

MYELOID NEOPLASIA

CEBPA mutations in 4708 patients with acute myeloid leukemia: differential impact of bZIP and TAD mutations on outcome

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KEY POINTS

- **CEBPA^{smbZIP}- and CEBPA^{bi}-mutant AML share clinical and mutational characteristics and are distinct from CEBPA^{smTAD}-mutant AML.**
- **Only in-frame mutations in CEBPA-bZIP are associated with favorable clinical response in monoallelic and biallelic constellations.**

Biallelic mutations of the CEBPA gene (CEBPA^{bi}) define a distinct entity associated with favorable prognosis; however, the role of monoallelic mutations (CEBPAsm) is poorly understood. We retrospectively analyzed 4708 adults with acute myeloid leukemia (AML) who had been recruited into the Study Alliance Leukemia trials, to investigate the prognostic impact of CEBPAsm. CEBPA mutations were identified in 240 patients (5.1%): 131 CEBPA^{bi} and 109 CEBPAsm (60 affecting the N-terminal transactivation domains [CEBPA^{smTAD}] and 49 the C-terminal DNA-binding or basic leucine zipper region [CEBPA^{smbZIP}]). Interestingly, patients carrying CEBPA^{bi} or CEBPA^{smbZIP} shared several clinical factors: they were significantly younger (median, 46 and 50 years, respectively) and had higher white blood cell (WBC) counts at diagnosis (median, 23.7 × 10⁹/L and 35.7 × 10⁹/L) than patients with CEBPA^{smTAD} (median age, 63 years, median WBC 13.1 × 10⁹/L; *P* < .001). Co-mutations were similar in both groups: GATA2 mutations (35.1% CEBPA^{bi}; 36.7% CEBPA^{smbZIP} vs 6.7% CEBPA^{smTAD}; *P* < .001) or NPM1 mutations (3.1% CEBPA^{bi}; 8.2% CEBPA^{smbZIP} vs 38.3% CEBPA^{smTAD}; *P* < .001). CEBPA^{bi} and CEBPA^{smbZIP}, but not CEBPA^{smTAD} were associated with significantly improved overall (OS; median 103 and 63

vs 13 months) and event-free survival (EFS; median, 20.7 and 17.1 months vs 5.7 months), in univariate and multivariable analyses. Additional analyses revealed that the clinical and molecular features as well as the favorable survival were confined to patients with in-frame mutations in bZIP (CEBPA^{bZIP-inf}). When patients were classified according to CEBPA^{bZIP-inf} and CEBPA^{other} (including CEBPA^{smTAD} and non-CEBPA^{bZIP-inf}), only patients bearing CEBPA^{bZIP-inf} showed superior complete remission rates and the longest median OS and EFS, arguing for a previously undefined prognostic role of this type of mutation.

Introduction

Patients with normal karyotype represent the largest subgroup of patients with acute myeloid leukemia (AML).¹ Research performed during the past 20 years has revealed several novel abnormalities that are particularly common in this subgroup of patients (eg, activating mutations of the FLT3 receptor tyrosine kinase²⁻⁵ and mutations of the *NPM1* gene encoding the nucleophosmin protein.⁶⁻⁸) In addition, mutations of the gene encoding CCAAT/enhancer-binding protein- α (*CEBPA*) have been described in patients with AML and normal karyotype.⁹⁻¹² The *CEBPA* gene encodes a transcription factor that serves as a master regulator of granulopoiesis.¹³ Targeted disruption of the gene in mice is associated with a block of granulocyte development and downregulation of target genes such as granulocyte colony-stimulating factor receptor.¹³ The intronless *CEBPA* gene on chromosome 19q13.1 encodes 2 major protein isoforms, the 42-kDa full-length protein (p42), which has the full transcriptional activity, and a shorter, 30-kDa isoform (p30), produced from a second, alternative start codon and shown to have complex functions, including an inhibitory effect on⁹ and increased degradation¹⁴ of the p42 full-length protein.

Since the initial report,⁹ several groups have investigated *CEBPA* mutations in AML.^{11,12,15-18} Besides the reproducible association with certain morphological and clinical features (eg, FAB M1 and M2 morphology, high CD34 expression on blasts, and predominance in normal karyotype), nearly all studies have shown that patients with *CEBPA* mutations have a more favorable outcome. However, more recent results have indicated that a good prognosis is confined to patients with biallelic or double mutations (*CEBPA*^{bi}),¹⁹⁻²¹ whereas patients with monoallelic *CEBPA* mutations (*CEBPA*sm) did not differ in their response to treatment from patients with wild-type *CEBPA* (*CEBPA*^{wt}) and had a less favorable outcome.¹⁹⁻²¹ This finding led to the inclusion of biallelic *CEBPA* mutations as an independent entity in the most recent World Health Organization classification,²² as well as a favorable prognostic group in the ELN2017 recommendations.²³ However, the impact of monoallelic *CEBPA* mutations has been investigated in more detail in only a few studies, especially in light of the different biological effects of N- and C-terminal mutations.^{21,24,25}

To investigate the prevalence and prognostic role of *CEBPA* mutations, in particular *CEBPA*sm mutations, in adult patients with AML, we studied 4708 patients with newly diagnosed AML. In contrast to many previous studies, we included patients of all age groups, as well as secondary AML (sAML) after prior myelodysplasia (MDS) or therapy-related AML (tAML). Because most *CEBPA* mutations reported so far consist of insertions or deletions,²⁶⁻²⁹ we used high-resolution fragment analysis for mutation screening and found *CEBPA* mutations in 5.1% of patients. The results of targeted next-generation sequencing (NGS) indicated profound differences in the co-mutational spectrum of individual *CEBPA* mutations. Our results point to a differential effect of *CEBPA*sm mutations that appear to be associated with similar clinical parameters, co-mutations, and outcome compared with *CEBPA*^{bi} mutations. If confirmed, these results, generated in one of the largest cohorts of patients with AML analyzed so far, could build the basis of a refined clinical classification of *CEBPA* mutations.

Patients, materials, and methods

Patients

We screened 4708 adult patients with newly diagnosed AML (n = 3729 with de novo AML; n = 644 with AML and a history of MDS; and n = 335 with tAML) for the presence of mutations in the *CEBPA* gene. Most individuals (n = 3104; 67.45%) were treated in prospective studies, including the AML96 (n = 1457), AML2003 (n = 1081), AML60⁺ (n = 359), and SORAML (n = 207) protocols of the Study Alliance Leukemia (SAL). The remaining patients (n = 1604) were recruited to the SAL registry and biorepository. Detailed treatment protocols have been published³⁰⁻³³ and are summarized in the supplemental Data (available on the *Blood* Web site), including the number of patients treated in each protocol. All studies involved risk-stratified consolidation therapy according to cytogenetic risk groups (*CEBPA* mutations were not used for risk stratification in any of those studies). Patients <60 years of age received standard cytosine arabinoside (Ara-C)/anthracycline (DA 3 + 7)-based, double-induction chemotherapy followed by consolidation with high-dose Ara-C in patients who had favorable risk. Patients in the intermediate- and high-risk groups had the option of upfront allogeneic transplantation and underwent autologous transplantation or chemotherapy-based consolidation in the absence of suitable donors. In older patients treated with curative intention, the chemotherapy regimen was adjusted according to predefined algorithms that integrate performance status and organ function.

This study was approved by the ethics board of the Technical University Dresden. Each patient gave written informed consent to participate in the respective study protocols.

Patient samples

All materials investigated were obtained at the time of diagnosis. Bone marrow was used whenever available; in all other cases, peripheral blood samples were examined. Genomic DNA was extracted from mononuclear cells by using standard procedures (DNA blood minikit; Qiagen, Hilden, Germany).

PCR for *CEBPA*

All polymerase chain reaction (PCR) analyses were performed on genomic DNA. Details of the PCR primers and cycling conditions are given in supplemental Tables 1 and 2 and supplemental Figure 1.

PCR-amplified mutant samples were purified and sequenced on an ABI3130xl instrument. Sequences were compared with the WT-*CEBPA* messenger RNA (mRNA) sequence (U34070).

NGS-based characterization of co-mutations in patients with *CEBPA* mutations

Profiling of mutations was achieved by targeted NGS-based resequencing with the TruSight Myeloid assay (Illumina, Chesterford, United Kingdom) covering 54 genes frequently mutated in AML, as described recently³⁴ (details in the supplemental Data). Data alignment of demultiplexed FastQ files, variant calling, and filtering were performed with the Sequence Pilot software package (JSI Medical Systems GmbH, Ettenheim, Germany), with default settings and a 5% variant allele frequency cutoff. *FLT3*-internal tandem duplication (ITD) and *NPM1* mutations were evaluated as reported previously.^{5,8}

RNA sequencing

RNA sequencing (RNA-Seq) was performed on total RNA isolated at diagnosis from 20 patients with a *CEBPA* mutation (5 *CEBPA*^{bi-inf}, 5 *CEBPA*^{sm-inf}, 5 *CEBPA*^{sm-other}, and 5 *CEBPA*^{bi-other}), by using strand-specific RNA-Seq library preparation (Ultra II Directional RNA Library Prep; New England Biolabs) and sequenced on an Illumina NovaSeq 6000 instrument. The complete workflow and the bioinformatic analyses are detailed in the supplemental Data.

Statistical analysis

Clinical variables across groups were compared by using the χ^2 test or 2-sided Fisher's exact test for categorical variables. The nonparametric Mann-Whitney *U* test was applied for continuous variables. *P* < .05 indicated a significant difference. Numerical variables are expressed as the median with interquartile range (IQR). Univariate analyses for the influence of the *CEBPA* mutational status on complete response (CR) rates were performed by using the χ^2 test. The log-rank test was used to evaluate OS and EFS. For multivariable analysis of prognostic factors, Cox proportional hazards regression models were used. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) was modeled as a time-dependent covariate. *P*-values of association analyses of *CEBPA* mutations with clinical variables and other molecular abnormalities were adjusted for multiplicity by using the Bonferroni-Holm correction. All statistical analyses were performed with the SPSS software package, version 26 (SPSS, Chicago, IL) and R version 3.5.3 (<https://www.R-project.org/>).

Results

A total of 371 individual *CEBPA* mutations were identified in 240 of the 4708 patients (5.1%) by the screening procedure. In all patients with a mutation identified in any part of *CEBPA*, Sanger sequencing of the entire gene was performed to assess the presence of additional mutations, including single nucleotide variants, and to confirm the alterations identified using fragment analysis. Patients showing the previously described 6-bp polymorphism in TAD2²⁶ were included in the WT-*CEBPA* group.

As illustrated in supplemental Figure 2 and supplemental Table 3 and reported in previous studies, mutations largely clustered

in the N-terminal first transactivation domain (TAD1) and the DNA-binding and basic leucine zipper region (bZIP) in the C-terminal part of *CEBPA*. In our cohort, 131 of 240 patients (54.6%) presented with 2 *CEBPA* mutations, mostly consisting of combined mutations in bZIP and TAD, denoted as *CEBPA*^{bi}. *CEBPA*sm mutations were found in 109 of 240 patients (45.4%), of which 60 had N-terminal (*CEBPA*^{smTAD}) and 49 C-terminal (*CEBPA*^{smbZIP}) mutations. As reported before, most of the mutations in the TAD domains caused a frameshift, whereas mutations in the bZIP-region were predominantly in-frame insertions and duplications. Eight patients had monoallelic mutations with high variant allele frequency (4 *CEBPA*^{smTAD} and 4 *CEBPA*^{smbZIP}), indicating a homozygous state.

CEBPA mutations and clinical characteristics

The association of clinical parameters according to the localization of the *CEBPA* mutation (ie, patients with single N- or C-terminal mutations and patients with biallelic mutations) is summarized in Table 1. Compared with patients bearing *CEBPA*^{wt}, those with *CEBPA*^{bi} were significantly younger (median age, 46 years; IQR, 38-59) at diagnosis, similar to those with *CEBPA*^{smbZIP} (median age, 50 years; IQR, 39-57), whereas patients with *CEBPA*^{smTAD} were significantly older (median age, 63 years; IQR, 55-69.3) and were more comparable to the *CEBPA*^{wt} group (median, 57 years; IQR, 46-67; *P* < .001). When patients were categorized in 10-year age intervals (Figure 1), a continuous decrease in *CEBPA*^{bi} and *CEBPA*^{smbZIP} mutations was seen with increasing age, whereas *CEBPA*^{smTAD} alterations increased. In line with this finding, only a single patient with a *CEBPA*^{smTAD} mutation was observed in the group of patients <30 years of age.

As outlined in Table 1, patients with *CEBPA*^{smTAD} also had significantly lower WBC counts and significantly lower rates of CD34 positivity, compared with the *CEBPA*^{bi} and *CEBPA*^{smbZIP} groups, and more commonly had prior myelodysplasia syndrome (MDS) or tAML that evolved as a secondary disease.

Association of CEBPA mutations with other molecular abnormalities

Based on the targeted NGS approach, additional mutations were identified in 208 of 240 patients with a *CEBPA*

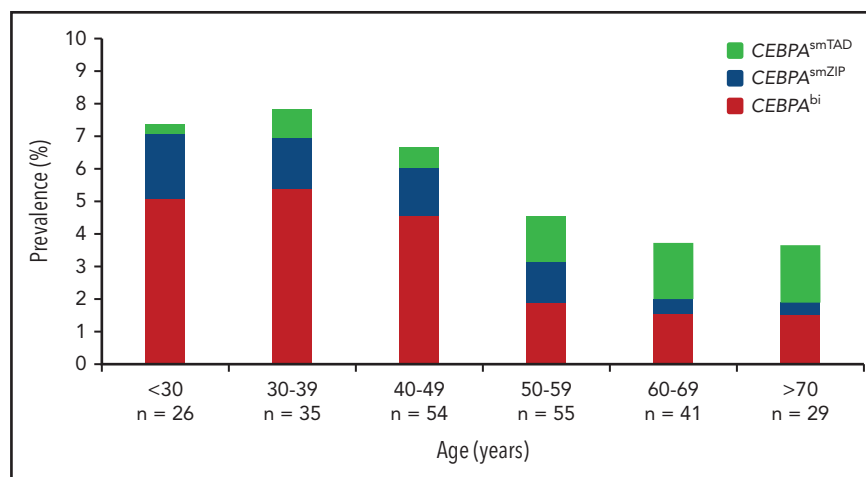


Figure 1. Age distribution of the 240 *CEBPA* mutations (*CEBPA*^{bi}, *CEBPA*^{smTAD}, *CEBPA*^{smbZIP}) identified in this study.

Table 1. Clinical and cytogenetic characteristics *CEBPA*^{wt} compared with *CEBPA*^{bi}, *CEBPA*^{smbZIP} and *CEBPA*^{smTAD} mutant patients

	<i>CEBPA</i> ^{wt} n = 4468	<i>CEBPA</i> ^{smTAD} n = 60	<i>CEBPA</i> ^{smbZIP} n = 49	<i>CEBPA</i> ^{bi} n = 131	P (adj.)
Age, y, median (IQR)	57 (46-67)	63 (55-69)	50 (39-57)	46 (38-59)	<.001
Sex, n (%)					
Female	2170 (49)	32 (53)	16 (33)	70 (53)	.26
Male	2298 (51)	28 (47)	33 (67)	61 (47)	
AML type, n (%)					
de novo	3510 (78)	50 (84)	47 (96)	122 (93)	.001
sAML	630 (14)	8 (13)	2(4)	4 (3)	
tAML	328 (7)	2 (3)	—	5 (4)	
Laboratory, median (IQR)					
BM blasts, %	60 (38-80)	60 (43.3-72.8)	65.3 (43.9-80)	64 (50-87)	.823
CD34-positivity, %	16 (2-47)	19.2 (3.8-65)	45 (19.5-68)	55.6 (32.9-77)	<.001
WBC, ×10 ⁹ /L	11.7 (3-45.7)	13.2 (3.3-51.2)	35.7 (11.5-93)	23.7 (9.3-64.7)	<.001
LDH, U/L	412.9 (272-727)	412 (291-569)	491 (344-941)	445 (292-679)	.99
FAB, n (%)					
M0	223 (5)	—	1 (2)	—	<.001
M1/M2	2040 (46)	40 (67)	39 (80)	101 (77)	
M4-M7	1535 (34)	15 (25)	8 (16)	19 (15)	
Unknown	670 (15)	5 (8)	1 (2)	10 (8)	
Cytogenetics, n (%)					
Normal karyotype	1923 (43)	37 (62)	34 (72)	102 (78)	<.001
Aberrant karyotype	2359 (53)	23 (38)	15 (28)	29 (22)	
Unknown	186 (4)	—	—	—	
Risk, n (%)					
Favorable risk (MRC)	353 (8)	1 (2)	1 (2)	—	<.001
Intermediate risk (MRC)	2928 (68)	54 (90)	43 (92)	119 (98)	
Adverse risk (MRC)	1001 (23)	5 (8)	3 (6)	2 (2)	
Treatment, n (%)					
Primary allo-HCT	747 (17)	3 (5)	10 (20)	36 (27)	<.001
Salvage allo-HCT	915 (20)	13 (22)	8 (16)	22 (17)	.189

Bold P-values indicate statistically significant results.

BM, bone marrow; FAB, French-American-British; LDH, lactate dehydrogenase; MRC, medical research council.

mutation (86.7%). The most frequently mutated genes were *TET2* (70 of 240; 29.2%), *GATA2* (68 of 240; 28.3%), *DNMT3A* (45 of 240; 18.8%), *FLT3-ITD* (39 of 240; 16.3%), *NPM1* and *NRAS* (31 of 240; 12.9% each), and *WT1* (30 of 240; 12.5%).

Figure 2 illustrates the distribution of co-mutations in the 3 *CEBPA* subgroups. Significant differences were observed for several genes, the most striking being *GATA2*, which was mutated in 35.1% of patients with *CEBPA*^{bi}, 36.7% of those with *CEBPA*^{smbZIP}, but in only 6.7% of those with *CEBPA*^{smTAD} ($P = .001$), and *NPM1*, which was mutated in 3.1% of patients with

CEBPA^{bi} and 8.2% of those with *CEBPA*^{smbZIP}, but in 38.3% of patients with *CEBPA*^{smTAD} ($P < .001$). Significant differences were also found for mutations in *DNMT3A*, *FLT3-TKD*, *IDH1*, and *IDH2*, as well as in *SRSF2* and *WT1* (Figure 2B). In general, the spectrum of mutations of *CEBPA*^{smbZIP} was more comparable to that of patients with *CEBPA*^{bi} and differed markedly from the *CEBPA*^{smTAD} group, the latter being more similar to patients with *CEBPA*^{wt}, who frequently carried mutations in genes associated with AML after prior MDS, such as spliceosome mutations (ie, *SRSF2*, *SF3B1*, or *U2AF1*) and alterations associated with DNA methylation (ie, *DNMT3A*, *ASXL1*, *TET2*, *IDH1*, and *IDH2*). Interestingly, only 7 patients in this analysis

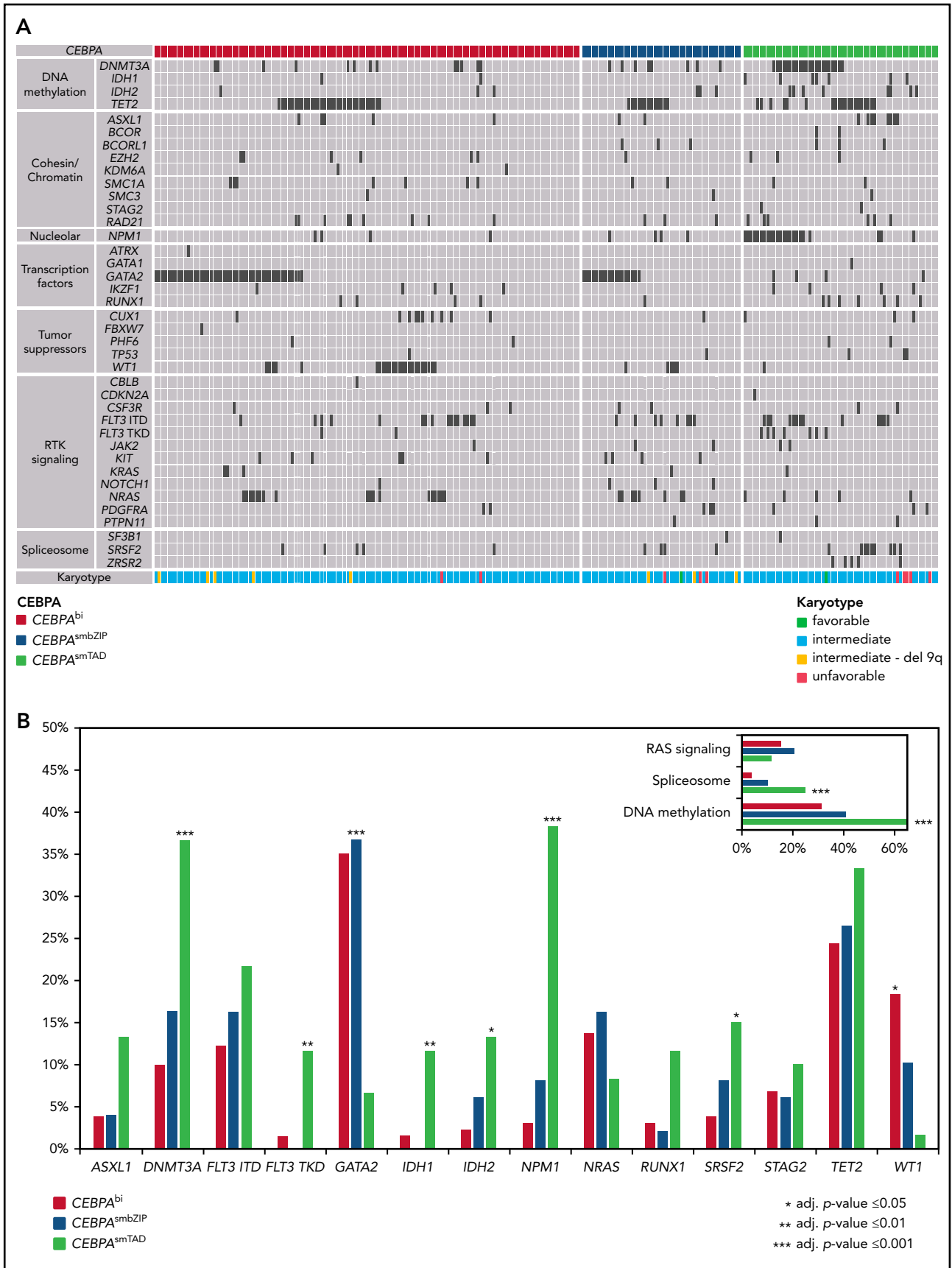


Figure 2.

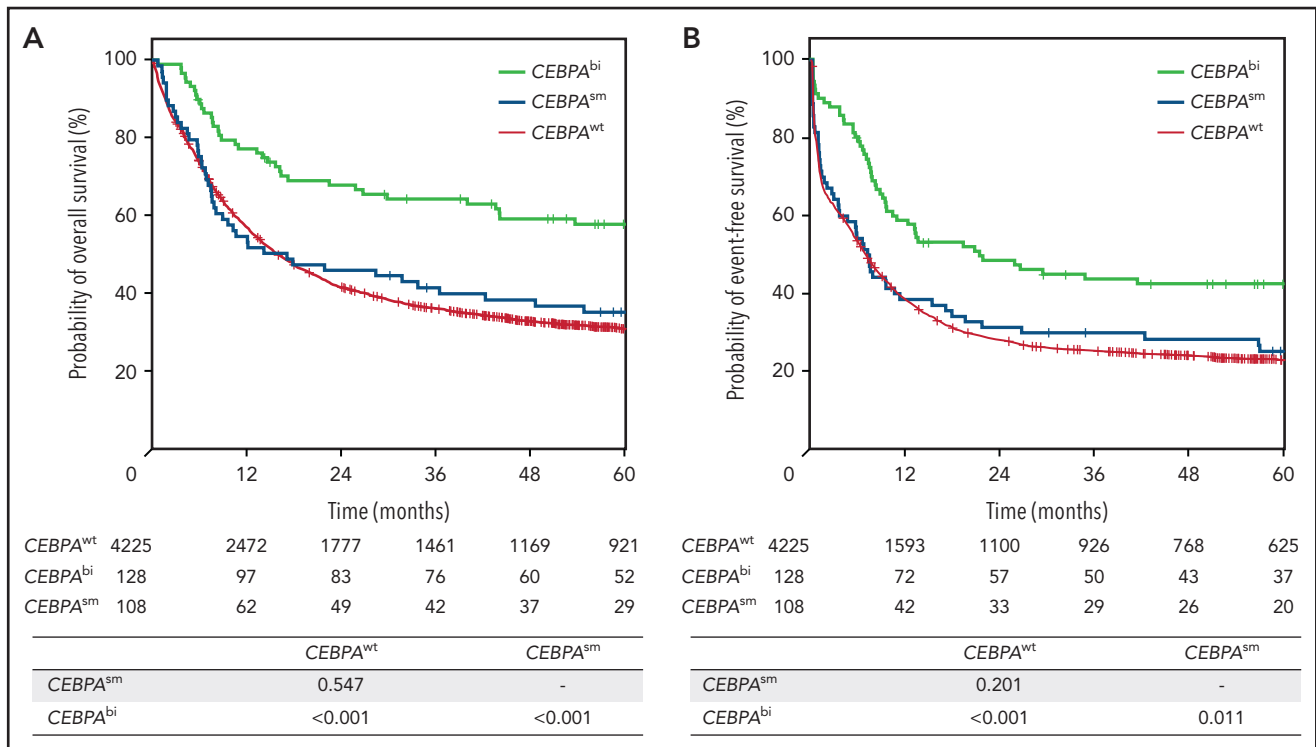


Figure 3. Survival analysis according to CEBPA mutation status (CEBPA^{wt}, CEBPA^{bi}, and CEBPAsm) within the cohort of 4461 patients receiving intensive treatment. Kaplan-Meier plots showing OS (A) and EFS (B). Numbers below the figures denote the patients at risk. The tables provide the results of pairwise univariate analyses.

had mutations in *CSF3R*, with no significant differences found between the groups (3.1% CEBPA^{bi}, 2% CEBPA^{smbZIP}, and 3.3% CEBPA^{smTAD}).

CEBPA mutations and outcome

The prognostic relevance of CEBPA mutations was analyzed in 4461 intensively treated patients, with a median follow-up time of patients remaining alive of 61 months (IQR, 36-96 months). When analyzed according to the current recommendations (ie, separating only CEBPA^{bi} and CEBPAsm), only patients with CEBPA^{bi} had a significantly higher rate of complete remission (CR; CEBPA^{bi}, 95% vs CEBPAsm, 75% and CEBPA^{wt}, 73%; $P < .001$), as well as a higher median OS (CEBPA^{bi}, 103.2 months; 95% confidence interval [CI], 70.7-inf vs CEBPAsm, 21.9 months; 95% CI, 12.7-54, and CEBPA^{wt}, 19.3 months; 95% CI, 17.9-21; $P < .001$) and EFS (CEBPA^{bi}, 20.7 months; 95% CI, 13.1-101.1 vs CEBPAsm, 9.4 months; 95% CI, 5.7-15.3, and CEBPA^{wt}, 7.0 months; 95% CI, 6.5-7.6; $P < .001$; Figure 3), which was confirmed in multivariable analyses (Table 2).

Given the marked differences in the clinical and molecular alterations associated with the different localization of single-mutant CEBPA, we also looked for the impact of the localization of CEBPAsm on outcome. Patients carrying CEBPA^{smbZIP} showed a

significantly higher CR rate (CEBPA^{bi} 95% vs CEBPA^{smbZIP} 86% vs CEBPA^{smTAD} 66% vs CEBPA^{wt} 73%) and a significantly longer OS (CEBPA^{bi} 103.2 months; 95% CI, 70.7-inf, vs CEBPA^{smbZIP}, 63.3 months; 95% CI, 20.5-inf, vs CEBPA^{smTAD}, 12.7 months; 95% CI, 7.5-36.5, and CEBPA^{wt}, 17.9 months; 95% CI, 17.9-21) and EFS (CEBPA^{bi}, 20.7 months; 95% CI, 13.1-101.1 vs CEBPA^{smbZIP}, 17.1 months; 95% CI, 8.3-inf vs CEBPA^{smTAD}, 5.7 months; 95% CI, 2.6-10, and CEBPA^{wt}, 7.0 months; 95% CI, 6.5-7.6; Figure 4A-B). Multivariable analysis confirmed that CEBPA^{smbZIP} mutations represented an independent favorable risk for outcome (Table 3).

Effect of co-mutations on outcome

We compared the effect of co-mutations on the survival of the patients according to the mutational status of the 3 most common mutations previously associated with outcome (ie, *GATA2*, *TET2*, and *WT1* in the 3 CEBPA mutation subgroups (CEBPA^{bi}, CEBPA^{smbZIP}, and CEBPA^{smTAD}). Although the presence of *GATA2* mutations was associated with improved OS and EFS in the CEBPA^{bi} and CEBPA^{smbZIP} groups (Figure 5A-B); however, these differences were not significant in pairwise comparisons (all P -values were adjusted for multiple testing with the Bonferroni-Holm procedure). However, for *TET2*^{mut} (Figure 5B-C) a significant difference was found between CEBPA^{bi}/TET2^{wt}

Figure 2. Alignment of additional gene mutations for 240 patients with CEBPA^{mut}. (A) Co-mutations organized by categories of related genes, as labeled on the left. Patients are shown in order by CEBPA subgroup. The heat map includes all mutated genes in patients with CEBPA^{bi}, CEBPA^{smbZIP}, or CEBPA^{smTAD}. Each column represents one of the 240 analyzed samples. Mutations in the investigated genes are shown by black bars, light gray bars indicate WT status. (B) Frequency distribution of additional gene mutations identified in patients with CEBPA^{bi}, CEBPA^{smbZIP}, or CEBPA^{smTAD} mutations (frequency of at least 10% in 1 subgroup). RAS signaling including *KRAS*, *NRAS*, *PTPN11*, and *CBLB*; spliceosome, including *SF3B1*, *SRSF2*, and *ZRSR2*; and methylation, including *DNMT3A*, *IDH1*, *IDH2*, and *TET2*.

Table 2. Results of the multivariable analysis for CEBPA^{bi} vs CEBPAsm

	OS (HR)	P	95% CI	EFS (HR)	P	95% CI	CR1 (OR)	P	95% CI
Intermediate karyotype	1			1			1		
Favorable karyotype	0.427	<.001	(0.35-0.52)	0.400	<.001	(0.34-0.47)	3.063	<.001	(2.07-4.54)
Adverse karyotype*	1.858	<.001	(1.68-2.05)	1.633	<.001	(1.49-1.79)	0.477	<.001	(0.40-0.57)
Age	1.032	<.001	(1.03-1.04)	1.023	<.001	(1.02-1.03)	0.948	<.001	(0.94-0.95)
Log ₁₀ WBC	1.155	<.001	(1.08-1.24)	1.228	<.001	(1.15-1.31)	0.754	<.001	(0.66-0.87)
De novo AML	1			1			1		
sAML	1.086	.131	(0.98-1.21)	1.069	.196	(0.97-1.18)	0.733	.002	(0.60-0.90)
tAML	1.386	<.001	(1.20-1.60)	1.098	.186	(0.96-1.26)	0.691	.009	(0.52-0.91)
No FLT3-ITD ^{mut}	1			1			1		
FLT3-ITD ^{mut}	1.206	<.001	(1.09-1.33)	1.242	<.001	(1.14-1.36)	1.017	.870	(0.83-1.25)
No NPM1 ^{mut}	1			1			1		
NPM1 ^{mut}	0.629	<.001	(0.57-0.69)	0.535	<.001	(0.49-0.59)	2.127	<.001	(1.75-2.59)
CEBPA ^{wt}	1			1			1		
CEBPA ^{bi}	0.510	<.001	(0.39-0.67)	0.534	<.001	(0.42-0.68)	6.328	<.001	(2.73-14.67)
CEBPA sm	0.825	.122	(0.65-1.05)	0.746	.011	(0.56-0.94)	1.117	.644	(0.70-1.78)
No allo-HSCT in CR1	1			1			1		
Allo-HSCT in CR1	0.773	<.001	(0.69-0.87)	0.630	<.001	(0.56-0.79)	—	—	—

The multivariable analysis performed includes the different study regimens as strata.

*Adverse karyotype according to ELN 2017.

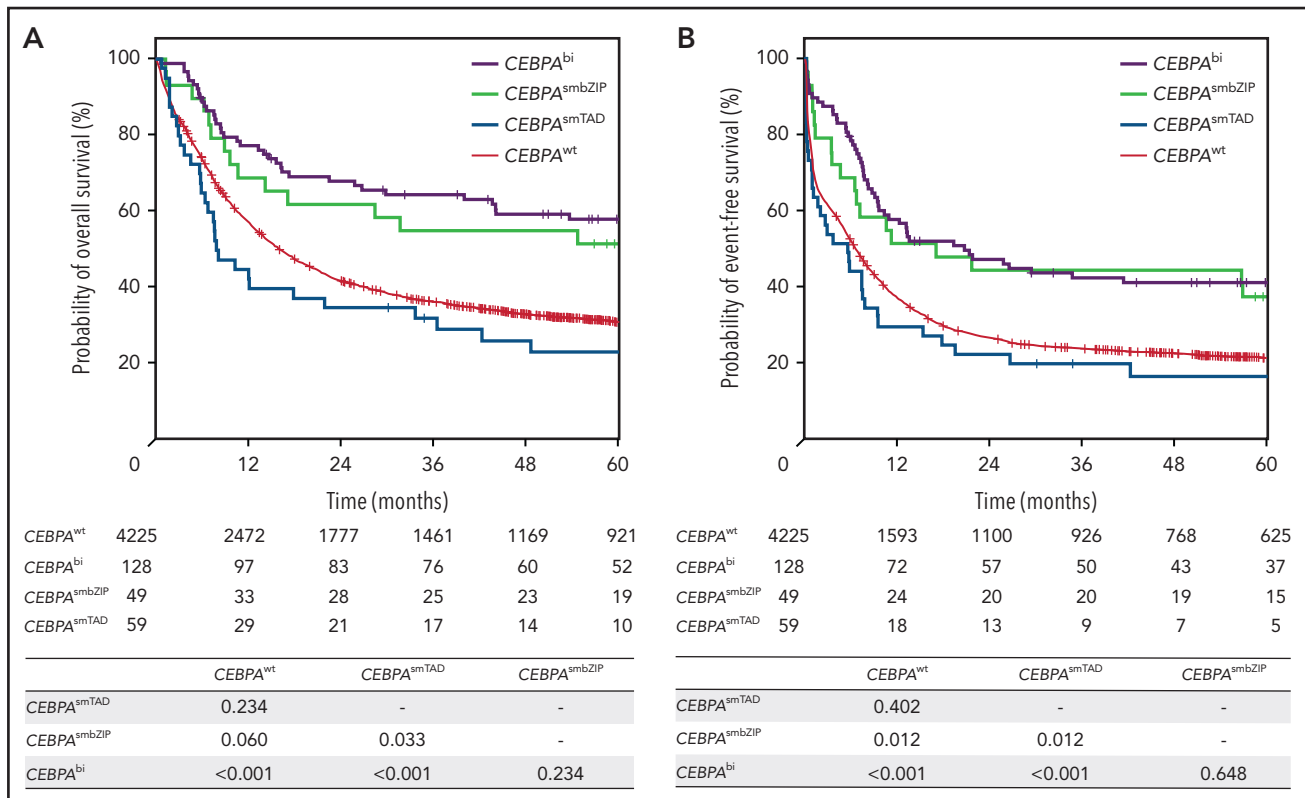


Figure 4. Survival analysis according to CEBPA mutation status (CEBPA^{bi}, CEBPA^{smTAD}, CEBPA^{smbZIP}, and CEBPA^{wt}) within the cohort of 4461 patients receiving intensive treatment. Kaplan-Meier plots showing OS (A) and EFS (B). Numbers of patients at risk and results of log-rank tests from pairwise comparisons are provided below the x-axis.

and *TET2*^{mut} (median OS: CEBPA^{bi}/*TET2*^{wt}, not reached; 95% CI, 101.1-inf, vs CEBPA^{bi}/*TET2*^{mut}, 22.5 months; 95% CI, 13.9-inf; *P* = .012; median EFS: CEBPA^{bi}/*TET2*^{wt}, 34.7 months; 95% CI, 13.5-inf vs CEBPA^{bi}/*TET2*^{mut}, 9.5 months; 95% CI, 5.23-50.2; *P* = .02) and a trend for a different EFS in patients with CEBPA^{smbZIP}/*TET2*^{wt} or CEBPA^{smbZIP}/*TET2*^{mut} (median EFS CEBPA^{smbZIP}/*TET2*^{wt}, not reached; 95% CI, 9.59-inf vs CEBPA^{smbZIP}/*TET2*^{mut}, 9.2 months; 95% CI, 2.40-66.2; *P* = .06). *WT1* mutations were associated with lower probability of survival (supplemental Figure 4); however, the comparison between the groups did not reach statistical significance.

NPM1 mutations, the most common co-mutation in patients carrying CEBPA^{smTAD}, were associated with slightly better OS and EFS; however, these differences were also not significant (supplemental Figure 5).

Presence of “typical” bZIP-mutations and outcome

Mutations in the CEBPA-bZIP region are typically in-frame (ie, multiples of 3 bp) and affect the DNA-binding-, fork-, or bZIP-region (for simplicity, summarized as bZIP) between amino acid positions 278 and 345 of the CEBPA protein (supplemental Figure 3), whereas frameshift mutations, the hallmark of mutations affecting the TAD1 and TAD2 domains, are less common in C-terminal mutations. To investigate whether the presence of typical in-frame bZIP mutations (irrespective of the biallelic or monoallelic status) actually represents the decisive molecular factor for the favorable outcome observed, we regrouped the 240

patients with CEBPA^{mut} according to the presence or absence of these mutations. Typical bZIP mutations according to this classification were found in 118 of 131 who had CEBPA^{bi} and 39 of 49 of those with CEBPA^{smbZIP} (denoted CEBPA^{bi-inf} and CEBPA^{sm-inf}). Thirteen of 131 CEBPA^{bi} had nontypical biallelic mutations (eg, consisting of 2 different TAD domain mutations or a combination of TAD mutations and frameshift or nonsense bZIP mutations, denoted CEBPA^{bi-other}). Patients with mutation in TAD as well as patients with a nontypical single-allele bZIP mutation were grouped as “other single-allele mutations” (CEBPA^{sm-other}). We again observed profound differences in the age distribution, with typical bZIP mutations (double and single allele) predominating in younger adults, whereas other mutations, especially the few nontypical biallelic mutations, were predominantly detected in patients >50 years of age (supplemental Table 4; supplemental Figure 6).

To further characterize potential biological similarities between CEBPA^{bi-inf} and CEBPA^{sm-inf}, we performed RNA-seq of 20 samples from patients with CEBPA^{bi-inf}, CEBPA^{sm-inf}, CEBPA^{bi-other}, and CEBPA^{sm-other} (5 samples per group). As illustrated in the volcano-plots in supplemental Figure 7, we found between 34 and 129 differentially expressed genes in pairwise comparisons between all groups, the only exception being CEBPA^{bi-inf} and CEBPA^{sm-inf}, where no significant differences were found at a false discovery rate of 5% (supplemental Figure 7C).

The survival analysis performed also revealed profound differences. Superimposable, favorable outcomes (OS and EFS) were

Table 3. Results of the multivariable analysis for CEBPA^{bi} vs CEBPA^{smbZIP} vs CEBPA^{smtAD}

	OS (HR)	P	95% CI	EFS (HR)	P	95% CI	CR1 (OR)	P	95% CI
Intermediate karyotype	1			1			1		
Favorable karyotype	0.426	<.001	(0.35-0.51)	0.400	<.001	(0.34-0.47)	3.076	<.001	(2.08-4.56)
Adverse karyotype*	1.853	<.001	(1.68-2.04)	1.625	<.001	(1.49-1.78)	0.476	<.001	(0.40-0.58)
Age	1.032	<.001	(1.03-1.04)	1.023	<.001	(1.02-1.03)	0.948	<.001	(0.94-0.95)
Log ₁₀ WBC	1.158	<.001	(1.08-1.24)	1.231	<.001	(1.16-1.31)	0.751	<.001	(0.66-0.86)
De novo AML	1			1			1		
sAML	1.088	.127	(0.98-1.21)	1.069	.189	(0.97-1.18)	0.735	.003	(0.60-0.90)
tAML	1.380	<.001	(1.19-1.60)	1.098	.186	(0.96-1.26)	0.697	.009	(0.53-0.91)
No FLT3-ITD ^{mut}	1			1			1		
FLT3-ITD ^{mut}	1.204	<.001	(1.09-1.33)	1.238	<.001	(1.13-1.35)	1.018	.866	(0.83-1.25)
No NPM1 ^{mut}	1			1			1		
NPM1 ^{mut}	0.625	<.001	(0.57-0.69)	0.531	<.001	(0.49-0.58)	2.151	<.001	(1.77-2.62)
CEBPA ^{wt}	1			1			1		
CEBPA ^{bi}	0.507	<.001	(0.39-0.67)	0.530	<.001	(0.42-0.67)	6.367	<.001	(2.75-14.77)
CEBPA ^{smbZIP}	0.620	.019	(0.42-0.92)	0.537	.001	(0.37-0.77)	1.876	.139	(0.81-4.32)
CEBPA ^{smtAD}	1.024	.878	(0.75-1.39)	0.981	.891	(0.74-1.31)	0.825	.515	(0.46-1.48)
No allo-HSCT in CRT	1			1			1		
Allo-HSCT in CRT	0.776	<.001	(0.69-0.87)	0.632	<.001	(0.56-0.71)	—	—	—

The multivariable analysis performed includes the different study regimens as strata.

*Adverse karyotype according to ELN 2017.

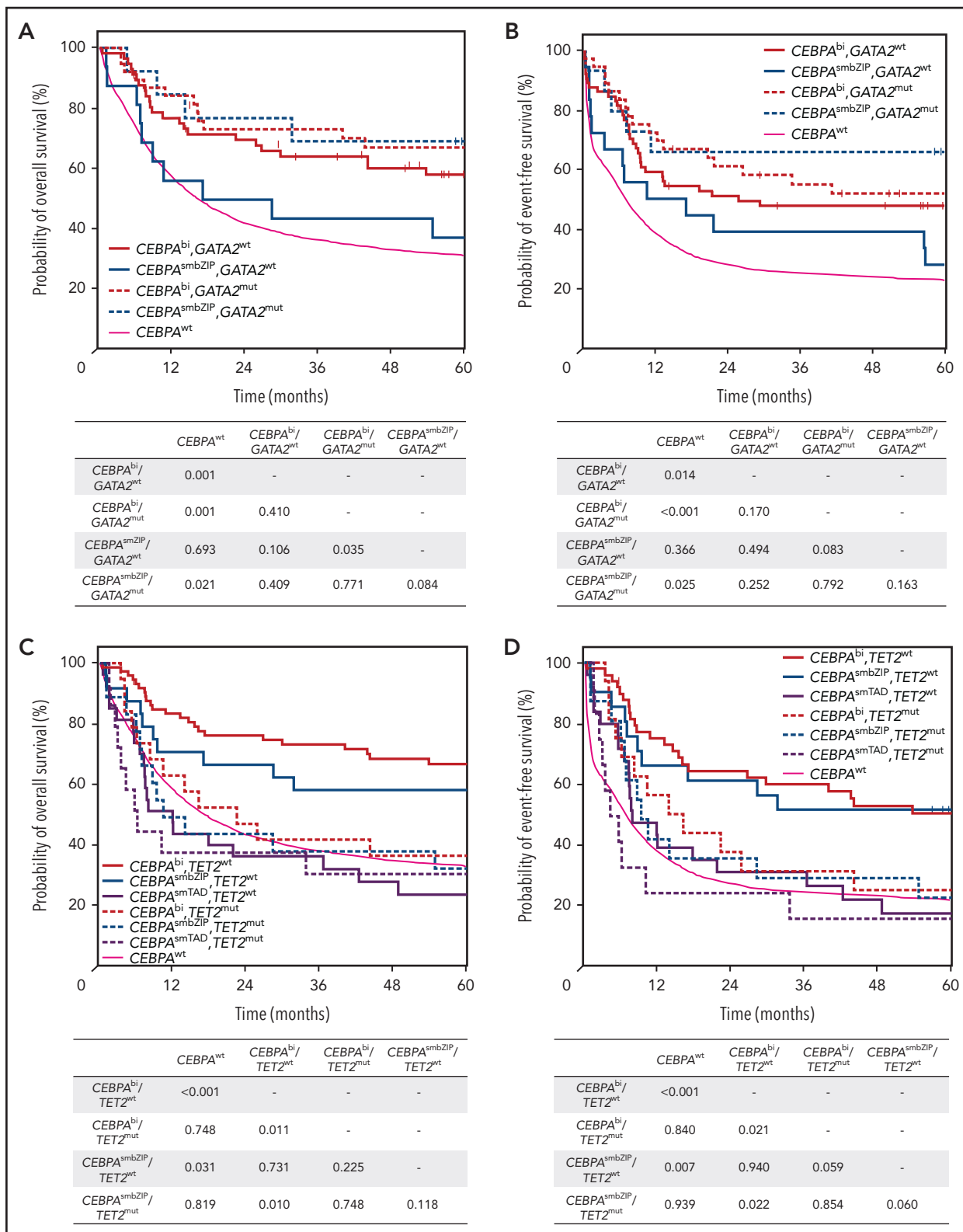


Figure 5. Survival analysis according to CEBPA and GATA2 mutational status within the cohort of 4461 patients receiving intensive treatment. Kaplan-Meier plots showing OS (A) and EFS (B) of $CEBPA^{wt}$, compared with $CEBPA^{bi}/GATA2^{wt}$ (n = 82), $CEBPA^{bi}/GATA2^{mut}$ (n = 46), $CEBPA^{smbZIP}/GATA2^{wt}$ (n = 31), $CEBPA^{smbZIP}/GATA2^{mut}$ (n = 18) mutant cases. Survival analysis according to CEBPA mutation status and TET2 status within a cohort of 4461 patients. Kaplan-Meier plots showing OS (C) and EFS (D) of $CEBPA^{wt}$, compared with $CEBPA^{bi}/TET2^{wt}$ (n = 98), $CEBPA^{bi}/TET2^{mut}$ (n = 30), $CEBPA^{smbZIP}/TET2^{wt}$ (n = 33), $CEBPA^{smbZIP}/TET2^{mut}$ (n = 16) mutant cases. Numbers of patients at risk and results of log-rank tests from pairwise comparisons are provided below the x-axis.

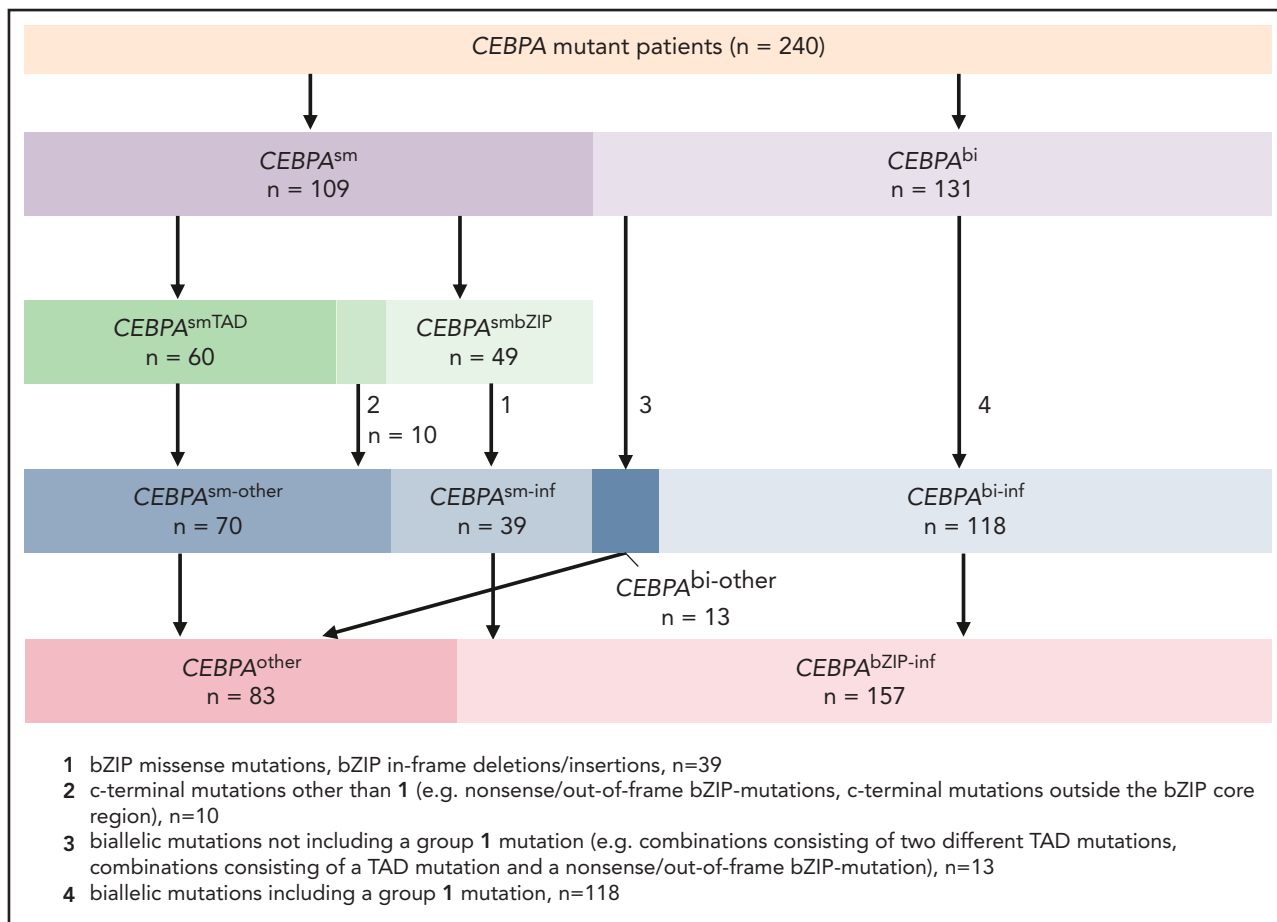


Figure 6. Grouping of CEBPA mutations according to localization and type.

confined to patients in the *CEBPA*^{bi-inf} and the *CEBPA*^{sm-inf} groups, whereas patients with other mutational constellations, in particular patients with nontypical biallelic *CEBPA* mutations, showed an inferior outcome (supplemental Table 5; supplemental Figure 8). Co-mutations retained their prognostic impact in patients carrying *CEBPA*^{bi-inf} and *CEBPA*^{sm-inf} (as illustrated for the most common *GATA2* mutations in supplemental Figure 9).

Prognostic classification for CEBPA mutations

Because these results strongly support the notion that mutation constellations containing typical in-frame bZIP mutations (ie, *CEBPA*^{bi-inf} and *CEBPA*^{sm-inf}) are comparable with respect to most clinical and molecular factors studied, we combined these 2 mutation types (denoted *CEBPA*^{bZIP-inf}) and compared this new group to those patients without these mutations (*CEBPA*^{other}; allocation summarized in Figure 6).

As illustrated in Table 4, the clinical associations already observed for the individual groups were even more pronounced in this analysis, especially the highly divergent median age (46 vs 62 years; $P < .001$), the difference with respect to the association with sAML (4% vs 17%; $P < .001$), the median CD34 positivity (51% vs 34%; $P < .001$), and the median WBC counts ($25 \times 10^9/L$ vs $14.7 \times 10^9/L$; $P < .001$). As expected, the mutational spectrum of patients belonging to these 2 subgroups showed highly significant differences. Only 1 of 157 patients with

CEBPA^{bZIP-inf} (0.6%) showed an *NPM1* mutation, compared with 30 of 83 patients with *CEBPA*^{other} mutations (36.1%; $P < .001$; Figure 7C; supplemental Figure 9). Other mutations that were significantly more common in the *CEBPA*^{other} subgroup were alterations affecting DNA methylation (43 of 157, 27.4% vs 60 of 83, 72.3%; $P < .001$) and the spliceosome (4 of 157, 2.6% vs 21 of 83, 25.3%; $P < .001$).

Outcome analyses performed for *CEBPA*^{bZIP-inf} patients confirmed a strong prognostic impact of this subgroup, whereas *CEBPA* mutations in the other patients did not show significant differences compared with *CEBPA*^{wt} (Figure 7A-B). Interestingly, patients with *CEBPA*^{bZIP-inf} appear not to benefit from HSCT performed in the first complete remission (CR1; supplemental Figure 10). Multivariable analysis performed for this classification confirmed that *CEBPA*^{bZIP-inf} positivity was the strongest predictor for achievement of CR (OR: 6.06; 95% CI, 2.78-13.23, $P < .001$) and a strong prognostic factor for OS (hazards ratio [HR], 0.57; 95% CI, 0.46-0.71; $P < .001$) and EFS (HR, 0.53; 95% CI, 0.43-0.64, $P < .001$; Table 5). We also looked for the effect of mutant *GATA2*, *TET2*, and *WT1* on outcome in the novel subgroups. As observed in the previous analyses, mutant *GATA2* and the absence of *TET2* and *WT1* mutations were associated with improved OS and EFS in patients carrying *CEBPA*^{bZIP-inf} but not in those with *CEBPA*^{other}, although these differences were not significant (supplemental Figure 11).

Table 4. Clinical variables in patients with *CEBPA*^{bZIP-inf} and *CEBPA*^{other} mutations

	<i>CEBPA</i> ^{wt} n = 4468	<i>CEBPA</i> ^{bZIP-inf} n = 157	<i>CEBPA</i> ^{other} n = 83	P (adj.)
Age in years, median (IQR)	57 (46-67)	46 (36-57)	62 (55-69)	<.001
Sex, n (%)				
Female	2170 (49)	76 (48)	42 (51)	.885
Male	2298 (51)	81 (52)	41 (49)	
AML type, n (%)				
De novo	3510 (78)	149 (96)	69 (83)	<.001
sAML	630 (14)	5 (3)	9 (11)	
tAML	328 (7)	2 (1)	5 (6)	
FAB subtype, n (%)				
M0	223 (5)	1 (1)	/	<.001
M1/M2	2040 (46)	127 (81)	53 (65)	
M4-M7	1535 (34)	22 (14)	20 (24)	
Unknown	670 (15)	7 (4)	9 (11)	
Laboratory, median (IQR)				
BM blasts in %	60 (38-80)	64 (50-78)	62 (43.5-78)	.853
CD34-positivity in %	16 (2-47)	51 (29-75.2)	34 (6.6-72.2)	<.001
WBC in 10 ⁹ /l	11.7 (3-45.7)	25 (9.2-70)	14.7 (4.6-53.4)	.001
LDH in U/l	412.9 (272-727)	445 (295-758)	449 (296-601)	.242
Treatment, n (%)				
Primary allo-HCT	747 (17)	52 (33)	5 (6)	<.001
Salvage allo-HCT	915 (20)	21 (13)	16 (19)	.209

Bold P-values indicate statically significant results.

BM, bone marrow; LDH, lactate dehydrogenase; FAB, French-American-British; MRC, medical research council.

Discussion

We analyzed 4708 adult patients with newly diagnosed AML for *CEBPA* alterations and identified mutations in *CEBPA* in 5.1%. Our cohort differed from patient cohorts in previous studies investigating the role of *CEBPA* mutations,^{9-12,15,21,24,35-42} because we did not select for age, cytogenetic subgroups, or disease status. Within the entire cohort, 49% showed an aberrant karyotype, and the median patient age at diagnosis was 57 years (IQR, 46-67 years), more accurately reflecting the entirety of patients with AML in general. This fact may explain why the prevalence of *CEBPA* mutations found in our study is at the lower end of the previously reported range of 4% to 20%.^{9-12,15,21,24,35-42}

Our main focus was the role of monoallelic *CEBPA* mutations; therefore, we performed an extensive evaluation of this subgroup and a detailed analysis of the individual type and localization of the mutation.

A first aspect observed with respect to the different mutational subgroups (*CEBPA*^{bi}, *CEBPA*^{smbZIP}, and *CEBPA*^{smTAD}) was the highly significant difference in the age distribution. Whereas *CEBPA*^{bi} and *CEBPA*^{smbZIP} were predominantly found in younger adults and decreased with age, *CEBPA*^{smTAD} mutations were rare in patients up to the age of 40 years and were

particularly common in older individuals. The lower age of patients with *CEBPA*^{bi} mutations has already been reported in previous studies.^{21,24,41} In contrast, the major age difference between *CEBPA*^{smTAD} and *CEBPA*^{smbZIP} mutations has not been reported before. Interestingly, a very recently published work in pediatric AML found only monoallelic *CEBPA*^{bZIP} mutations in their analysis, indicating that these mutations are indeed significantly more prevalent in younger individuals.⁴³ *CEBPA*^{bi} and *CEBPA*^{smbZIP} also showed overlapping laboratory profiles, with higher rates of CD34 positivity and higher WBC counts.⁴³ We also observed a favorable prognostic impact of *CEBPA*^{smbZIP} comparable to biallelic *CEBPA* mutations. This finding again is in concordance with the data reported by Tarlock et al in pediatric AML, showing that the outcome of patients with *CEBPA*^{bi} or *CEBPA*^{smbZIP} mutations did not differ.⁴³

Our results indicate a similar spectrum of co-mutations for *CEBPA*^{bi} and *CEBPA*^{smbZIP} (ie, significantly higher rates of *GATA2* and *WT1* mutations and a mere lack of *NPM1* alterations). *GATA2* mutations in *CEBPA*^{bi} have been described previously,⁴⁴ whereas a similar association of *CEBPA*^{smbZIP} with *GATA2* mutations has not been reported so far. The presence of *GATA2*, *TET2*, and *WT1* mutations significantly affected the outcome of patients carrying *CEBPA*^{bi}, whereas *CEBPA*^{smbZIP} mutations showed a significant effect on outcome only for mutations in *GATA2*. The prognostic impact of mutations in *GATA2*,

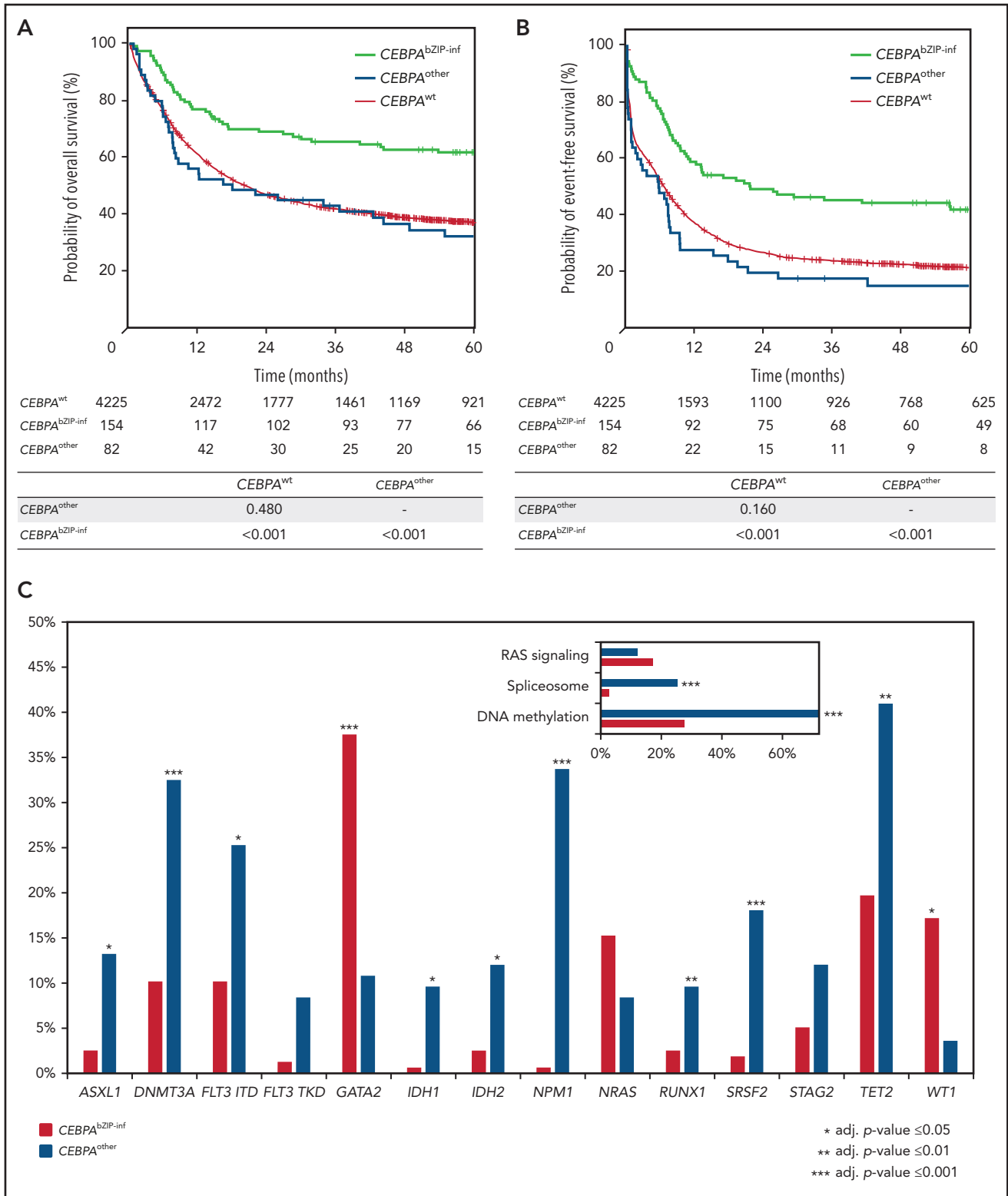


Figure 7. Survival analysis according to *CEBPA* mutation status (*CEBPA*^{wt}, *CEBPA*^{bZIP-inf}, and *CEBPA*^{others}) within the cohort of 4461 patients receiving intensive treatment. Kaplan-Meier plots showing OS (A) and EFS (B). (C) Frequency distribution of additional gene mutations identified in patients with *CEBPA*^{bZIP-inf} and *CEBPA*^{others} mutant genes (frequency of at least 5% in one subgroup). RAS signaling, including *KRAS*, *NRAS*, *PTPN11*, *CBLB*; spliceosome, including *SF3B1*, *SRSF2*, and *ZRSR2*; and methylation, including *DNMT3A*, *IDH1*, *IDH2*, and *TET2*. Numbers of patients at risk and results of log-rank tests from pairwise comparisons are provided below the x-axis.

TET2, and *WT1* has been described recently,^{45,46} but was confined to biallelic *CEBPA* mutations. In contrast, our data indicate that these prognostic associations also apply to patients with

CEBPA^{smbZIP}, but not to those with *CEBPA*^{smTAD}. Clustering of co-mutations according to functional groups further highlighted a differential spectrum of co-occurring mutations, with

Table 5. Results of the multivariable analysis for CEBPA^{bZIP-inf} vs CEBPA^{other}

	OS (HR)	P	95% CI	EFS (HR)	P	95% CI	CR1 (OR)	P	95% CI
Intermediate karyotype	1			1			1		
Favorable karyotype	0.456	<.001	(0.38-0.55)	0.436	<.001	(0.38-0.51)	3.072	<.001	(2.07-4.55)
Adverse karyotype*	1.823	<.001	(1.67-2.00)	1.590	<.001	(1.46-1.73)	0.478	<.001	(0.40-0.58)
Age	1.033	<.001	(1.03-1.04)	1.025	<.001	(1.02-1.03)	0.948	<.001	(0.94-0.95)
Log ₁₀ WBC	1.159	<.001	(1.09-1.24)	1.228	<.001	(1.16-1.31)	0.753	<.001	(0.66-0.86)
De novo AML	1			1			1		
sAML	1.050	.356	(0.95-1.16)	1.020	.683	(0.93-1.12)	0.734	.003	(0.60-0.90)
tAML	1.312	<.001	(1.15-1.50)	1.056	.413	(0.93-1.20)	0.704	.012	(0.53-0.93)
No FLT3-ITD ^{mut}	1			1			1		
FLT3-ITD ^{mut}	1.179	<.001	(1.08-1.29)	1.193	<.001	(1.10-1.30)	1.032	.767	(0.84-1.27)
No NPM1 ^{mut}	1			1			1		
NPM1 ^{mut}	0.635	<.001	(0.58-0.70)	0.553	<.001	(0.51-0.60)	2.139	<.001	(1.76-2.60)
CEBPA ^{wt}	1			1			1		
CEBPA ^{bZIP-inf}	0.570	<.001	(0.46-0.71)	0.525	<.001	(0.43-0.64)	6.061	<.001	(2.78-13.23)
CEBPA ^{other}	0.897	.410	(0.69-1.16)	0.964	.762	(0.76-1.22)	1.003	.989	(0.61-1.65)
No allo-HSCT in CR1	1			1			1		
Allo-HSCT in CR1	0.671	<.001	(0.60-0.75)	0.446	<.001	(0.40-0.50)	—	—	—

The multivariable analysis performed includes the different study regimens as strata.

CR1, first complete remission.

*Adverse karyotype according to ELN 2017.

CEBPA^{smTAD} mutations showing a significantly higher prevalence of mutations affecting proteins involved in DNA methylation (ie, DNMT3A, TET2, IDH1, and IDH2) as well as RNA splicing (ie, SRSF2, SF3B1, and ZRSR2), which was not seen in patients with *CEBPA*^{bi} and *CEBPA*^{smbZIP} (Figure 4B). Additional analyses looking in more detail for the individual mutations in *CEBPA* in our patients suggested that the identified clinical and molecular associations as well as the association with outcome were restricted to typical in-frame mutations within the bZIP region.

The reason for this differential behavior is unclear at present, but based on previous in vitro as well as animal experiments, TAD1 and bZIP mutations have clear functional differences (reviewed in Pulikkan et al⁴⁷). The frameshift mutations typically observed in the TAD1 domain induce the consecutive translation of the shorter *CEBPA*^{p30} protein, instead of the *CEBPA*^{p42} full-length protein. In contrast, with both *CEBPA*^{p42} and *CEBPA*^{p30}, mutations in the DBD and bZIP domains result in loss of DNA binding as well as dimerization. Recent animal data modeling the disease suggest functional disparity of different bZIP mutations. Lethally irradiated mice undergoing transplant of hematopoietic stem cells homozygous for a point mutation in bZIP (designated BRM2) start to develop a myeloproliferative disease that transforms into overt AML.⁴⁸ By transplanting transgenic cells carrying the most common mutation in bZIP (K313dup; K allele), alone or in combination with a TAD1 mutation (designated L-allele), Bereshchenko et al⁴⁹ documented that cells from mice carrying either the K/K or the K/L genotype, showed similarities in their mRNA expression profiles and higher expansion of immature blasts in the BM, which was distinct from the L/L genotype, as well as *CEBPA*-WT cells. *CEBPA*-mutated AML is characterized by specific RNA,⁵⁰ as well as miRNA^{35,51} expression profiles. The expression of several key miRNAs, such as *miR-34*, *miR-182*, and *miR-223*, which are involved in stem cell self-renewal, cell migration, and granulocytic differentiation, are physiologically regulated by *CEBPA* (reviewed in Stavast et al⁵²). Interestingly, recent data suggest that bZIP-mutant *CEBPA* does not downregulate *miR-182*, leading to a block of granulocytic differentiation.⁵³ This incapability appears to be mainly restricted to typical in-frame bZIP mutations clustering around the core mutated amino acids 312 and 313 of the *CEBPA* protein.⁵³ In support of this, one of the genes we found most highly deregulated between *CEBPA*^{bi-inf}/*CEBPA*^{sm-inf} and *CEBPA*^{sm-other}, *OSTL/RNF217*, coding for a highly conserved RING-finger ubiquitin ligase overexpressed in various leukemia entities,⁵⁴ has been shown to be regulated by the miRNA cluster *miR-183-96-182*.⁵⁵

Taken together, the *CEBPA*^{bZIP-inf} genotype identified in our analysis describes a subgroup of *CEBPA* mutations predominantly found in younger adults, indicating characteristics of a more immature and proliferative disease that has an overall prevalence of 3.3% in adult patients with AML, but is found in up to 7% of patients ≤ 40 years, thus representing a relevant subgroup, especially in younger adults. The mere absence of *NPM1* mutations (1 of 157 patients; 0.6%) and the high prevalence of *GATA2* mutations in these patients, which was found to be associated with an even better prognosis and a long-term survival in up to 80%, further highlights the special biology of these leukemias. The 2016 World Health Organization classification defines the *CEBPA* mutational class by the presence of a biallelic mutation, regardless of the localization within the gene. Given that

90% of the patients with the biallelic *CEBPA* mutation in this analysis actually carried typical bZIP *CEBPA* mutations, there is obviously a high degree of overlap, which may explain why this difference was undetected in most previous analyses, although there was some evidence in another study.²⁵ However, that only the 90% of patients with biallelic *CEBPA* mutations containing typical bZIP mutations in fact show a better outcome indicates that only those should be assigned to the favorable risk group.

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Authorship

Contribution: F.T., J.A.G., and C.T. designed the study; performed the research; collected, assembled, analyzed, interpreted the data; and wrote the manuscript; F.T., J.A.G., S. Stasik., A.P., R.M.-L., and P.J.M.V., performed the molecular analyses and analyzed the data; S.H. performed the molecular analyses; M. Kramer and J.S. performed the statistical analyses; J.M.M., C.R., U.K., A.K., S. Scholl, A.H., T.H.B., R. Naumann, B.S., H.E., M.S., A.B., A.N., K.S.-E., C.S., S.W.K., M.H., R. Nopenney., U.K., C.D.B., M. Kaufmann, F.S., K.S., M.v.B., C.M.-T., U.P., W.E.B., H.S., G.E., M.B., and J.S., treated the patients and collected the clinical data; and all authors approved the final version of the manuscript.

Conflict-of-interest disclosure: C.T. is CEO and co-owner of AgenDix GmbH. The remaining authors declare no competing financial interests.

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Footnotes

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Original data can be obtained by e-mail request to the corresponding author (christian.thiede@uniklinikum-dresden.de).

The online version of this article contains a data supplement.

There is a *Blood* Commentary on this article in this issue.

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Appendix: participating centers of the Study Alliance Leukemia (SAL)

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