an individual AML patient [216]. GEP showed only 11 out of 20 cells selected for the analysis were putative blasts, i.e., *CD34*-positive, or HLA-DRA- and CD117-positive. Moreover, two of them were outliers in PCA. Complementary targeted DNA sequencing revealed the presence of mutations in *DNMT3A* and *NPM1*, and *FLT3*-ITD in the analyzed AML sample. However, RNA-seq identified only *DNMT3A* mutation in only one single cell. Possible explanations are not sufficient coverage (although *NPM1* and *FLT3* genes are usually highly expressed in AML), imperfect algorithms for mutation detection in RNA-seq data, and mutational heterogeneity of blast cells. An argument for the last explanation can be found in the report of Shlush et al. [217] who identified in highly purified preleukemic stem cells *DNMT3A* mutation at high allele frequency, but did not detected concomitant *NPM1* mutation. Therefore, the authors concluded *DNMT3A* arose early in AML evolution. The preliminary experiences with single-cell sequencing show this technology demands optimization, but seems to be a strategy of future.

32. Commercial Solutions for AML Research and Clinics

As a result of years of transcriptome-scale studies, a few commercial tools dedicated to AML and other hematologic malignancies were developed. Affymetrix technology, which conquered the microarray market, was used by SkylineDx (https://www.skylinedx.com), high-tech commercial-stage biotech company headquartered in Rotterdam, the Netherlands, to design AMLprofiler, a qualitative *in vitro* diagnostic and prognostic microarray supporting the choice of optimal therapy strategy [218]. AMLprofiler combines seven separate assays used for the purpose of cytogenetics, mutation detection, and gene expression analysis, reducing the time between sampling and diagnosis from 4 weeks to 3 days. The time of diagnostic report generation does not exceed 15 min.

Illumina (https://www.illumina.com), the company headquartered in San Diego, California, USA, who practically monopolized the NGS market, released the MiSeqDx system, the first FDA-regulated, CE-IVD-marked, NGS platform for *in vitro* diagnostic testing. Although AML-dedicated kits do not exist for MiSeqDx, the system is customizable and a number of partners collaborates with the company to develop new clinical assays. At present, kits for target sequencing are available for other Illumina systems, e.g., TruSight Myeloid Sequencing Panel, targeting 54 genes, including *CEBPA*, *NPM1* and *FLT3*-ITD, or AmpliSeq for Illumina Myeloid Panel, targeting 40 DNA genes, 29 RNA fusion driver genes, and 5 gene expression levels associated with myeloid cancers, including AML.

Other integrated diagnostic platforms are being developed and validated, e.g., rapid and sensitive NGS-based assay combining karyotyping and mutational screening of AML [219]. Here, three NGS libraries are generated: two DNA-based libraries—for whole genome sequencing and selected variant identification—and one RNA-based library for fusion transcript detection. The whole workflow can be completed within 5 days.

33. Conclusions

Within the last two decades, an explosion of AML studies, driven by technological progress, could be observed. AML

picture emerging from transcriptome research is very complex and dynamic. AML transcriptome, affected by cytogenetic and genetic variability, resembles a mosaic, composed of many elements interacting with each other. AML subtypes present unique patterns of protein-coding gene and microRNA expression. Moreover, a spectrum of fusion genes, alternative transcripts and newly discovered chimeric RNAs, as well as a lot of noncoding RNAs, including long linear and circular forms, contribute to the complexity of leukemic transcriptome.

Transcriptome data cannot be interpreted separately from genetic and genomic data. Even a sole point mutation can affect expression of numerous genes, e.g., when it occurs in a transcription factor, epigenetic regulator, or a crucial member of a signaling pathway. Therefore, genome, exome, methylome sequencing, or targeted resequencing is often combined with RNA-seq. In some cases transcriptome analyses helped to define which mutation was the driver one. It must be remembered that a disease is not limited to leukemic blasts. Recent studies on BM microenvironment underlined its role in AML initiation, progression, and relapse. Recognition of molecular interplay between LSCs and BM niche not only is necessary to understand the AML biology but also opens novel AML treatment directions.

Many reports proved the power of global transcriptome profiling and proposed application of GEP for AML diagnosis and prognosis. A single microarray-based test usually classified AML subgroups properly without any a priori knowledge. The following studies utilized more advanced generations of microarrays, composed of an increasing number of probes, and included more numerous cohorts of patients. Of note, the results obtained by different groups were largely overlapping. As gene expression-based prediction of the major cytogenetic subgroups was efficient in both, pediatric and adult, AMLs, GEP appears as an attractive alternative to classical cytogenetics which is laborious and time-consuming. On the other hand, there is no need to harness high-throughput technology when simple, e.g., PCR-based, diagnostic tests can easily confirm the presence of the fusion genes.

Because some genes are significantly overexpressed in AML, not only in leukemic blasts but also in BM microenvironment, and their expression affects treatment response, transcript measurement at the time of diagnosis should be obligatory in AML diagnostics. In my opinion, reliable quantitative PCR techniques, e.g., ddPCR, have currently the highest potential to be routinely applied in clinical practice to analyze selected transcript levels. To analyze higher number of transcripts, gene expression arrays or RNA-seq may be applied instead. Although both high-throughput GEP approaches demand specialized, expensive equipment, and well-trained staff, RNA-seq seems to be a superior technology, offering more information at a comparable cost. Within the last few years, small personal, relatively cheap, and portable sequencers started to appear which makes NGS-based tests more available. Moreover, alternative, third-generation sequencing technologies are becoming more and more popular. I believe that future clinical diagnostic laboratories will offer NGS services, not limited to mutation detection, as a standard. Even if accepting GEP as the only diagnostic test would be difficult, it could at least serve as a first screening or a complementary tool.

Summarizing, although gene expression studies were not directly translated into clinical practice up to date, they helped us to understand the biology of tumors and undoubtedly contributed to the improvement of classification of hematological malignancies and risk estimation, which is crucial for optimal treatment decision and directs us towards personalized medicine.

Conflicts of Interest

The author declares that she has no conflicts of interest.

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Supplementary Materials

Supplementary Table 1 presents the results of PubMed search demonstrating a great effort of the scientific community put into acute myeloid leukemia research. All terms used for searching are included, along with the corresponding numbers of papers found, with two additional filters applied. Part of the information presented in Supplementary Table 1 was used to generate Figure 1 plots. Supplementary Table 2 lists all AML transcriptome papers cited in the manuscript. The following information is included: title, first, and last author, laboratory, name of the journal, year of publication, AML sample size, sample description, and technology applied. All papers are divided according to the technology (microarray/PCR or NGS) and then ordered chronologically. *(Supplementary Materials)*

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Research Article

HDAC and HMT Inhibitors in Combination with Conventional Therapy: A Novel Treatment Option for Acute Promyelocytic Leukemia

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Acute promyelocytic leukemia (APL) is characterized by *PML-RARA* translocation, which causes the blockage of promyelocyte differentiation. Conventional treatment with Retinoic acid and chemotherapeutics is quite satisfactory. However, there are still patients who relapse or develop resistance to conventional treatment. To propose new possibilities for acute leukemia treatment, we studied the potential of histone deacetylase (HDAC) inhibitor and histone methyl transferase (HMT) inhibitor to enhance conventional therapy *in vitro* and *ex vivo*. NB4 and HL60 cell lines were used as an *in vitro* model; APL patient bone marrow mononuclear cells were used as an *ex vivo* model. Cell samples were treated with Belinostat (HDAC inhibitor) and 3-Deazaneplanocin A (HMT inhibitor) in combination with conventional treatment (Retinoic acid and Idarubicin). We demonstrated that the combined treatment used in the study had slightly higher effect on cell proliferation inhibition than conventional treatment. Also, enhanced treatment showed stronger effect on induction of apoptosis and on suppression of metabolism. Moreover, the treatment accelerated granulocytic cell differentiation and caused chromatin remodelling (increased H3K14 and H4 acetylation levels). *In vitro* and *ex vivo* models showed similar response to the treatment with different combinations of 3-Deazaneplanocin A, Belinostat, Retinoic acid, and Idarubicin. In conclusion, we suggest that 3-Deazaneplanocin A and Belinostat enhanced conventional acute promyelocytic leukemia treatment and could be considered for further investigations for clinical use.

1. Introduction

Acute promyelocytic leukemia (APL) is a subgroup of acute myeloid leukemia, most commonly characterized by chromosomal translocation that generates PML-RARα fusion protein. This protein is responsible for the blockage of promyelocyte differentiation and thus for promyelocyte proliferation and accumulation in the blood [1, 2]. A discovery that all-trans-retinoic acid (RA) targets PML-RARα protein and thereby induces promyelocytic differentiation revolutionized APL treatment. A vast majority of patients achieve complete remission after treatment with various combinations of Retinoic acid with arsenic trioxide and chemotherapeutics [3]. However, a small proportion of APL patients are resistant or develop resistance to RA treatment, which is considered as a critical problem [4].

Therefore, the development of novel treatment strategies is necessary. There is a growing interest in epigenetic therapy. Epigenetic changes such as altered DNA methylation and histone modifications deregulate gene expression and can lead to the induction and maintenance of cancer. Many processes in the cell, for instance, the differentiation blockade

and malignant cell proliferation, are influenced by epigenetic alterations [5, 6]. A number of mutated epigenetic modifier genes account for myeloproliferative neoplasms and leukemias [7]. Thus, epigenetic drugs against chromatin regulators are an important tool for cancer treatment [5, 6]. It was demonstrated that, in APL, PML-RARα fusion protein binds DNA and multimerize through its PML domain. Moreover, this aberrant protein recruits various other partners and forms a large protein complex. Among recruited complex proteins, there are various chromatin regulators such as histone deacetylases (HDACs), histone methyltransferases (HMTs), DNA methyltransferases, and polycomb repressive complexes (PRCs) 1 and 2[8]. Thus, targeting not only PML-RARα but also other members of the aberrant complex, such as HDAC and HMT, might potentially improve conventional APL therapy.

HDAC inhibition facilitates chromatin decondensation, which leads to activated gene expression. HDAC inhibitor Belinostat was shown to be effective for relapsed or refractory peripheral T-cell lymphoma treatment in clinical trials. In 2014, it was approved by FDA for this cancer type treatment [9]. There are some widely known HMTs to be involved in carcinogenesis; for example, histone methyl transferase EZH2 is overexpressed in various cancers and it was demonstrated to inhibit acute myeloid leukemia cell differentiation [10]. Epigenetic agent 3-Deazaneplanocin A is an inhibitor of S-adenosyl-L-methionine-dependent HMTs, including EZH2. In preclinical studies, it was shown to inhibit cell proliferation and cause apoptosis in various cancer types [11, 12].

Recently, we showed that epigenetic modifiers 3-Deazaneplanocin A and Belinostat in combination with RA inhibited APL cell proliferation, caused apoptosis, enhanced cell differentiation, and caused chromatin remodelling *in vitro* [13]. Furthermore, in the study with murine xenograft model, we demonstrated that this combined treatment prolonged APL xenograft mice survival and prevented tumour formation [14]. The purpose of this study was to determine the effect of 3-Deazaneplanocin A and Belinostat in combination with conventional treatment (RA + Idarubicin) on NB4 and HL60 cells *in vitro* and on APL patient promyelocytes possessing *PML-RARA* translocation *ex vivo*. We examined the effect of the proposed new epigenetic treatment in combination with conventional treatment on leukemic cell proliferation and granulocytic differentiation potential, changes in oncogene expression, histone modifications involved in chromatin remodelling, and proapoptotic protein expression.

2. Materials and Methods

2.1. NB4, HL60, and APL Patient Cell Cultivation. NB4 and HL60 cell lines were purchased from DSMZ (Braunschweig, Germany). Bone marrow sample was obtained from a patient diagnosed with APL (promyelocytes consisted of 70% of bone marrow karyocytes; *PML-RARA* translocation was detected). White mononuclear cells were purified from bone marrow aspirate by Ficoll-Paque PLUS density gradient centrifugation (GE Healthcare Chicago, IL, USA). Ethical permission from Vilnius Regional Biomedical Research Ethics Committee (approval no. 158200-16-824-356) and informed consent of the patients were obtained. NB4 cells and freshly purified APL patient cells were seeded at density 0.5×10^6 cells/ml and cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Carlsbad, CA, USA) at 37°C in a humidified 5% CO_2 atmosphere.

2.2. Cell Treatment and Proliferation, Survival, Apoptosis, and Cell Cycle Assays. Cell samples were treated with 1 μM Retinoic acid (Sigma-Aldrich, St. Louis, MO, USA), 2 nM or 8 nM Idarubicin (Sigma-Aldrich), 0.5 μM 3-Deazaneplanocin A (Cayman Chemical Company, Ann Arbour, MI, USA), and 0.2 μM Belinostat (PXD101) (Selleckchem, Munich, Germany) in different combinations. Cell proliferation and survival were evaluated by trypan blue exclusion test. Cells were mixed with 0.2% of trypan blue dye (final concentration). Viable and dead (blue coloured) cell numbers were determined by counting the cells in a haemocytometer under the light microscope. For the detection of apoptosis, we used the assay "ApoFlowEx® FITC Kit" (Exbio, Vestec, Czech Republic) according to the manufacturer's instructions. This assay detects viable, early apoptotic, and late apoptotic or necrotic cells according to how they get stained by Annexin V-FITC and Propidium Iodide. Stained cells were analysed on the BD FACS Canto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Cell cycle distribution was analysed using standard propidium iodide staining procedure [15].

2.3. Mitochondrial Respiration and Glycolytic Activity Measurement. Cell mitochondrial respiration and glycolytic activity were measured using "Agilent Seahorse XF Cell Energy Phenotype Test Kit" (Agilent Dako, Santa Clara, CA, USA) according to the manufacturer's instructions. Plate wells were coated with poly-D-lysine 24 hours before measurement. NB4 cells were seeded at 3 x 10^4 cells/well, centrifuged for 1 min at 300xg at room temperature, and incubated for 30 min at 37°C without CO_2. After incubation, cell metabolic phenotype was measured on the Agilent Seahorse XF Extracellular Flux Analyzer (Agilent). Determined oxygen consumption rate (OCR) demonstrates mitochondrial respiration and extracellular acidification rate (ECAR), rate of glycolysis of the cells.

2.4. Granulocytic Cell Differentiation Assay. The degree of granulocytic differentiation was evaluated by the ability of cells to reduce soluble nitro blue tetrazolium (NBT) (Sigma-Aldrich) to insoluble blue-black formazan after stimulation with phorbol myristate acetate (PMA) (Sigma-Aldrich). Cells were mixed with 0.1% of NBT and with 100 ng/ml PMA (final concentrations) and were incubated at 37°C for 30 min. Undifferentiated and differentiated (NBT+) cells were counted in a haemocytometer under the light microscope. Differentiated cell percentage was expressed as the NBT^+ cell number relative to viable cell number.

2.5. Gene Expression Analysis by RT-qPCR. Total RNA was purified using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), cDNA was synthesized using SensiFAST™ cDNA Synthesis Kit (Bioline, Memphis, TN, USA), and qPCR was

Table 1: Primers used for RT-qPCR analysis.

Gene	Forward and reverse primers
ATM	F: CTCTGAGTGGCAGCTGGAAGA
	R: TTTAGGCTGGGATTGTTCGCT
CCNE2	GATGGAACTCATTATATTAAAGGCTTT
	AGGAGCATCTTTAAGAGCATCAACTT
CEBPE	F: CAGCCGAGGCAGCTACAATC
	R: AGCCGGTACTCAAGGCTATCT
CSF3	F: GCTGCTTGAGCCAACTCCATA
	R: GAACGCGGTACGACACCT C
CSF3R	F: CTTGTGGCCTATAACTCAGCC
	R: CCCACTCAATCACATAGCCCT
GAPDH	F: AGTCCCTGCCACACTCAG
	R: TACTTTATTGATGGTACATGACAAGG
MYC	F: AATGAAAAGGCCCCCAAGGTAGTTATCC
	R: GTCGTTTCCGCAACAAGTCCTCTTC
p21	F: GGCAGACCAGCATGACAGATT
	R: GCGGATTAGGGCTTCCTCT
P27	F: TAATTGGGGCTCCGGCTAACT
	R: TGCAGGTCGCTTCCTTATTCC
p53	F: TAACAGTTCCTGCATGGGCGGC
	R: AGGACAGGCACAAACACGCACC
PPARG	F: GCTCTAGAATGACCATGGTTGAC
	R: ATAAGGTGGAGATGCAGGCTG
Rb	GCAGTATGCTTCCACCAGGC
	AAGGGCTTCGAGGAATGTGAG
TERT	F: CGTACAGGTTTCACGCATGTG
	R: ATGACGCGCAGGAAAAATG
WT1	F: GGCATCTGAGACCAGTGAGAA
	R: GAGAGTCAGACTTGAAAGCAGT

performed using SensiFAST™ SYBR® No-ROX Kit (Bioline) on the RotorGene 6000 system (Corbett Life Science, QIAGEN, Hilden, Germany). Primer sequences (Metabion international AG, Planegg/Steinkirchen, Germany) are presented in Table 1. mRNA levels were normalized to GAPDH expression. Relative gene expression was calculated using ΔΔCt method.

2.6. Immunoblotting. Cell lysates were prepared as described previously [15]. Proteins were fractionated in 7.5-15% SDS-PAGE gradient electrophoresis gel and transferred on PVDF membrane. Primary antibodies against WT1 (mouse, clone 6F-H2) (Thermo Fisher Scientific, Waltham, MA, USA), Bcl-2 (mouse, clone 100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), BAX (rabbit, 2D2) (Santa Cruz Biotechnology), H3K27me3 (rabbit, polyclonal) (Millipore, Billerica, MA, USA), H3K14Ac (rabbit, polyclonal) (Millipore), H4 hyper Ac (rabbit, polyclonal) (Millipore), Caspase-3 (rabbit, clone H-277) (Santa Cruz Biotechnology), GAPDH (mouse, clone 6C5) (Abcam, Cambridge, UK), HRP-conjugated secondary antibodies against mouse immunoglobulins (goat, polyclonal) (Agilent Dako, Santa Clara, CA, USA), and rabbit immunoglobulins (goat, polyclonal) (Agilent Dako) were used according to the manufacturer's instructions. GAPDH was used as loading control. "Clarity Western ECL Substrate" (BIORAD, Hercules, CA, USA) was used for chemiluminescent detection. Signal detection was carried out on ChemiDoc™ XRS+ System (BIORAD). Quantitative evaluation was performed using ImageJ software.

2.7. Statistical Analysis. Data are expressed as mean ± standard deviation (S.D.). One-way ANOVA and two-tailed Student's *t*-test were used to calculate the significance of difference between treated and untreated samples; significance was set at $P \leq 0.05$ (∗).

3. Results

3.1. Enhanced Treatment Affected APL Cell Proliferation and Death. Bone marrow cells from APL patient possessing *PML-RARA* translocation were purified for white mononuclear cells. NB4 cell line, HL60 cell line, and APL patient white mononuclear cells (70% of blast cells) were treated with 1 μM Retinoic acid, 2 nM or 8 nM Idarubicin, 0.5 μM 3-Deazaneplanocin A, and 0.2 μM Belinostat in different combinations for 72 hours. Drug concentrations were chosen based on previously published work [13] and on unpublished data. Previously, we demonstrated that combined treatment with Belinostat and 3-Deazaneplanocin A had stronger effect on leukemic cells in comparison to their individual effects *in vitro* and *in vivo* [13, 14]. Thus, in this *ex vivo* study, we did not test them separately. In order to compare epigenetic agents 3-Deazaneplanocin A (HMT inhibitor) and Belinostat (HDAC inhibitor) in combination with Idarubicin and Retinoic acid to conventional treatment alone (Idarubicin + Retinoic acid), treated cell proliferation and survival were evaluated every 24 hours (Figure 1(a)). Conventional treatment enhanced with epigenetic agents had slightly higher effect on NB4, HL60, and APL patient cell proliferation and survival compared to treatment with Idarubicin and Retinoic acid alone. However, the combination of 3-Deazaneplanocin A, Belinostat, Idarubicin, and Retinoic acid did not show highly increased cytotoxicity.

In addition, we tested the ability of 3-Deazaneplanocin A and Belinostat combination with Idarubicin and Retinoic acid to induce apoptosis. NB4, HL60, and APL patient cells were treated with different combinations of these agents for 72 hours. Antiapoptotic protein Bcl-2 expression decreased and proapoptotic protein BAX expression increased after the treatment (Figure 4). NB4 cells were stained with Annexin V and Propidium Iodide and analysed (Figure 1(b)). The results show that epigenetic agents 3-Deazaneplanocin A and Belinostat enhanced Idarubicin and Retinoic acid effect; treatment with the combination showed the highest number of apoptotic cells. Moreover, Caspase-3 activation was assessed by immunoblot in NB4 and HL60 cell lines after the treatment (Figure 1(c)). The highest amount of activated (cleaved) Caspase-3 was detected after the treatment with Retinoic acid, 8 nM Idarubicin, 3-Deazaneplanocin A, and Belinostat combination.

Since actively proliferating cells are also active metabolically, we evaluated oxidative phosphorylation and glycolysis

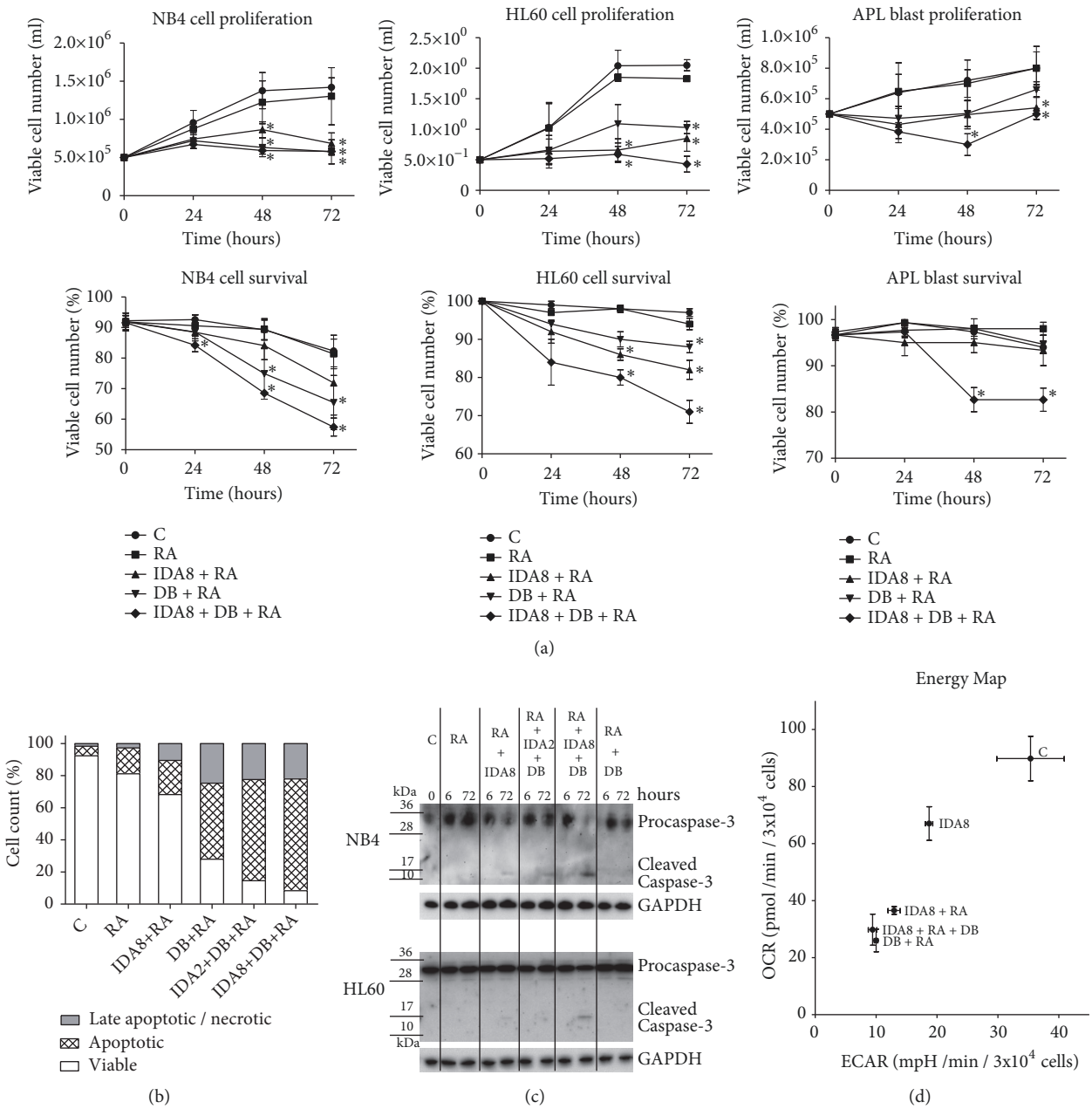

Figure 1: *Cell proliferation and survival after treatment with epigenetic agents in combination with conventional treatment*. NB4 and HL60 cells and APL patient promyelocytes were treated with 1 μM Retinoic acid (RA), 2 nM or 8 nM Idarubicin (IDA2 or IDA8), 0.5 μM 3-Deazaneplanocin A (D), and 0.2 μM Belinostat (B) in different combinations; C: untreated cells. (a) Treated cell proliferation and survival were analysed by trypan blue exclusion test. Results are mean ± S.D. (n = 3); $*P \leq 0.05$, calculated by Student's t-test to determine the significance of difference between groups of treated and untreated samples at the same incubation time. (b) Treated NB4 cell apoptosis was evaluated by staining with Annexin V and Propidium Iodide after 72 hours of treatment. (c) Caspase-3 activation was assessed by immunoblot analysis. (d) Metabolic changes in treated NB4 cells were measured using Agilent Seahorse XF Cell Energy Phenotype Test Kit. OCR: oxygen consumption rate (demonstrates cells mitochondrial respiration); ECAR: extracellular acidification rate (demonstrates rate of glycolysis of the cells); results are mean ± S.D. (n = 2).

rate changes after NB4 cell treatment (Figure 1(d)). Combinations with 3-Deazaneplanocin A and Belinostat had the highest effect on metabolic activity impairment. These findings support proliferation and survival results described above. Taken together, our proposed enhancement of conventional treatment (Idarubicin and Retinoic acid together with epigenetic agents 3-Deazaneplanocin A and Belinostat) had higher effect on cell proliferation and survival inhibition and on apoptosis induction.

3.2. Combined Treatment Affected Cell Cycle Progression. Cell cycle distribution after NB4 and HL60 cell treatment

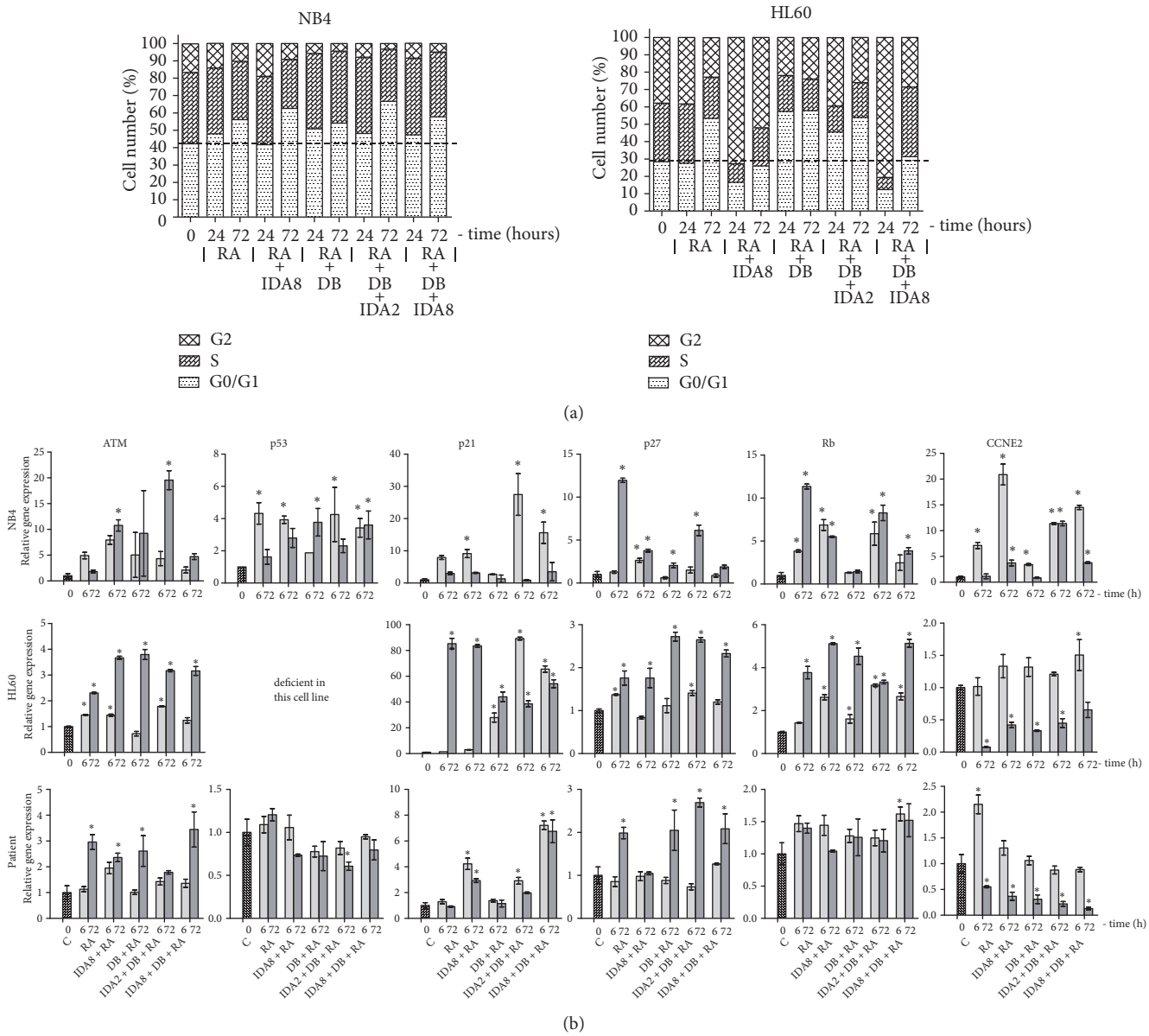

Figure 2: *Cell cycle progression analysis after treatment with epigenetic agents in combination with conventional treatment*. NB4 and HL60 cells and APL patient promyelocytes were treated with 1 μM Retinoic acid (RA), 2 nM or 8 nM Idarubicin (IDA2 or IDA8), 0.5 μM 3-Deazaneplanocin A (D), and 0.2 μM Belinostat (B) in different combinations; C: untreated cells. (a) Cell cycle distribution was analysed using standard propidium iodide method. (b) Cell cycle inhibition related gene expression changes after treatment were measured using RT-qPCR $\Delta\Delta$Ct method. GAPDH was used as a "housekeeping" gene; results are presented as changes in comparison to untreated cells; results are mean ± S.D. (n = 3); $*P \leq 0.05$, calculated by one-way ANOVA statistical test.

with Retinoic acid, Idarubicin, 3-Deazaneplanocin A, and Belinostat combinations was evaluated using standard propidium iodide staining procedure (Figure 2(a)). All treatment combinations caused NB4 cell cycle arrest at the phase G0/G1. Retinoic acid, 3-Deazaneplanocin A, and Belinostat combination with 2 nM Idarubicin had slightly higher effect on cell accumulation at the phase G0/G1 compared to the same combination with higher dose of Idarubicin (8 nM). Meanwhile, HL60 cell treatment with higher dose of Idarubicin (8 nM) in combinations for 24 hours caused cell accumulation in G2 cell cycle phase. However, after 72 hours, cell cycle distribution changed and started showing similar tendency as after treatment with other combinations (cell accumulation at the phase G0/G1).

In order to further analyse the proposed combination effect on cell cycle, we assessed cell cycle related gene expression changes by RT-qPCR. Gene expression changes of cell cycle inhibitors *ATM*, *p53*, *p21*, *p27*, and *Rb* and cell cycle activator *CCNE2* (cyclin E2) were analysed in NB4, HL60, and APL patient cells after 6 and 72 hours of treatment with 3-Deazaneplanocin A and Belinostat in different combinations with Retinoic acid and Idarubicin (Figure 2(b)). NB4 cells

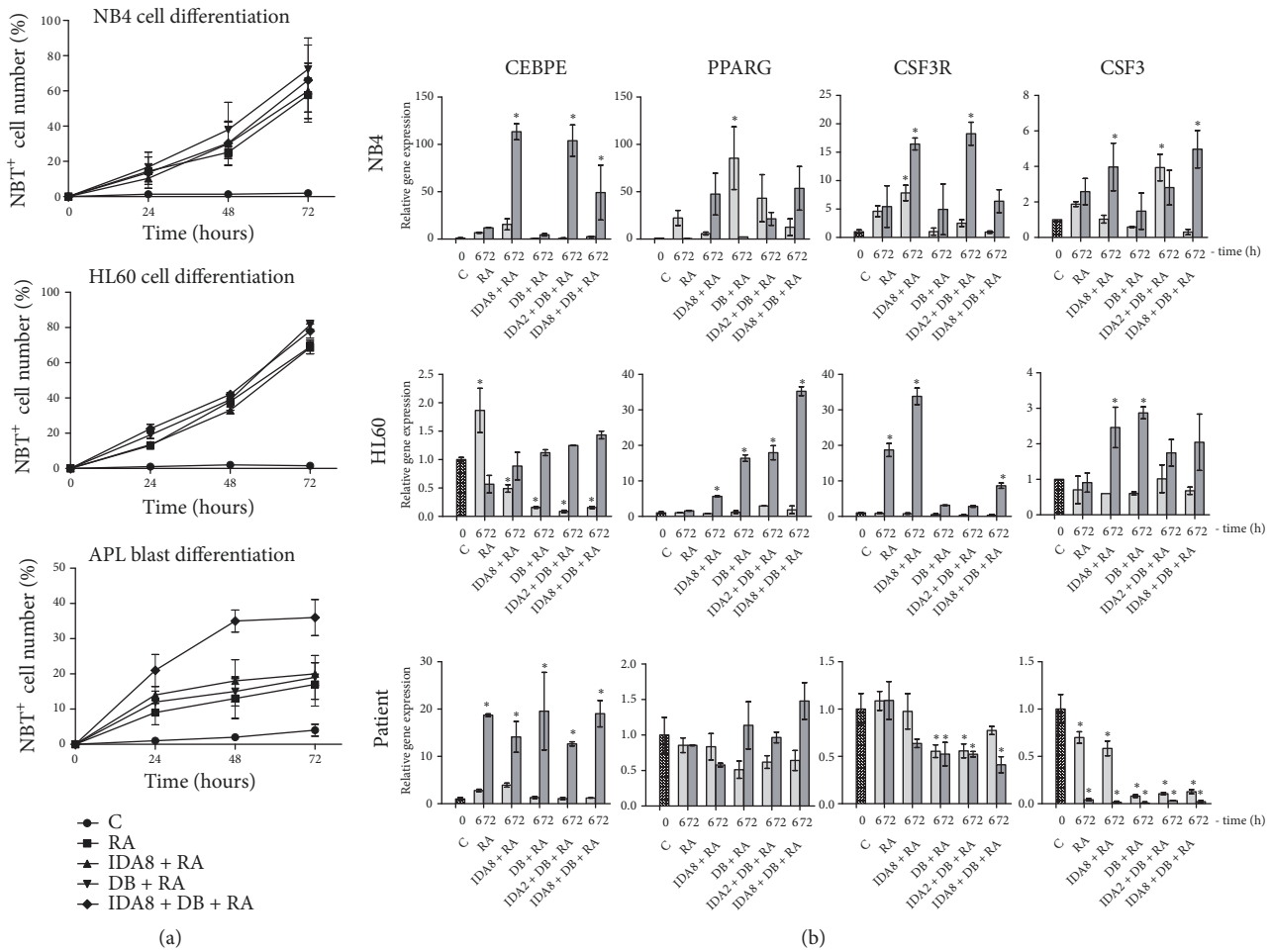

Figure 3: *Granulocytic differentiation potential assessment after treatment with epigenetic agents in combination with conventional treatment.* NB4 and HL60 cells and APL patient promyelocytes were treated with 1 μM Retinoic acid (RA), 2 nM or 8 nM Idarubicin (IDA2 or IDA8), 0.5 μM 3-Deazaneplanocin A (D), and 0.2 μM Belinostat (B) in different combinations for 72 hours; C: untreated cells. (a) Treated cell differentiation was evaluated by nitro blue tetrazolium (NBT) assay. (b) Cell differentiation related gene expression changes after treatment were measured using RT-qPCR $\Delta\Delta$Ct method. GAPDH was used as a "housekeeping" gene; results are presented as changes in comparison to untreated cells; results are mean ± S.D. (n = 3); $*P \leq 0.05$, calculated by one-way ANOVA statistical test.

possess mutated *p53* which is incapable of binding DNA [16]; HL60 cells lack any *p53* expression due to major deletions in the gene [17]. APL patient *p53* mutation status was not tested; however its expression did not increase after the treatment. This demonstrates that cell cycle inhibition in our tested cells might be regulated by p53-independent pathways. Other tested cell cycle inhibitors' genes expression increased in NB4, HL60, and APL patient cells. Cell cycle activator *CCNE2* (cyclin E2) gene expression in HL60 cells after the treatment with 8 nM Idarubicin in combination with Retinoic acid, 3-Deazaneplanocin A, and Belinostat correlated with cell cycle distribution analysis results (Figure 2(a)); initially *CCNE2* expression increased, but, later, after 72 hours, it decreased.

3.3. Enhanced Treatment Caused Cell Differentiation. NB4, HL60, and APL patient cells' capacity to differentiate into granulocytes was determined by NBT assay. Cells were treated with new epigenetic modifiers in combination with conventional treatment (3-Deazaneplanocin A and Belinostat combined with Idarubicin and Retinoic acid) and NBT test was performed every 24 hours (Figure 3(a)). NB4 and HL60 cells showed higher capacity to differentiate than APL patient blasts, which could be explained by not as pure APL patient blast population (70% of the population are blasts). Granulocytic cell differentiation occurred in a similar extent in the cases of NB4 and HL60 cells and in even higher extent in the case of APL patient cells after treatment with our proposed new combination compared to conventional treatment. It means that even though more cells entered apoptosis after enhanced treatment, this did not impair differentiation efficiency.

Also, we evaluated differentiation associated *CEBPE*, *PPARG*, *CSF3R*, and *CSF3* genes expression changes after NB4, HL60, and APL patient blasts' treatment with different combinations of 3-Deazaneplanocin A, Belinostat, Idarubicin, and Retinoic acid for 6 hours and for 72 hours

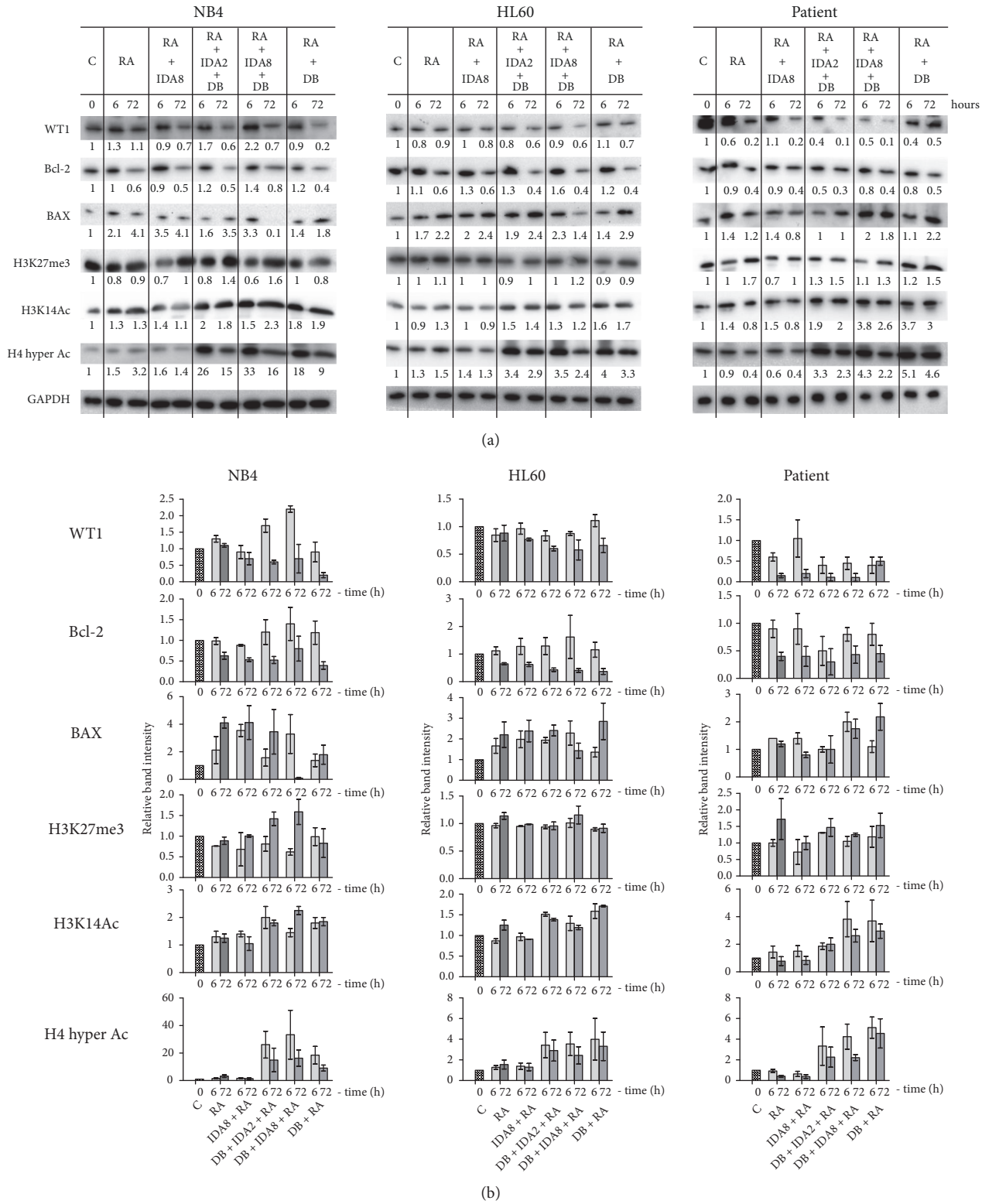

Figure 4: *Protein expression changes after NB4, HL60, and APL patient's cell treatment with epigenetic agents in combination with conventional treatment*. Cell samples were treated with 1 μM Retinoic acid (RA), 2 nM or 8 nM Idarubicin (IDA2 or IDA8), 0.5 μM 3-Deazaneplanocin A (D), and 0.2 μM Belinostat (B) in different combinations for 6 and 72 hours; C: untreated cells. (a) Protein level changes were assessed by immunoblot analysis. Intensity of protein bands was measured using ImageJ software and normalized to the GAPDH loading control; results are presented as changes in comparison to untreated cells. (b) Graphical visualization of relative band intensity of detected protein levels as measured using ImageJ software and normalized to the GAPDH loading control; results are mean ± S.D. (n=2).

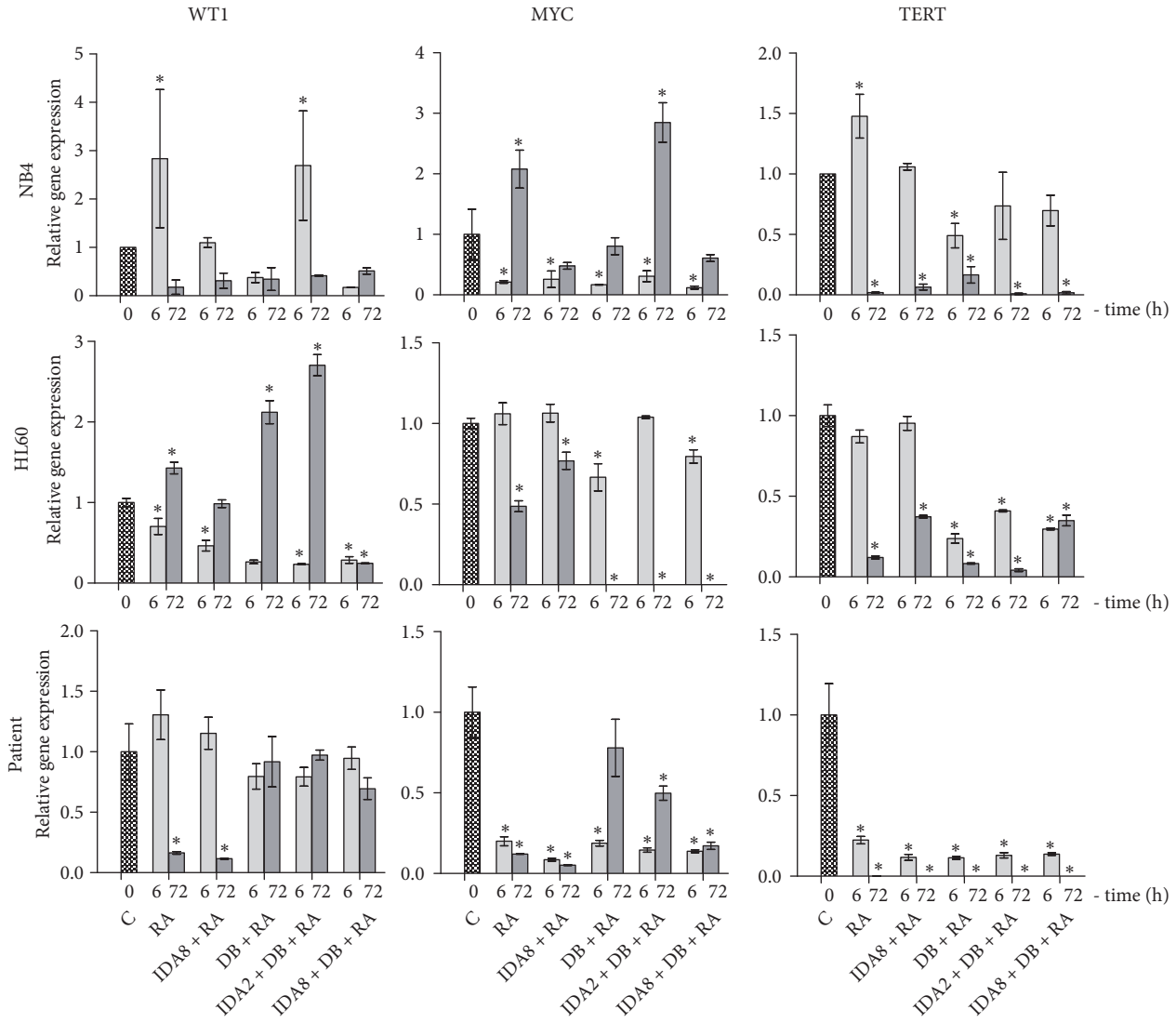

Figure 5: *Evaluation of oncogene expression changes after NB4, HL60, and APL patient's cell treatment with epigenetic agents in combination with conventional treatment*. Cell samples were treated with 1 μM Retinoic acid (RA), 2 nM or 8 nM Idarubicin (IDA2 or IDA8), 0.5 μM 3-Deazaneplanocin A (D), and 0.2 μM Belinostat (B) in different combinations for 6 and 72 hours; C: untreated cells. Oncogene expression changes after treatment were measured using RT-qPCR $\Delta\Delta$Ct method. GAPDH was used as a "housekeeping" gene; results are presented as changes in comparison to untreated cells; results are mean ± S.D. (n = 3); $*P \leq 0.05$, calculated by one-way ANOVA statistical test.

(Figure 3(b)). Interestingly, differentiation associated gene expression response to the treatment was different in APL patient cells compared to NB4 and HL60 cells. All tested genes' expression significantly elevated after NB4 and HL60 cell treatment, while APL patient cells treatment caused elevation of only *CEBPE* gene, but *CSF3R*, *CSF3* gene expression significantly decreased. Thus, enhanced treatment did not impair differentiation efficiency but caused different molecular response in NB4 and APL patient blasts.

3.4. Enhanced Treatment Caused Epigenetic Remodelling. Since 3-Deazaneplanocin A and Belinostat are epigenetic modifiers, we evaluated their potency to induce epigenetic changes; we tested H3K27 methylation, H3K14, and H4 acetylation level changes after NB4, HL60, and APL patient cell treatment with different combinations of 3-Deazaneplanocin A, Belinostat, Idarubicin, and Retinoic acid (Figure 4). Although we did not detect the decrease in H3K27me3 level, we observed significantly increased acetylation of histones H3K14 and H4 after treatment with agent combinations containing 3-Deazaneplanocin A and Belinostat.

3.5. Enhanced Treatment Decreased Oncogene Expression. In this study, we analysed oncogenes *WT1*, *MYC*, and *TERT* relative gene expression after 6 and 72 hours of treatment. All tested oncogenes' expression decreased in NB4, HL60, and APL patient cells, as assessed using RT-qPCR method (Figure 5). We further demonstrated that WT1 protein level also decreased after cell treatment (Figure 4), which

confirmed *WT1* gene downregulation. Conventional treatment alone (Idarubicin + Retinoic acid) was sufficient for oncogene downregulation: enhancement with epigenetic agents 3-Deazaneplanocin A and Belinostat did not increase the effect. On the other hand, it is beneficial to elucidate that epigenetic remodelling by HMT and HDAC inhibitors did not deregulate tested oncogene expression.

4. Discussion

In the study, we investigated the potential of conventional treatment (Idarubicin + Retinoic acid) that enhanced with epigenetic agents to treat APL. We tested HMT inhibitor 3-Deazaneplanocin A and HDAC inhibitor Belinostat in combination with Idarubicin and Retinoic acid *in vitro* (NB4 cell line) and *ex vivo* (APL patient bone marrow cell samples). HDAC inhibitors are widely researched to treat various disorders such as cancer, neurodegenerative disorders, and immune disorders [18]. Some of them are already approved as drugs (Vorinostat, Belinostat, Panobinostat, and Romidepsin) [19]. In general, HDAC inhibitors were shown to inhibit cancer cell growth, cause apoptosis, and induce cell differentiation [19]. It was demonstrated that low acetylation levels correlate with negative outcomes [20]. HMT enzymes have not been studied as extensively; however, their deregulation was recognized as a hallmark of cancer. It has been demonstrated that reversible histone lysine methylation is involved in cell proliferation, differentiation, DNA repair, and recombination [21]. Thus, histone methyltransferases are also perspective drug targets and their inhibitors are an interesting approach for leukemia treatment [22].

Combinations of various therapies usually have synergistic or additive effects. Thus, epigenetic agents tend to be combined with other therapeutics [19]. It was demonstrated that the combination of 3-Deazaneplanocin A (HMT inhibitor) and Vorinostat (HDAC inhibitor) had synergistic effect on non-small-cell lung carcinoma cells, which might be explained by the fact that HDAC activity is required for histone methyltransferase EZH2 caused transcriptional repression (EZH2 interacts with HDACs through PRC2 protein EED) [23]. Cotreatment with 3-Deazaneplanocin A and Panobinostat (HDAC inhibitor) was also more effective for acute myeloid leukemia treatment in mice models [24]. We have previously demonstrated additive effect of 3-Deazaneplanocin A and Belinostat on APL treatment with Retinoic acid *in vitro* and *in vivo* [13, 14]. Here, we revealed that conventional treatment (Retinoic acid + Idarubicin) enhancement with epigenetic agents triggered chromatin remodelling (significantly increased acetylation of histones H3K14 and H4). Aberrant HDAC expression, which causes histone deacetylation, was observed in various cancer types. As HDACs are involved in numerous cancer development important processes (such as apoptosis, senescence, differentiation, and angiogenesis), their inhibition is an attractive therapeutic approach [25]. In our study, significantly increased histone acetylation after combined treatment shows that the treatment likely caused enhanced gene transcription coherent to cancer cell proliferation inhibition and induction of differentiation.

Moreover, we demonstrated that 3-Deazaneplanocin A and Belinostat enhanced conventional treatment (Idarubicin + Retinoic acid), causing inhibition of APL cell proliferation and survival. Cytotoxicity did not increase highly. Staining with Annexin V / Propidium Iodide and detected downregulation of antiapoptotic protein Bcl-2, upregulation of proapoptotic protein BAX, and activation of Caspase-3 showed that our proposed new combination intensified cell apoptosis. Also, treatment with 3-Deazaneplanocin A, Belinostat, Retinoic acid, and Idarubicin combination had the highest effect on metabolic activity impairment. It has been demonstrated by many scientists before that 3-Deazaneplanocin A inhibited proliferation and caused apoptosis in various cancer cells. For example, one study showed that 3-Deazaneplanocin A inhibited growth, induced apoptosis, caused senescence, and changed cell cycle related protein expression in colon cancer cells [26]. Another study demonstrated that 3-Deazaneplanocin A blocked malignant peripheral nerve sheath tumour cell growth and survival in mouse xenograft models *in vivo* [27]. Moreover, 3-Deazaneplanocin A was shown not to alter mice behaviour and of all tested organs it had irreversible side effects only on testis (caused effects are commonly found in most chemotherapy treatments) [28]. Similarly, Belinostat inhibited cell growth and induced apoptosis in various human cells and in APL cell lines NB4 and HL60 [15]. Belinostat is already approved as a drug; it is safe and is generally well tolerated [29]. To conclude, our proposed combination (3-Deazaneplanocin A, Belinostat, Retinoic acid, and Idarubicin) had higher effect on inhibition of tested cell proliferation and survival and on induction of apoptosis than conventional treatment alone (Retinoic acid + Idarubicin).

Cell cycle analysis revealed that the proposed combination caused cell cycle arrest in G0/G1 phase except that HL60 treatment with combinations with higher dose of Idarubicin (8 nM) for 24 hours caused cell cycle arrest in the phase G2. It is known that Idarubicin inhibits DNA topoisomerase II, thus disrupting DNA synthesis and arresting cells in the phase G2 [30]. Meanwhile, Retinoic acid and Belinostat caused NB4 and HL60 cell cycle arrest [15] and 3-Deazaneplanocin A caused gastric cancer cell accumulation at the phase G0/G1 [31]. Thus, the combination of 3-Deazaneplanocin A, Belinostat, and Retinoic acid with lower dose of Idarubicin (2 nM) also arrested NB4 and HL60 cell cycle at the phase G0/G1. Gene expression analysis of cell cycle inhibitors *ATM*, *p21*, *p27*, and *Rb* and cell cycle activator *CCNE2* supported these findings. Since *p53* is mutated in NB4 and HL60 cells [16, 17] and its expression did not increase in APL patient cells (Figure 2(b)), cell cycle inhibition in our tested cells might be regulated by p53-independent pathways.

We also determined that newly proposed combination accelerated granulocytic cell differentiation in higher extent compared to conventional treatment. Granulocytic differentiation results obtained by nitro blue tetrazolium (NBT) assay were supported by elevated CEBPE and PPARG gene expression. It complies with previous findings that 3-Deazaneplanocin A and Belinostat enhanced Retinoic acid induced granulocytic differentiation [13]. However, *CSF3R* and *CSF3* gene expression changes were opposite

to each other: while their expression increased in NB4 and HL60 cells, it significantly decreased in APL patient blasts (Figure 3(b)). *CSF3* codes for the granulocyte colony-stimulating factor (G-CSF); *CSF3R* codes its receptor. G-CSF induces myeloid cell proliferation and survival, followed by neutrophilic differentiation [32]. The differences could be explained by high variance among blasts from different patients. For some subsets of patients, leukemic blasts proliferate spontaneously, whereas for other patients exogenous growth factors/cytokines are required for blast proliferation and differentiation [33]. APL patient cells were cultured under the same conditions as NB4 and HL60 cells (without exogenous growth factors); thus, this might explain lower proliferation and differentiation levels and the decrease of *CSF3* and *CSF3R* gene expression in APL patient cells. Although combined treatment caused different molecular response in APL blasts as compared to tested cell lines, differentiation was induced successfully in all tested cells.

One of the reasons of blocked cell differentiation might be elevated oncogene expression, which is very common in cancer cells. For example, transcription factor MYC promotes cell survival and drug resistance [34]. Its deregulation might cause uncontrolled cell proliferation, inhibit myeloid cell differentiation, and introduce other cancerous changes. *MYC* is overexpressed in many cancer cases. Therefore, it is an attractive target for cancer therapeutics [35]. Retinoic acid was demonstrated to restrict MYC level [36]. Another oncogene, *WT1*, is also overexpressed in the majority of acute myeloid leukemia patients. Increased *WT1* levels are associated with resistance to therapy, a higher incidence of relapse, and poor overall survival [37]. *TERT* codes telomerase catalytic subunit, which usually is overexpressed in hematologic malignancies. It correlates with resistance to apoptosis and senescence [38]. Thus, downregulation of these genes is important for successful treatment results. We demonstrated that expression of these oncogenes was significantly downregulated after cell treatment with Retinoic acid and Idarubicin. Although, treatment enhancement with 3-Deazaneplanocin A and Belinostat did not increase the effect, these epigenetic agents did not deregulate tested oncogene expression.

5. Conclusions

In this study, we demonstrated that HMT inhibitor 3-Deazaneplanocin A and HDAC inhibitor Belinostat enhanced conventional treatment (Retinoic acid + Idarubicin) for acute promyelocytic leukemia *in vitro* and *ex vivo*. Treatment enhancement with epigenetic agents caused chromatin remodelling which is associated with chromatin relaxation and enhanced transcription. Such changes might upregulate genes important for leukemia treatment. This is illustrated by our results demonstrating to a greater extent inhibited cell proliferation and survival, induced apoptosis, reduced metabolic activity, and accelerated granulocytic differentiation after treatment with 3-Deazaneplanocin A, Belinostat, Retinoic acid, and Idarubicin combination. We also revealed that different experimental models, NB4 and HL60 cell lines (*in vitro*) and APL patient blasts (*ex vivo*), displayed similar response to the treatment.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Acknowledgments

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Research Article

Complications of Intrathecal Chemotherapy in Adults: Single-Institution Experience in 109 Consecutive Patients

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Acute lymphoblastic leukemia and other aggressive lymphoid malignancies like Burkitt leukemia/lymphoma have high incidence of central nervous system (CNS) involvement. Various solid tumors, most notably breast cancer, can also metastasize into the CNS as a late stage complication causing devastating effects. Intrathecal (IT) chemotherapy consisting of methotrexate, cytarabine, or the two in combination is frequently used for the prophylaxis and treatment of CNS metastasis. Because of the high toxicity of these chemotherapeutic agents, however, their side effect profiles are potentially catastrophic. The incidence of neurotoxicity secondary to IT chemotherapy is well defined in the pediatric literature but is poorly reported in adults. Here, we investigated the incidence of neurologic and nonneurologic side effects secondary to IT chemotherapy in 109 consecutive adult patients over a two-year time period at hospitals associated with our institution. Of 355 IT chemotherapy treatments received by these patients, 11 (3.10%) resulted in paresthesias or paralysis, which we defined as significant neurologic events in our analysis. We also examined minor events that arose after IT chemotherapy, including back pain, headache, fever, vomiting, and asthenia. At least one of these occurred after 30.70% of IT chemotherapy doses. Clinicians involved in the care of patients receiving IT chemotherapy should be aware of these findings and consider treatment options lower rate of neurotoxicity such as high-dose systemic methotrexate.

1. Introduction

Advances in the treatment of many hematological and solid tumor malignancies have improved disease-free survival rates. Unfortunately, such improvements have come with increased frequency of relapses in the leptomeninges or CNS parenchyma, most commonly in aggressive lymphoid malignancies such as acute lymphoblastic leukemia (ALL), Burkitt lymphoma/leukemia, and lymphoblastic lymphoma [1–4]. Leptomeningeal metastasis can also complicate solid tumors, breast carcinoma being the most commonly associated [5]. Several treatments have been developed to target malignant cells in the CNS to prevent these events, most commonly intrathecal (IT) chemotherapy. The chemotherapeutic agents approved for intrathecal use in the United States include methotrexate, cytarabine, liposomal cytarabine, and thiotepa [1, 5, 6]. The scheduling and dosing of these medications varies depending on whether they are used for prophylaxis or treatment. Corticosteroids are frequently included with IT chemotherapy, most commonly hydrocortisone, to increase cytotoxicity and to decrease the risk of chemical arachnoiditis [1]. Most prophylactic regimens for leukemia and lymphomas contain methotrexate, either as a single agent or in combination with cytarabine.

The goal of IT chemotherapy is to maximize CNS drug exposure through direct CSF introduction, while reducing systemic drug toxicities [2]. The narrow therapeutic index and high potential toxicities of these agents mean

IT administration can have potentially catastrophic consequences. Chemical arachnoiditis, an acute syndrome occurring hours after injection and characterized by headache, backache, vomiting, fever, meningismus, and cerebral fluid pleocytosis, is among the most common and potentially serious effects [1, 7]. More severe symptoms also have been reported including cauda equina syndrome, encephalitis, papilledema, myelopathy, paraplegia, cranial nerve palsies, and seizures [7–11]. It is possible that the incidence of neurological complications in this setting is underestimated because cases may go unrecognized or unreported. To better characterize the incidence of neurological complications secondary to IT therapy, we examined sequential adult patients who received it over a two-year period at our institution. We documented signs and symptoms of neurotoxicity not present before administration that developed acutely thereafter. We provide illustrative case example followed by analysis of events in 109 consecutive patients.

2. Methods

We included adult patients with hematologic and solid tumors who received IT chemotherapy between January 2014 and December 2015 at Jackson Memorial Hospital and Sylvester Comprehensive Cancer Center. Primary endpoint studied was development of new symptoms indicative of neurotoxicity and/or arachnoiditis within fourteen days of administration of IT chemotherapy, specifically paralysis, paresthesias, headache, back pain/nuchal rigidity, asthenia, fever, nausea, or vomiting. Additional sensory and sphincter disturbances, which may also be associated with arachnoiditis, were not reliably documented and were excluded from analysis. We defined paralysis and paraesthesias as significant neurologic events for the purposes of analysis and the other side effects as minor events. This division was to allow distinction between more serious neurologic toxicities associated with IT chemotherapy from events with less impact on quality of life and/or of a more systemic nature.

Known CNS involvement was defined as follows: (1) CSF positive for malignancy by cytology and/or flow cytometry from samples collected at the time of administration or previously in association with the patient's current malignancy; (2) contrast enhanced MRI of the brain and/or spinal cord showing leptomeningeal carcinomatosis according to attending radiologist's report [12].

Patient and disease variables were compared between treatment modalities using the chi-square test for categorical data and the Mann-Whitney U test for continuous data. Relative risk was estimated using univariate Poisson regression models to assess the effect of these characteristics on a specific adverse event with respect to the treatment modalities. Tests were two-sided, and findings were considered statistically significant at $p<0.05$. All analyses were performed using SAS and R software.

This study was a retrospective chart review that did not involve any interaction with patients and therefore specific informed consent from each patient was not required by our Institutional Review Board. Any information that might lead to the identification of individual patients has been excluded. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

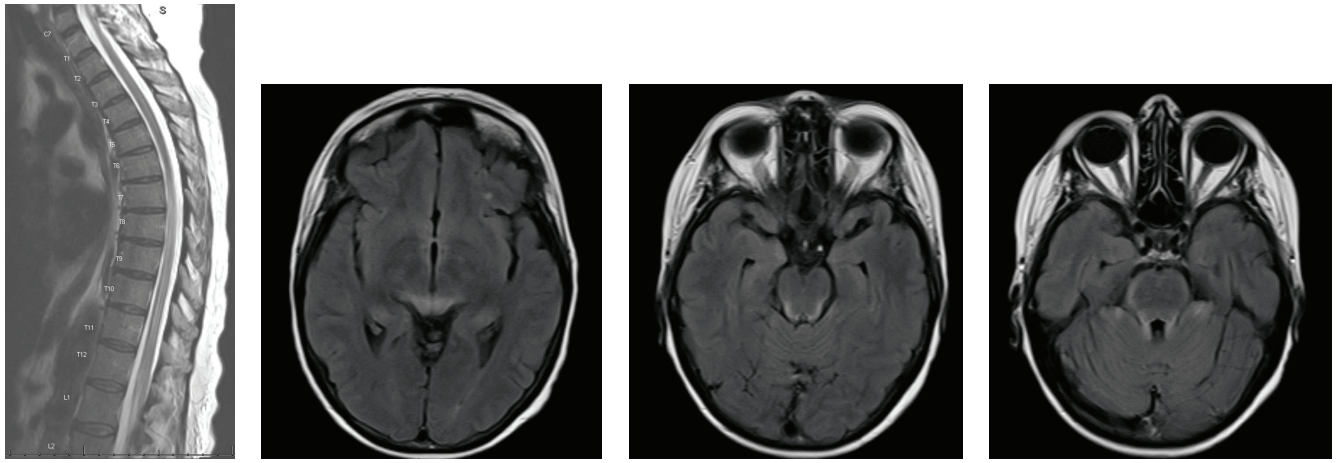
3. Results

3.1. Illustrative Case, Severe Neurologic Complications of IT Chemotherapy. A 61-year-old Hispanic woman with a history of stage IV diffuse large B-cell lymphoma (DLBCL) was admitted for salvage therapy with rituximab, dexamethasone, high-dose cytarabine, and cisplatin (R-DHAP). She previously had completed six cycles of rituximab, cyclophosphamide, adriamycin, vincristine, and prednisone (R-CHOP) with twelve milligrams of intrathecal (IT) methotrexate (MTX) prophylactically in each cycle.

On day 1 of R-DHAP the patient received IT MTX 12 mg with cytarabine 50 mg. Flow cytometry and cytology from the lumbar puncture were negative for malignancy. The following day the patient complained of a nonpositional headache rated as 7 out of 10 in intensity. The headache was associated with photophobia, nausea without vomiting, and double vision. She denied neck stiffness or fever. Acetaminophen did not relieve the pain but sumatriptan provided mild relief. On day three she reported bilateral lower extremity weakness, right greater than left. She also reported inability to ambulate secondary to the weakness, rectal incontinence, and urinary retention. Weakness progressed in the days that followed to bilateral lower extremity paralysis.

Neurologic exam was significant for right lateral rectus paresis (with the rest of the cranial nerve exam unremarkable), decreased strength of all muscle groups in the bilateral lower extremities, diminished reflexes in the bilateral patellar and Achilles tendons, positive Babinski on the right, and diminished sensation to light touch over the sacrum, posterior thighs, and perineum. The physical exam findings were not present prior to administration of IT chemotherapy. Six days after symptom onset repeat LP again yielded negative cerebrospinal fluid (CSF) studies for involvement by malignant cells. CSF total protein was elevated to 131 mg/dL, glucose and total cell count were within normal limits, and gram stain and culture were negative. Lumbar and thoracic spine MRIs revealed mild enhancement of the ventral and dorsal nerve roots of the cauda equina, particularly at T12-L3, and diffuse central spinal cord signal abnormality most prominent from T6-L2 (Figure 1(a)). Brain magnetic resonance imaging (MRI) showed symmetric FLAIR signal abnormality in the brainstem and cerebellum without diffusion restriction or abnormal enhancement (Figures 1(b)-1(d)).

No other etiology for her symptoms was identified, and they were attributed to intrathecal chemotherapy-induced neurotoxicity. The patient did not recover neurologic function, and her systemic lymphoma unfortunately progressed soon thereafter. She entered hospice care and died secondary to complications of her systemic lymphoma.



(a) (b) (c) (d)

Figure 1: *MRI images of severe IT chemotherapy complications.* (a) Increased T2 signal throughout the spinal cord, most prominent from T6 to the conus. There is some associated cord expansion. The cord signal involves almost the entire diameter most noted at T8. (b-d) Brain images showing symmetric FLAIR signal abnormality in the brainstem, cerebellum, and possibly the thalami without diffusion restriction or abnormal enhancement.

3.2. Consecutive Case Series. During the study period, 109 patients received IT chemotherapy, of whom 74 (68%) were male and 35 (32%) were female. Forty-four (40%) were Hispanic. The median patient age was 50 years old; the age range was 20 to 88 years old. The most common diagnosis was diffuse large B-cell lymphoma (40%), followed by B-cell ALL (28%), T-cell ALL (8%), and Burkitt lymphoma (8%). Sixteen (15%) of patients were HIV positive and 3 (2.8%) had chronic renal failure. At time of treatment, 33 (30%) patients had CNS involvement (Table 1). The median number of IT chemotherapy doses per patient was 2 (range 1-12).

Therapy consisted of methotrexate alone, cytarabine alone, or methotrexate + cytarabine. Neither thiotepa nor topotecan was used at either institution in adult patients during the time period in question. The total number of IT doses recorded was 355. There were 150 doses of methotrexate alone, 18 of cytarabine alone, and 187 of cytarabine + methotrexate.

Rates of each symptom per administration of IT chemotherapy are shown in Table 2 and Figure 2. We also determined the rate at which each symptom occurred per patient over the all doses administered (Table 2). The significant neurologic events paralysis and/or paresthesias occurred after 11 doses (3.10%) affecting 9 patients (8.26%). Minor events occurred after 109 doses (30.70%) affecting 29 patients (26.61%). We documented only new-onset symptoms because the systemic changes fever, nausea, vomiting, and asthenia, which may be associated with chemical arachnoiditis, may also occur for other reasons in this patient population (see Discussion). We compared rates of adverse events among the three treatment modalities but did not detect any significant differences (Table 2). When liposomal cytarabine (Depocyte) was compared to the nonliposomal formulation, there was again no significant difference in rates of adverse neurologic events. There was

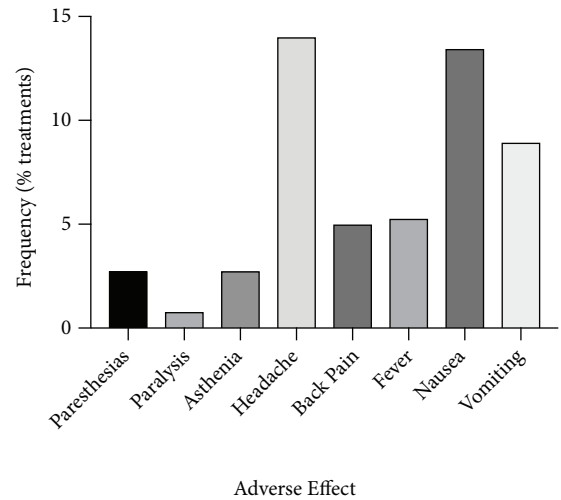


Figure 2: *Rates of adverse effects of IT chemotherapy.* Percentage of 355 IT chemo doses associated with each side effect in a series of 109 consecutive patients.

significant correlation, however, between the number of IT treatments a patient received and likelihood of experiencing at least one adverse effect (correlation coefficient 0.35, p=0.001).

We also examined correlation between adverse neurologic events and independent variables, specifically HIV status, renal failure, and known CNS involvement. Patients with known CNS involvement were found to have risk of any event with borderline statistical significance (RR=2.9, 95%CI=0.99-8.49, p=0.052) but with high significance when considering only minor events (RR=4.35, 95%CI=1.85-10.24, p=0.0008). There were no differences detected in patients who had renal failure or HIV.

Table 1: Patient population and disease characteristics.

Variable	N	%
Gender		
Female	35	32.1
Male	74	67.9
Race/Ethnicity		
Non-Hispanic White	20	18.3
Non-Hispanic Black	17	15.6
Hispanic White	42	38.5
Hispanic Black	2	1.8
Asian	2	1.8
Haitian	4	3.7
Other/unknown	22	20.2
Type of Cancer		
DLBCL	43	39.4
B-Cell ALL	31	28.4
T-Cell ALL	8	7.3
Burkitt Lymphoma	8	7.3
Breast Cancer	3	2.8
Other Non-Hodgkin	7	6.3
CML	1	0.9
CLL	1	0.9
Other	4	3.7
Chronic Renal Failure		
Yes	3	2.8
No	106	97.2
HIV		
Yes	16	14.7
No	93	85.3
CNS Involvement		
Yes	33	30.3
No	76	69.7

4. Discussion

Overall incidence of acute neurotoxicity from IT MTX in children is 3-11% [13], but review of the literature reveals rates are not well defined in adults. We report rates of significant adverse neurologic events following IT chemotherapy used as either prophylaxis or treatment for known leptomeningeal involvement at our medical center over a two-year period. We found these events occurred after 3.1% of IT chemotherapy doses, affecting 8.26% of the patients in our consecutive case series. Minor side effects were more common, occurring after 30.70% of doses and affecting 26.61% of patients at least once during the course of their therapy. We found a strong correlation between number of IT treatments received and likelihood of suffering at least one adverse effect. Headache, nausea, vomiting, back pain, and fever were most common—all of which are known symptoms of chemical arachnoiditis. Our results indicate an incidence of neurologic side effects secondary to intrathecal chemotherapy in adults significantly higher than for children and which may be higher than what is commonly perceived by practitioners.

Although symptoms such as headache and back pain related to lumbar puncture are not uncommon, clinicians should also be cognizant that these symptoms may signify impending onset of more significant toxicity. Methotrexate is typically assumed to be the major cause of such neurotoxicities [13], but cytarabine is also a known major cause [14–16]. Our series did not reveal any differences in relative risk of neurologic side effects between MTX, cytarabine, or the two in combination. Jabbour et al. evaluated neurologic complications secondary to IT liposomal cytarabine in combination with high-dose methotrexate as prophylactic treatment in patients with ALL and found the incidence of severe complications to be 16% [8]. A smaller retrospective review by Gállego Pérez-Larraya et al. studying IT liposomal cytarabine given as prophylaxis in patients with non-Hodgkin lymphoma reported 28% of patients developed moderate or severe neurotoxicity [14]. Pretreatment with oral dexamethasone has been found to decrease adverse side effects of depot form of cytarabine (DTC) [17].

IT chemotherapy for both therapy and prophylaxis of CNS involvement has been a mainstay for medical

Table 2: Adverse neurologic events.

	No. of patients affected (%)	No. of Events (occurrence rate per IT treatment)	Treatment	RR	95% CI	P Value
Significant Neuro Events	9 (8.26)	11 (3.10)				
Overall∗			MTX	1	-	-
			CYT	1.67	0.19-14.27	0.641
			MTX and CYT	1.16	0.50-2.71	0.728
Paresthesias	8 (7.34)	10 (2.82)				
			MTX	1	-	-
			CYT	1.67	0.19-14.27	0.64
			MTX and CYT	0.64	0.17-2.39	0.51
Paralysis∗∗	2 (1.83)	3 (0.85)				
			MTX	1	-	-
			CYT	8.33	0.52-133.23	0.134
			MTX and CYT	0.80	0.05-12.82	0.876
Minor Events	29 (26.61)	109 (30.70)				
Overall∗			MTX	1	-	-
			CYT	1.53	0.75-3.12	0.241
			MTX and CYT	0.97	0.66-1.41	0.857
Asthenia	4 (3.67)	10 (2.82)				
			MTX	1	-	-
			CYT	2.78	0.56-13.76	0.21
			MTX and CYT	0.80	0.26-2.49	0.703
Headache	15 (13.76)	50 (14.08)				
			MTX	1	-	-
			CYT	1.23	0.43-3.53	0.694
			MTX and CYT	0.68	0.39-1.19	0.180
Back pain	9 (8.26)	18 (5.07)				
			MTX	1	-	-
			CYT	2.08	0.44-9.81	0.353
			MTX and CYT	1.00	0.40-2.54	0.996
Fever	5 (4.59)	19 (5.35)				
			MTX	1	-	-
			CYT	2.78	0.56-13.78	0.211
			MTX and CYT	1.47	0.54-3.98	0.447
Nausea	13 (11.93)	48 (13.52)				
			MTX	1	-	-
			CYT	0.93	0.21-3.99	0.918
			MTX and CYT	1.25	0.69-2.26	0.464

Table 2: Continued.

	No. of patients affected (%)	No. of Events (occurrence rate per IT treatment)	Treatment	RR	95% CI	P Value
Vomiting	6 (5.50)	32 (9.01)				
			MTX	1	-	-
			CYT	1.11	0.25-4.86	0.889
			MTX and CYT	0.91	0.45-1.82	0.788

∗Multiple major or minor events simultaneously are counted only once in overall (overall numbers are therefore less than total of subcategories).
∗∗One patient had both paresthesias and paralysis in two events. To avoid double-counting, these events were included only in paresthesias for the RR calculation.

management of leukemia and lymphoma throughout the world for several decades and for patients with leptomeningeal involvement by solid tumors [7]. Survival for patients with leptomeningeal spread of disease, however, is low while the incidence of early and late complications associated with IT chemotherapy can be high [18–20]. Several studies have suggested that systemic high-dose (HD) MTX may improve the response rate or survival of patients with CNS involvement [18, 19]. Glantz et al. treated 16 patients with neoplastic meningitis from a variety of solid tumors and lymphoma with HD MTX alone and retrospectively compared outcomes to 15 patients treated with standard IT chemotherapy [19]. Significantly longer median survival was found in patients who received HD IV MTX (13.8 months) compared to those who received IT MTX (2.3 months; P=0.003). Daily CSF samples were collected and MTX concentrations were measured, revealing that IV administration achieved superior CSF MTX levels. As the flow of CSF is from the ependymal cells in the brain down to the cauda equina where it is resorbed, the administration of IT MTX to the area between L2 and L3 was suboptimal. IT topotecan has been used with some success in pediatric malignancies based on favorable pharmacokinetic properties [21, 22]. In adults, however, IT topotecan as a single agent did not produce clinical benefit over standard therapies in a multicenter phase 2 trial for patients with meningeal involvement by any malignancy [23]. A later case series, however, showed anecdotally particular patients may achieve clinical benefit lasting up to 12 months from IT topotecan [24].

Olmos-Jimenez et al. performed an observational and prospective study in Spain evaluating standardized triple intrathecal chemotherapy in adult hematology-oncology patients over an 18-month period [25]. Similar to our study, adverse events occurring after administration of IT chemotherapy was recorded; however this study was substantially smaller containing only 20 patients and 56 treatments. The study population was 75% male, 50% of the patients had non-Hodgkin lymphoma, and 5% had pre-existing leptomeningeal disease. Adverse events occurred after 39.3% of 56 doses recorded. The vast majority of events (96.7%) were grades 1-2 with only one event being grade 3. As in our study, the adverse event recorded most frequently was headache, followed by vomiting and vertigo. One administration event (1.8%) resulted in grade 2 sensorimotor polyneuropathy.

5. Limitations

Because our study was retrospective, the ability to determine causality of symptoms is limited and we acknowledge that the population studied had additional reasons to develop some of the symptoms reported. Given the disparate underlying malignancies of the patients in our series, we did not investigate systemic chemotherapy as a confounder; future studies should investigate whether concurrent administration of agents that may cause neuropathy, such as vincristine, may lead to increased rates of neurologic events with administration of IT chemotherapy. We believe for the great majority of incidents we analyzed here, however, the timing of the symptoms in relation to IT chemotherapy administration reflects a likely causative link. An additional caveat of this study is that we could not specifically separate out prophylactic measures such as administration of hydrocortisone as independent variables in our analysis due to low numbers and incomplete documentation of nonpharmacologic interventions. Questions regarding efficacy of interventions designed to prevent IT chemo toxicities are likely best answered through prospective studies. Use of the Common Toxicity Criteria (CTC) to define symptoms was considered and would have made the side effects we report more easily comparable to experiences at other institutions. This was not possible for the purposes of this retrospective analysis, however, in which patient charting did not consistently follow the criteria. This is another matter for which a prospective study would be well suited.

6. Conclusion

The most common approach for prevention of CNS spread of hematologic tumors is IT chemotherapy, with or without radiation. Its use should not be discarded, but practitioners should be aware of the potential complications and a frequency that may be higher than commonly perceived. Consideration of alternate, less toxic forms of therapy such as systemic HD MTX may be warranted and, as highlighted, could be more effective in some cases.

Data Availability

The pooled statistical data used to support the findings of this study are included within the article. Additional details used

to support the findings of this study are available from the corresponding author upon request, but we cannot release data that could lead to identification of specific patients.

Conflicts of Interest

The authors declare no conflicts of interest relevant to this work.

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