Integrative analysis of type-I and type-II aberrations underscores the genetic heterogeneity of pediatric acute myeloid leukemia

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ABSTRACT

Background
Several studies of pediatric acute myeloid leukemia have described the various type-I or type-II aberrations and their relationship with clinical outcome. However, there has been no recent comprehensive overview of these genetic aberrations in one large pediatric acute myeloid leukemia cohort.

Design and Methods
We studied the different genetic aberrations, their associations and their impact on prognosis in a large pediatric acute myeloid leukemia series (n=506). Karyotypes were studied, and hotspot regions of *NPM1*, *CEPBA*, *MLL*, *WT1*, *FLT3*, *N-RAS*, *K-RAS*, *PTPN11* and *KIT* were screened for mutations of available samples. The mutational status of all type-I and type-II aberrations was available in 330 and 263 cases, respectively. Survival analysis was performed in a subset (n=385) treated on consecutive acute myeloid leukemia Berlin-Frankfurt-Munster Study Group and Dutch Childhood Oncology Group treatment protocols.

Results
Genetic aberrations were associated with specific clinical characteristics, e.g. significantly higher diagnostic white blood cell counts in *MLL*-rearranged, *WT1*-mutated and *FLT3*-ITD-positive acute myeloid leukemia. Furthermore, there was a significant difference in the distribution of these aberrations between children below and above the age of two years. Non-random associations, e.g. *KIT* mutations with core-binding factor acute myeloid leukemia, and *FLT3*-ITD with t(15;17)(q22;q21), *NPM1*- and *WT1*-mutated acute myeloid leukemia, respectively, were observed. Multivariate analysis revealed a 'favorable karyotype', i.e. t(15;17)(q22;q21), t(8;21)(q22;q22) and inv(16)(p13q22)/t(16;16)(p13;q22). *NPM1* and *CEBPA* double mutations were independent factors for favorable event-free survival. *WT1* mutations combined with *FLT3*-ITD showed the worst outcome for 5-year overall survival (22±14%) and 5-year event-free survival (20±13%), although it was not an independent factor in multivariate analysis.

Conclusions
Integrative analysis of type-I and type-II aberrations provides an insight into the frequencies, non-random associations and prognostic impact of the various aberrations, reflecting the heterogeneity of pediatric acute myeloid leukemia. These aberrations are likely to guide the stratification of pediatric acute myeloid leukemia and may direct the development of targeted therapies.

Key words: pediatric AML, type I/II aberrations, mutation, prognostic factor.

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The online version of this article has a Supplementary Appendix.

Introduction

Acute myeloid leukemia (AML) accounts for 15-20% of pediatric leukemias.[1] Despite intensification of chemotherapy over the last decades, only approximately 60-70% of children with AML are cured.[2] AML is not a single disease entity, and its heterogeneity is reflected by differences in morphology, immunophenotype, as well as cytogenetic and molecular aberrations.[3] Moreover, recurrent (cyto)genetic aberrations are important prognostic factors in pediatric AML and an increasing number of study groups are using them for risk group stratification.[4-5]

Gilliland *et al.* hypothesized that the development of AML requires at least two types of genetic events.[6] Type-I aberrations occur as mutations in hotspots of specific genes involved in signal transduction pathways (*FLT3*, *KIT*, *NRAS*, *KRAS* and *PTPN11*) which lead to uncontrolled proliferation and/or survival of leukemic cells. Type-II aberrations are often chromosomal rearrangements of transcription factors resulting in the translation of fusion proteins leading to impaired differentiation of the leukemic cells, including *PML-RARα* [t(15;17)(q22;q21)], *AML1-ETO* [t(8;21)(q22;q22)], *CBFB-MYH11* [inv(16)(p13q22)/t(16;16)(p13;q22)] and 11q23/*MLL*-rearrangements. This hypothesis was further strengthened by observations from mouse models that one aberration is not sufficient to induce leukemia, but that cooperative events are needed to develop frank leukemia. For example, knock-in of FLT3-ITD leads to the development of a myeloproliferative disorder but lacks the maturation arrest typical of acute leukemia,[7] whereas co-expression with inv(16)(p13q22) or t(15;17)(q22;q21) resulted in AML.[8-9]

In pediatric AML, the individual type-I or type-II aberrations and their relationship with clinical outcome have been described in several studies.[4-5, 10-16] However, there has been no comprehensive overview of the associations and the prognostic impact of type-I and type-II aberrations in one large cohort of pediatric AML patients. Furthermore, over the last decade, novel molecular genetic aberrations in pediatric AML, such as mutations in the *CEBPA*, *NPM1* and *WT1* genes, as well as partial tandem duplications in the *MLL* gene (*MLL*-PTD), have been identified.[11,13,17] The prognostic impact of these newly identified aberrations all together in one large pediatric AML series has not so far been reported. Identifying prognostic factors in pediatric AML may lead to improved risk-group stratification, and may, therefore, have a direct impact on current and future treatment protocols. Secondly, specific leukemogenic aberrations may guide the development of targeted therapy approaches for selected patient groups.

We, therefore, performed a study on type-I and type-II aberrations in the largest pediatric AML series so far, which also focused on their association with clinical characteristics and outcome.

Design and Methods

Study cohort

This study included 506 pediatric patients with *de novo* AML, the data for whom were provided by the Dutch Childhood Oncology Group (DCOG), the AML 'Berlin-Frankfurt-Münster' Study Group (AML-BFM-SG), the Czech Pediatric Hematology (CPH) group, and the St. Louis Hospital in Paris, France. Institutional review board approval for these studies and informed consent was obtained according to local laws and regulations. Each study group performed a central review of the morphology according to the WHO/FAB classification.[18] Clinical and cell-biological data, including cytogenetic results, were obtained from these study groups and institutes.

Survival analysis was restricted to a subset of 385 AML patients who received relatively homogenous treatment according to DCOG/AML-BFM 87, DCOG 92-94/AML-BFM 93, AML-BFM 98, AML-BFM 04 and MRC-12/15 protocols. Details of these treatment protocols and overall outcome data have already been published.[19-24] Treatment consisted of 4 to 5 blocks of intensive chemotherapy, using a standard cytarabine and anthracycline backbone. Stem cell transplantation in first complete remission was only performed in a small number of selected high-risk patients.

Definition of gene mutations as type-I and type-II aberrations.

Screening of gene mutations was carried out according to the availability of material. Mutations were determined in the hotspot regions of *NPM1* (n=337), *CEPBA* (n=282), *MLL* (i.e. partial tandem duplications - PTD; n=309), *WT1* (n=330), *FLT3* (i.e. internal tandem duplications - ITD; n=372) and tyrosine kinase domain mutations (TKD; n=330), *N-RAS* and *K-RAS* (n=353), *PTPN11* (n=350) and *KIT* (n=368), as previously described.[13-14,25-29] This resulted in screening of all type-I aberrations in 330 cases and all type-II aberrations in 263 cases. A complete list of screened regions per gene, primers and PCR conditions is provided in the *Online Supplementary Table S1*. The 'fusion gene' type-II aberrations, i.e. *MLL*-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22)/t(16;16)(p13;q22), t(15;17)(q22;q21), were mutually exclusive with *NPM1* mutations, *CEBPA* double mutations and *MLL*-PTD aberrations, which suggests that these latter mutations can be considered as type-II aberrations. This is further strengthened by evidence that these aberrations result in a maturation arrest; e.g. targeted disruption of C/EBPα results in a selective early block of granulocyte differentiation,[30] while *NPM1* mutations and *MLL*-PTD disrupt the controlled expression of *HOX*-genes resulting in impaired differentiation of the hematopoietic cells.[31-33] Hence *NPM1* mutations, *CEBPA* double mutations, and *MLL*-PTD aberrations were considered to be type-II aberrations, whereas mutations in *FLT3*, *N/K-RAS*, *PTPN11* and *KIT* were considered to be type-I aberrations. As the leukemogenic mechanism of *WT1* mutations still needs to be clarified,[34] these mutations were arbitrarily categorized as type-I aberrations for the purposes of this study because they overlapped with the type-II defined subtypes.

Further information concerning DNA and RNA isolation, cytogenetic analysis, definition of cytogenetic groups and statistical analysis is available in the *Online Supplementary Appendix*.

Results

Study cohort

Characteristics of the study cohort are presented in Table 1. Sex distribution was 57% male *versus* 43% female. Median age was 8.7 years (range 0-18 years), and the distribution according to the age categories under two years, 2-9 years and ten years and over was 18%, 38% and 44%, respectively. The median white blood cell count (WBC) at diagnosis was 34×10^9/L (range 0-585×10^9/L). FAB-M2, -M4 and -M5 were the most common morphological subtypes in this cohort; these were 23%, 24% and 24%, respectively. Distribution according to sex, age, WBC and FAB-morphology was comparable with the

AML-BFM trials, i.e. the AML-BFM 93 (n=471) and 98 (n=473) trials, indicating that our cohort is representative for pediatric AML (*Online Supplementary Tables S2 and S3*). The 385 pediatric AML cases included in the survival analysis had a 5-year probability of event-free survival (pEFS) and overall survival (pOS) of 42±3% and 60±3%, respectively. These survival rates are in a similar range to those of previously published studies.[35]

Characteristics of pediatric AML with specific cytogenetic subtypes

Patients were assigned to the following cytogenetic groups: *MLL*-rearranged AML (24%; 122/506), t(8;21)(q22;q22) (13%; 64/506), inv(16)(p13q22)/t(16;16)(p13;q22) (10%; 48/506), t(15;17)(q22;q21) (6%; 28/506), t(7;12)(q36;p13) (1%; 7/506), t(6;9)(p23;q34) (1%; 7/506), complex karyotype (6%; 30/506), monosomy 7 (1%; 6/506), trisomy 8 (2%; 12/506), CN-AML (17%; 84/506), and 'other karyotype' (13%; 65/506) (Table 1A). In 7% (33/506) of the cases, conventional karyotyping failed, and neither RT-PCR nor FISH led to classification of these patients; these cases were, therefore, assigned to the 'unknown' cytogenetic group. An overview of the cytogenetic group assignment and the mutational status of the investigated genes of all individual patients is provided in the *Online Supplementary Table S4*.

No difference in sex distribution was seen between the different cytogenetic groups (Table 1A). Patients with t(8;21)(q22;q22) presented with a significantly lower WBC (median 13×10^9/L; $P<0.001$), and *MLL*-rearranged AML patients with a significantly higher WBC (median 63×10^9/L; $P=0.001$) compared with the other cytogenetic groups (Table 1A). The median ages of children with *MLL*-rearranged AML (3.7 years; $P<0.001$), with t(7;12)(q36;p13) (0.3 years; $P<0.001$) and with a complex karyotype (2.5 years; $P<0.001$) were all significantly lower compared with the other cytogenetic groups. In contrast, children with t(8;21)(q22;q22) (11.5 years; $P<0.001$), t(15;17)(q22;q21) (10.2 years; $P=0.03$), and t(6;9)(p23;q34) (12.8 years; $P=0.03$) were significantly older compared with the other cytogenetic groups (Table 1A).

Table 1. Overview of baseline clinical characteristics per cytogenetic (A) and molecular (B) aberration.

(A)

Cytogenetic aberration	Frequency n. (%)	Age (yr) n.	median	Range	*P* value*	Sex n.	% F	*P* value*	WBC (x10⁹/L) n.	median	range	*P* value
MLL-rearrangements	122 (24.1)	122	3.7	0.0-17.3	**<0.001**	122	38	0.16	96	63	1-585	**0.001**
t(8;21)(q22;q22)	64 (12.6)	64	11.5	2.6-18.5	**<0.001**	64	42	0.85	57	13	2-320	**<0.001**
inv(16)(p13q22)	48 (9.5)	48	10.5	0.7-17.3	0.13	48	46	0.71	42	68	3-234	0.10
t(15;17)(q22;q21)	28 (5.5)	28	10.2	1.9-17.7	**0.03**	28	54	0.26	24	26	1-247	0.36
t(7;12)(q36;p13)	7 (1.4)	7	0.3	0.2-1.5	**<0.001**	7	71	0.25	6	55	14-227	0.38
t(6;9)(p23;q34)	7 (1.4)	7	12.8	10.3-14.9	**0.03**	7	43	1.00	4	96	24-120	0.21
Complex (≥3 aberrations)	30 (5.9)	30	2.5	0.1-14.4	**<0.001**	30	47	0.70	24	21	3-320	0.24
Monosomy 7	6 (1.2)	6	10.3	4.2-14.1	0.70	6	33	0.70	3	55	3-66	0.76
Trisomy 8	12 (2.4)	12	12.0	1.0-16.8	0.11	12	33	0.48	8	77	8-302	0.13
CN-AML	84 (16.6)	84	10.7	0.1-18.8	0.01	84	41	0.57	73	43	1-535	0.15
Other	65 (12.9)	65	8.8	0.0-18.4	0.89	64	48	0.37	54	29	1-452	0.27
Unknown	33 (6.5)	33	9.0	0.7-18.0	0.19	32	47	0.67	26	37	0-483	0.49
All cases	506 (100)	506	8.7	0.0-18.8	-	504	43	-	417	34	0-585	-

*CN: cytogenetically normal; WBC: white blood cell count at diagnosis; Yr: years; %F: % female; *P values refer to the comparison of the variable between the genetic subgroup vs. all others. Bold P values are statistically significant.*

(B)

Molecular aberration	Frequency (%)	Age (yr) n.	median	Range	*P* value*	Sex n.	% F	*P* value*	WBC (x10⁹/L) n.	median	range	*P* value
NPM1 mut (n=337)	7.7	26	11.0	3.6-18.8	**0.03**	26	58	0.14	25	43	5-230	0.83
CEBPA double mut (n=282)	6.0	17	12.0	4.0-18.5	0.08	17	59	0.15	16	60	6-388	0.38
MLL-PTD (n=309)	2.3	7	7.5	4.8-18.0	0.64	6	50	0.70	5	73	45-170	0.10
WT1 mut (n=330)	8.8	29	9.2	1.9-17.8	0.47	29	38	0.52	26	86	3-354	**0.03**
FLT3-ITD (n=372)	18.0	67	10.1	1.6-18.8	**0.008**	66	46	0.88	59	74	5-535	**<0.001**
FLT3-TKD (n=330)	2.7	9	11.2	2.0-16.9	0.25	9	44	1.00	8	46	7-320	0.96
RAS pathway (n=348)	21.6	75	9.6	0.1-16.9	0.98	75	37	0.14	61	42	3-483	0.54
- *N-RAS* mut (n=353)	16.1	57	9.6	0.1-16.9	0.82	57	39	0.31	46	42	4-483	0.85
- *K-RAS* mut (n=353)	3.7	13	11.3	5.5-16.9	0.06	13	15	**0.03**	10	118	3-225	0.09
- *PTPN11* mut (n=350)	2.0	7	4.2	0.6-13.5	0.26	7	57	0.71	6	32	4-115	0.60
KIT mut (n=368)	8.4	31	10.3	0.2-16.7	0.35	31	39	0.58	29	37	2-234	0.48

*WBC: white blood cell count at diagnosis; yr: years; %F: % female; mut: mutation; *P values refer to the comparison of the variable between the genetic subgroup vs. the wildtypes. Bold P values are statistically significant.*

Characteristics of pediatric AML patients with type-II gene mutations

The following frequencies of gene mutations considered as type-II aberrations were found: *NPM1* (8%; 26/337), *CEBPA* double mutations (6%; 17/282) and *MLL*-PTD (2%; 7/309) (Table 1B). These aberrations were mainly present in patients with CN-AML, and were mutually exclusive with all other type-II aberrations. No differences were found in WBC or sex between patients carrying any of these aberrations and those without the indicated type-II aberration. Patients with *NPM1*-mutated AML were significantly older (median 11.0 years; *P*=0.03) compared to their wild-type counterparts (Table 1B).

In 180/506 (36%) cases, neither one of the type-II gene mutations in *NPM1*, *CEBPA* and *MLL*, nor one of the 'fusion gene' type-II aberrations, i.e. *MLL*-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22)/t(16;16)(p13;q22), t(15;17)(q22;q21), t(7;12)(q36;p13) and t(6;9)(p23;q34), were identified (Figure 1A). This percentage, however, is probably slightly lower (estimated ~33%) as we could only screen 118 of these 180 cases for *NPM1*, and 101 of the 180 cases for *CEBPA* and *MLL*-PTD mutations (*Online Supplementary Table S5*).

Characteristics of pediatric AML patients with type-I aberrations

For the classical type-I aberrations, i.e. mutations in *FLT3*, *N-* and *K-RAS*, *PTPN11* and *KIT*, the following frequencies were found: *FLT3*-ITD (18%; 67/372), *FLT3*-TKD (3%; 9/330), *N-RAS* (16%; 57/353), *K-RAS* (4%; 13/353), *PTPN11* (2%; 7/350) and *KIT* (8%; 31/368) (Table 1B). *WT1* mutations, which in this study were arbitrarily categorized as type-I aberrations, were found in 9% (29/330). Together, we identified type-I aberrations in 185/330 (56%) cases (Figure 1B). As far as sex distribution of the different type-I aberrations is concerned, *K-RAS* mutations were significantly associated with male sex (85% *vs.* 54% of *K-RAS* wild-type cases; *P*=0.03). *FLT3*-ITD-positive and *WT1*-mutated AML cases had a significantly higher WBC (median 74×10^9/L; *P*<0.001, and 86×10^9/L; *P*=0.03, respectively) compared to their wild-type counterparts. Patients with *FLT3*-ITD-positive AML had a significantly higher median age (10.1 years; *P*=0.008) compared to their wild-type counterparts (Table 1B).

Non-random associations between type-I and type-II aberrations in pediatric AML

An overview of the associations between the type-I and

Table 2A. Univariate analysis for survival parameters of pediatric AML.

	pEFS			pOS		
	Hazard Ratio	95% CI	*P* value	Hazard Ratio	95% CI	*P* value
Type-II aberration						
MLL-rearrangement	1.3	1.0-1.8	0.06	1.4	1.0-2.0	0.05
t(8;21)(q22;q22)	0.7	0.4-1.0	0.05	0.4	0.2-0.8	0.005
inv(16)(p13q22)	0.2	0.1-0.4	<0.001	0.1	0.0-0.4	0.001
t(15;17)(q22;q21)	0.7	0.3-1.3	0.24	0.7	0.3-1.7	0.44
NPM1 mutation	0.7	0.4-1.2	0.19	0.8	0.4-1.7	0.61
CEBPA double mutation	0.5	0.2-1.2	0.13	0.3	0.1-1.2	0.10
Type-I aberration						
FLT3-ITD	1.3	0.9-1.9	0.17	1.3	0.9-2.0	0.21
WT1 mutation	2.1	1.3-3.4	0.002	2.0	1.2-3.5	0.01
WT1 mutation & *FLT3*-ITD	1.1	1.0-1.1	0.009	1.1	1.0-1.2	0.007
RAS-pathway mutation	1.0	0.7-1.4	0.89	0.8	0.5-1.4	0.47
KIT mutation	0.6	0.4-1.1	0.11	0.6	0.3-1.3	0.18
WBC						
>50×10^9/L	1.4	1.0-1.8	0.03	1.6	1.2-2.3	0.006
Age						
≥2 years	0.9	0.7-1.3	0.72	0.8	0.6-1.2	0.37
≥10 years	0.9	0.7-1.1	0.31	0.9	0.6-1.2	0.47

pEFS: probability of event-free survival; pOS: probability of overall survival; WBC: white blood cell count at diagnosis.

Table 2B. Multivariate analysis for survival parameters of pediatric AML.

	pEFS			pOS		
	Hazard Ratio	95% CI	*P* value	Hazard Ratio	95% CI	*P* value
Type-II aberration						
Favorable karyotype	0.3	0.2-0.5	<0.001	0.2	0.1-0.5	<0.001
NPM1 mutation	0.4	0.2-0.9	0.02	0.6	0.3-1.4	0.21
CEBPA double mutation	0.3	0.1-0.8	0.02	0.2	0.1-0.9	0.03
MLL-rearrangement	1.2	0.7-1.9	0.58	1.5	0.9-2.6	0.16
Type-I aberration						
FLT3-ITD	1.2	0.8-2.0	0.40	1.3	0.7-2.3	0.43
WT1 mutation	1.7	0.8-3.3	0.14	1.7	0.8-3.8	0.17
WT1 & *FLT3*-ITD	1.0	0.9-1.1	0.62	1.0	0.9-1.1	0.78
WBC						
>50×10^9/L	1.2	0.8-1.7	0.40	1.5	0.9-2.3	0.09
Age						
≥10 years	1.2	0.9-1.8	0.27	1.3	0.8-1.9	0.32

pEFS: probability of event-free survival; pOS: probability of overall survival; WBC: white blood cell count at diagnosis; favorable karyotype includes t(8;21)(q22;q22), inv(16)(p13q22) and t(15;17)(q22;q21).

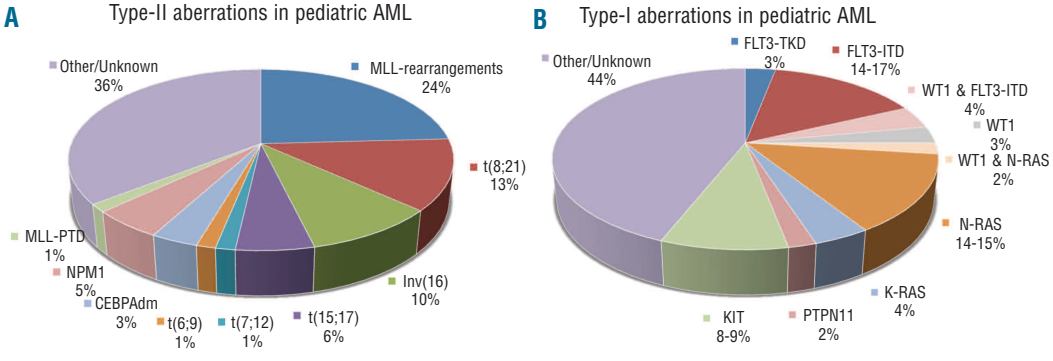

Figure 1. Distribution of the different type-I and type-II aberrations in pediatric AML. The heterogeneity of pediatric AML is reflected by the presence of the different type-I and type-II genetic aberrations. However, in a large number of cases the type-II (A) or type-I (B) aberrations have not yet been identified.

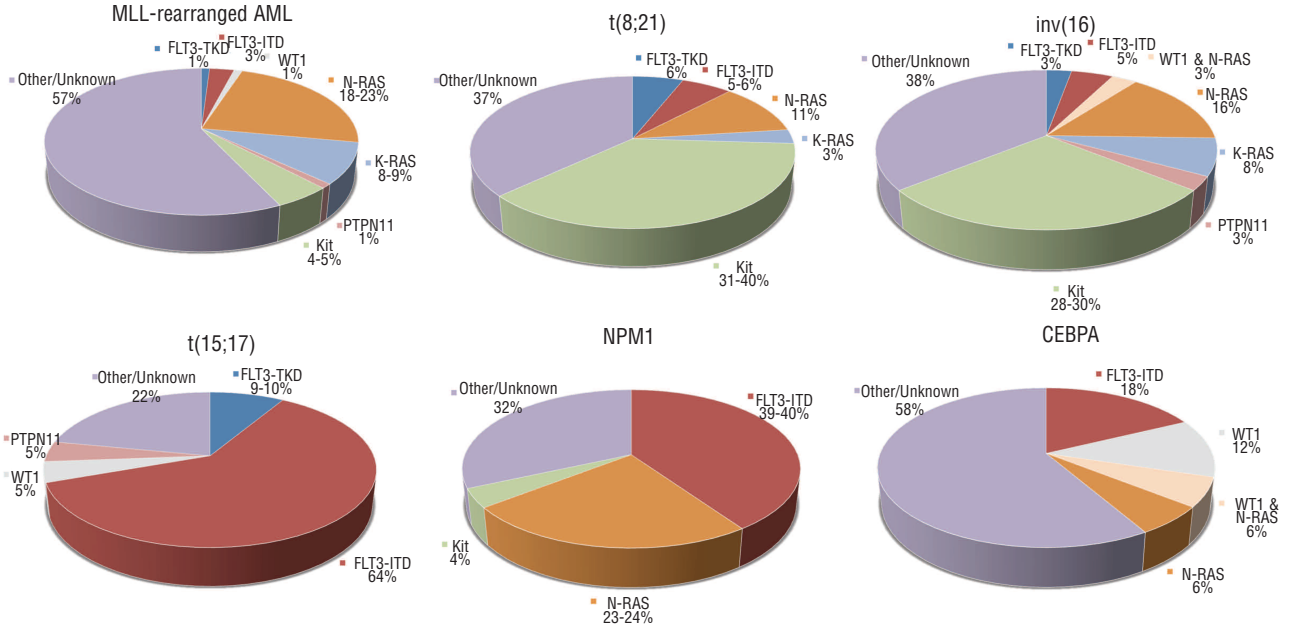

Figure 2. **Type-I aberrations per type-II defined subtype. Distribution of the different type-I aberrations according to the different type-II defined subtypes including more than 10 cases, i.e. *MLL*-rearrangements, t(8;21), inv(16), t(15;17), *NPM1*-mutated and *CEBPA* double mutated AML.**

type-II aberrations is presented in the *Online Supplementary Table S5*. Although *FLT3*-ITD mutations were identified in almost all type-II defined subtypes, the majority (42%) were restricted to cases with t(15;17)(q22;q21), *MLL*-PTD and *NPM1* mutations, in which 64%, 57% and 39%, respectively, harbored an *FLT3*-ITD (Figure 2). It is interesting to note that *FLT3*-ITD was also simultaneously present in 41% of the *WT1*-mutated cases. For *FLT3*-TKD mutations, which were far less frequent in pediatric AML, the highest frequencies were found in the *MLL*-PTD (17%) and t(15;17)(q22;q21) (10%) subtypes.

KIT mutations clearly associated with core-binding factor AML (CBF-AML), i.e. t(8;21)(q22;q22) and inv(16)(p13q22) /t(16;16)(p13;q22) (P<0.001). They were observed in 31% of t(8;21)(q22;q22) cases and in 28% of the inv(16)(p13q22) /t(16;16)(p13;q22) cases (Figure 2).

The *N-RAS*, *K-RAS* and *PTPN11* gene mutations, combined together as RAS-pathway activating mutations, showed an equal distribution among the patients with different type-II aberrations. The exception was the t(15;17)(q22;q21) subtype, in which no RAS-pathway mutations were observed, except for only one *PTPN11* mutation. Interestingly, *K-RAS* mutations, which were four times less frequent than *N-RAS* mutations, were more frequently seen in *MLL*-rearranged AML and in CBF-AML, while *N-RAS* mutations were most prevalent in t(6;9)(p23;q34) and *NPM1*-mutated AML. In *MLL*-rearranged AML, 43% carried one of the investigated type-I aberrations, of which the majority were RAS-pathway aberrations (33%; Figure 2).

WT1 mutations were mainly present in t(6;9)(p23;q34)-AML (33%), *CEBPA* double-mutant AML (18%) and the subtype with 'other/unknown' type-II aberrations (20%).

The distribution of genetic aberrations is highly correlated with age in pediatric AML

We investigated the frequency of genetic subtypes, according to the age categories 0-2 years, 2-5 years, 5-10 years, 10-15 years, and 15 years and older (*Online Supplementary Figure S1A-B*). The largest differences in genetic aberrations were detected between children under two years of age and children two years of age and over. We, therefore, focused further on these 2 age groups (*Online Supplementary Figure S1C-D*). In children under two years of age, significantly higher frequencies of *MLL*-rearrangements and complex karyotypes were observed when compared with children two years of age and over: 51% *versus* 18%; P<0.001, and 13% *versus* 4%; P=0.001, respectively. Furthermore, the youngest age category included all 7 patients with a t(7;12)(q36;p13) (P<0.001). In contrast, in this age category t(8;21)(q22;q22) was not observed, but this translocation was found in 15% (P<0.001) of children two years of age and over. The t(15;17)(q22;q21) was only present in a single case under two year of ages (1.9 years; 1%) but occurred significantly more often in children two years of age and over (7%; P=0.04). Furthermore, in children two years and over, a higher frequency of CN-AML was found compared with children under two years of age (respectively 19% *vs.* 6%, P=0.002) (*Online Supplementary Figure S1C*). *NPM1*, *CEBPA* and MLL-PTD aberrations were not detected in any of the patients under two years of age. In both age categories (<2 years and ≥2 years) the percentage of 'other/unknown' type-II aberrations was about one-third that of the AML, but there was a clear difference in the distribution of the different type-II aberrations (*Online Supplementary Figure S2A*).

In children two years of age and over, a significantly higher frequency of *FLT3*-ITD was found (21% *vs.* 3% in children <2 years; P=0.001), and a trend was observed for a higher frequency of *WT1* mutations in patients two years of age and over (10% *vs.* 2% in children <2 years; P=0.06). Furthermore, all *FLT3*-TKD (n=9) and *K-RAS* mutations (n=13) were found in children two years of age

and over, although this did not reach statistical significance. In contrast, both age categories included almost similar frequencies of *N-RAS* (15-20%), *PTPN11* (2-3%) and *KIT* mutations (5-10%) (*Online Supplementary Figures S1D and S2B*). When *RAS*-pathway aberrations were taken together, this pathway was affected at a similar frequency in both age categories (22% *vs*. 21% in children < 2 years and ≥2 years of age, respectively). In children under two years of age, 67% of the cases did not harbor one of the investigated type-I aberrations (i.e. *FLT3*-ITD, *FLT3*-TKD, *NRAS*, *KRAS*, *PTPN11*, *KIT* or *WT1*) *versus* only 40% in children two years of age and over ($P<0.001$). The difference between these age categories could largely be explained by the frequency of *FLT3*-ITD, which was only sporadically found in children under two years of age (*Online Supplementary Figure S2B*).

Clinical outcome of pediatric AML according to type-I and type-II aberrations

Survival analysis was only performed for the type-I and type-II defined subtypes containing more than 10 cases. For the type-II aberrations, this included *MLL*-rearrangements, t(8;21)(q22;q22), inv(16)(p13q22)/t(16;16)(p13;q22), t(15;17)(q22;q21), as well as *NPM1*- and *CEBPA*-double mutated cases. All other cases were grouped together and considered 'other/unknown' type-II aberrations (Table 2A). The Kaplan-Meier curves showed large differences between the different type-II aberrations for 5-year pOS, 5-year pEFS, and 5-year CIR (Figure 3A-C). Patients carrying an inv(16)(p13q22)/t(16;16)(p13;q22)-AML showed the most favorable outcome with a 5-year pOS, 5-year pEFS, and 5-year CIR of 97±3%, 76±8% and 19±7%, respectively. MLL-rearranged AML and the group with 'other/unknown' type-II aberrations showed the worst outcome with 5-year pOS of 56±5% and 40±5%, respectively, 5-year pEFS of 38±5% and 26±4%, respectively, and 5-year CIR of 38±5% and 51±5%, respectively. Interestingly, cases with a t(8;21)(q22;q22) had a relatively high 5-year CIR of 47±8%; this seemed to be related to concurrent *KIT* mutations, although numbers were too small to draw definitive conclusions. Cases with a t(15;17)(q22;q21) only had a 5-year CIR of 7±7%. For survival analysis of the type-I aberrations, cases with various RAS-pathway aberrations were combined. *WT1*-mutated AML cases were analyzed according to their *FLT3*-ITD status. All other cases were grouped together as 'other/unknown' type-I cases for the analysis. The Kaplan-Meier curves showed differences in 5-year pOS, 5-year pEFS, and 5-year CIR for the different type-I aberrations (Figure 3D-F). Cases with a combined *WT1* mutation and *FLT3*-ITD showed the worst prognosis with 5-year pOS of 22±14%, 5-year pEFS of 20±13%, and 5-year CIR of 50±17%.

Independent prognostic factors in pediatric AML

In order to reduce the number of variables in a multivariate Cox's proportional hazard model, CBF-AML and t(15;17)(q22;q21) were grouped together as the variable 'favorable karyotype' (Table 2B). The following variables, which were significant in univariate analyses and/or commonly used in pediatric AML (WBC>50×10^9/L and age >10 years), entered the model: 'favorable karyotype', *MLL*-rearrangements, *NPM1* mutations, *CEBPA* double mutations, *FLT3*-ITD, *WT1* mutations, the combination of *WT1* mutation plus an *FLT3*-ITD, WBC and age over ten years. This model identified favorable karyotype (hazard ratio (HR) 0.3, $P<0.001$), *NPM1* mutations (HR 0.4, $P=0.02$) and CEBPA double mutations (HR 0.3, $P=0.02$) as independent prognostic factors for pEFS. For pOS, favorable karyotype was an independent prognostic factor (HR 0.2, $P<0.001$). Furthermore, *CEBPA* double mutations (HR 0.2, $P=0.03$) independently predicted favorable pOS.

Discussion

Unraveling the genetics of pediatric AML provides a basis on which to improve risk group stratification. Furthermore, specific genetic aberrations could direct the development of targeted therapy approaches. The low incidence of pediatric AML makes it difficult to describe the relevance of these aberrations, and published data often focus on only one specific aberration. This study describes for the first time a large cohort of pediatric AML cases characterized for various cytogenetic and molecular genetic aberrations, allowing the comprehensive study of non-random associations, and their correlation with clinical characteristics and outcome.

We confirmed the non-random associations previously described between the different types of aberrations, e.g. *KIT* mutations with CBF-AML and *FLT3*-ITD with t(15;17)(q22;q21).[36-37] Moreover, *FLT3*-ITD significantly associated with *NPM1*-mutated (39%) and with *WT1*-mutated AML (41%). It is interesting to note that the association of *FLT3*/ITD was not correlated with a specific type of *NPM1* or *WT1* mutation (*data not shown*). As AML is likely to result from a multistep pathogenesis, it is conceivable that *FLT3*/ITD and *WT1* mutations are associated with additional aberrations, and it has recently been shown that their combination is frequently present in the rare subtype of adult AML harboring *NUP98*-rearrangments.[38] Overall, the majority of type-I aberrations showed an unequal distribution over the different type-II defined subtypes. Although *MLL*-rearranged AML harbored one of the lowest frequencies of type-I aberrations (43%), mutations in the RAS-signaling pathway interestingly represented the vast majority in *MLL*-rearranged AML.

Striking differences in genetic subtypes were found between children younger and older than two years at diagnosis of AML. Very young children with AML were characterized by a high frequency of *MLL*-rearrangements (51%), as previously reported.[5] Furthermore, they were characterized by a higher frequency of complex karyotypes, the exclusive presence of t(7;12)(q36;p13), and low frequencies or even total absence of t(8;21)(q22;q22), t(15;17)(q22;q21) and CN-AML. Moreover, the increasing incidence of CN-AML in childhood is continued into adulthood, in which CN-AML is present in approximately 45% of AML cases, whereas *MLL*-rearrangements are rare in adult AML.[39] We did not observe any difference in outcome between the age categories under two years and over two years. A recent large German study showed in more detail that adolescents (13-21 years) had a slightly inferior outcome compared to younger children, but no difference was seen between infants (0-2 years) and young children (2-13 years).[40] Although this does not suggest that different treatment strategies based on age are of any benefit, it is conceivable that the biological differences may lead to different treatment strategies for these age categories in the future.

Besides the different frequencies of several cytogenetic aberrations between pediatric AML and adult AML (see above), type-II gene mutations also displayed different frequencies within pediatric AML as well as between pediatric and adult AML. *NPM1* mutations, *CEBPA* double mutations and *MLL*-PTD did not occur in children below the age of two years. In line with this observation, *NPM1* mutations and *MLL*-PTD are less frequent in pediatric compared to adult AML (5-8% and 1-3% *vs.* 35% and 3-6%, respectively). In contrast, *CEBPA* double mutations display relatively similar frequencies (3-6% *vs.* 4-10%) between children and adults. Regarding type-I aberrations, pediatric AML cases harbor less frequently *FLT3*-ITD and *FLT3*-TKD, but RAS-pathway aberrations (*PTPN11*, *N-RAS* and *K-RAS* mutations) and *KIT* mutations have comparable frequencies.[41] *WT1* mutations seem to occur at a higher frequency in pediatric *versus* adult AML: 8-12% *versus* approximately 5-7%, respectively.

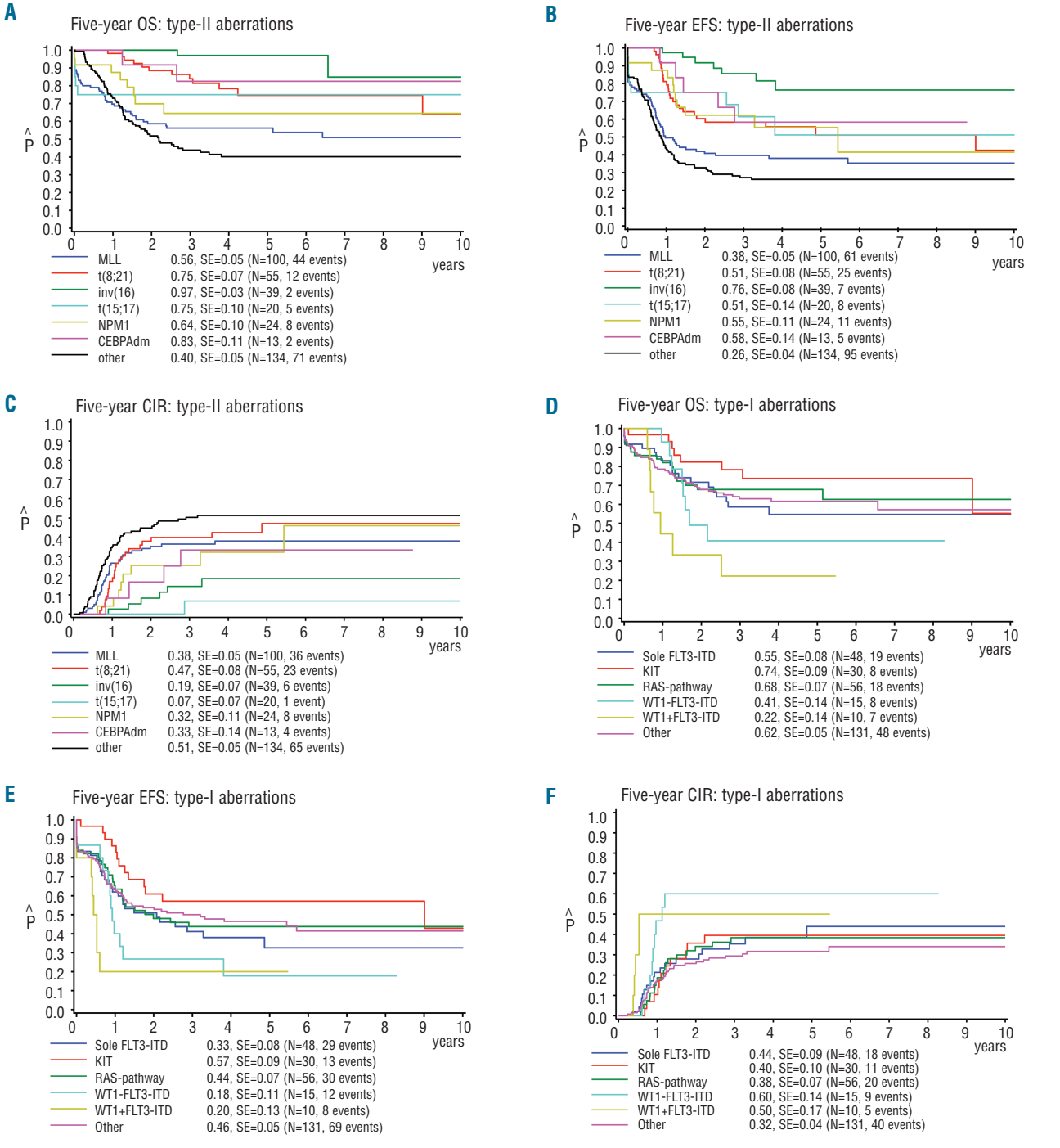

Figure 3. Survival analysis of the type-I and type-II aberrations in pediatric AML. Kaplan-Meier estimates for (A+D) pOS, (B+E) pEFS and (C+F) CIR for the different type-II and type-I aberrations, respectively.

Different type-I and type-II aberrations clearly had an impact on clinical outcome. In addition to the established favorable prognostic cytogenetic group including t(8;21)(q22;q22), inv(16)(p13q22)/t(16;16)(p13;q22), and t(15;17)(q22;q21), the type-II gene mutations *NPM1* and *CEBPA* double mutations were of independent prognostic relevance in pediatric AML when all prognostic factors were considered. From this, the established favorable cytogenetic risk group in pediatric AML can be extended with the molecular aberrations *NPM1* and *CEBPA* double mutations, and will now include approximately 35-40% of pediatric AML cases. *MLL*-rearrangements did not have any impact on clinical outcome, which is in agreement with our recent report that not *MLL*-rearrangements *per se*, but the specific *MLL*-translocation partners independently predict prognosis.[42] Regarding the type-I aberrations, *WT1* mutations and the combination of a *WT1* mutation and *FLT3*/ITD characterized poor prognostic subgroups in univariate analyses. These aberrations could not be shown to have independent prognostic significance in multivariate analyses, which might be influenced by the small numbers. We previously showed that this group with combined *FLT3*-ITD/*WT1* mutation had a dismal 5-year survival of 21%,[13] and this was confirmed by a large pediatric AML study from the COG.[12] *FLT3*-ITD did not have any prognostic value in our study, which might be influenced by the mutant/wild-type ratio. This has been shown to have a big influence on the prognostic impact,[16] or by its association with other favorable aberrations such as t(15;17)(q22;q21) and *NPM1* mutations. The investigation of the impact of the different type-I aberrations within specific subtypes of AML was restricted by small numbers, although *KIT* mutations seemed to be associated with the relatively high relapse rate in t(8;21)(q22;q22). However, in a large COG series, Pollard *et al.* recently showed that *KIT* mutations lacked prognostic significance in pediatric CBF-AML in contrast to adult CBF-AM.[43-44] This shows that further risk stratification in pediatric AML based on genetic aberrations has to be further validated by prospective pediatric studies.

Our study has implications for diagnostics in pediatric AML, and based on their frequency, impact on outcome, and possible target for therapy, we would currently suggest to screen for the fusion genes t(8;21)(q22;q22), inv(16)(p13q22)/t(16;16)(p13;q22), t(15;17)(q22;q21), and *MLL*-rearrangements [specifically t(6;11)(q27;q23), t(10;11)(p11.2/p12;q23) and t(1;11) (q21;q23)],[42] and for *NPM1*, *CEBPA*, *WT1* and *FLT3*/ITD mutations, as well as *KIT* mutations in CBF-AML.

Current pediatric AML treatment protocols consist of very intensive chemotherapy regimens which induce considerable toxicity. To further improve outcome in pediatric AML, new treatment strategies are needed. Different compounds targeting type-I aberrations are currently under development. The poor prognostic group combining an *FLT3*/ITD and a *WT1* mutation may potentially benefit from simultaneously targeting these aberrations. Activated *FLT3* can be targeted by compounds such as midostaurin, lestaurtanib, sorafenib, and other multi-targeted tyrosine kinase inhibitors.[45-46] However, so far monotherapy with these agents in adult AML has shown limited clinical activity.[47-48] In combination with chemotherapy, upregulation of FLT3-ligand might be a newly identified resistance mechanism.[49] Moreover, a recent randomized placebo-controlled trial of sorafenib did not show any benefit in patients in the experimental arm.[50] Therefore, we still need to see whether this strategy turns out to be successful. Compounds targeting *WT1* mutations are currently not available. Still, high expression of the *WT1* gene is found in most AML cases, and all *WT1*-mutated cases show high *WT1* expression.[51] Immunotherapy using a *WT1*-peptide vaccine is being developed, and a phase II trial in adult AML showed promising results.[52] In general, due to the different cooperating genetic events in AML, monotherapy, as with imatinib in CML (where a single fusion gene drives the disease), does not seem to be feasible,[45] and combinations of inhibitors may be required to efficiently kill the leukemic cells.

Intriguingly, in approximately 44% and 33-36% of pediatric AML cases, respectively, none of the investigated type-I or type-II aberrations were present. It needs to be mentioned that we may have missed mutations outside the screened hotspot regions, although from previous studies we expect them to be relatively rare. Furthermore, *RUNX1* mutations were not determined but a recent report suggests they are infrequent events in pediatric AML.[53] Over the last decade efforts have been made to identify the remaining genetic aberrations with high-throughput screening techniques, e.g. by genome-wide copy number analyses (using high resolution array-CGH and SNP-arrays), and by re-sequencing candidate genes such as all kinase-coding genes.[25, 54-56] Although the former led to the discovery of *ASXL1* and *TET2* mutations, it also revealed that AML harbored only a small number of genomic alterations compared with other cancers.[56] High-throughput sequencing of the first whole genomes of adult AML identified mutations in the metabolites *IDH1* and *IDH2*, and recently in the DNA methyltransferase gene *DNMT3A*, which both seemed frequent in adult AML.[57-58] Interestingly, in agreement with the hypothesis that AML results from a multistep pathogenesis, these aberrations might add an additional class of mutations as aberrant *TET2*, *IDH1* and -2 and *DNMT3A* have been shown to affect the epigenetic landscape of AML. Recent studies, however, indicated that these mutations might be rare in pediatric AML,[59-61] stressing the need for separate pediatric studies to discover the remaining genetic aberrations, including aberrations in miRNA-coding genes or in methylation of genes or their promoter regions.

In conclusion, the heterogeneity of pediatric AML is reflected by the presence of different age-dependent and clinically relevant genetic aberrations, allowing prognostically relevant groups to be identified. In addition, several non-random associations between genetic aberrations have been observed. The addition of these aberrations will help us to stratify pediatric AML and to direct further development towards targeted therapies.

Authorship and Disclosures

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