INTRODUCTION

Mutations of FLT3 (fms-related tyrosine kinase 3) are common gene mutations in acute myeloid leukemia (AML). They are found in 10–30% of patients, with a decreasing prevalence with older age [1, 2]. FLT3 encodes a receptor-tyrosine kinase that is expressed on hematopoietic stem and progenitor cells and regulates cellular proliferation and apoptosis through downstream signaling pathways such as STATS, RAS/MAPK, and PI3K/Akt [3]. Mutations of FLT3 encompass internal tandem duplications (ITD), most frequently located in the juxtamembrane region, as well as point mutations in the tyrosine kinase domain (TKD), both promoting ligand-independent, constitutive receptor activation [4]. ITD but not TKD mutations have been consistently linked to a higher relapse rate and an inferior overall survival [5–7]. Yet, the prognostic impact of FLT3-ITD is context-dependent and influenced by the mutational background, for example whether a concurrent NPM1 mutation is present or not, the location of the ITD insertion site, and the mutant to wildtype allelic ratio (AR) [5, 8–14]. The prognostic significance of the NPM1 mutated status and of the FLT3-ITD AR is implemented in the 2017 European LeukemiaNet (ELN) risk stratification [15].

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The successful development of first- and next-generation FLT3 inhibitors has contributed to a new era for precision medicine in AML [16]. In the international CALGB 10603/RATIFY trial, the addition of the oral multi-targeted small molecule FLT3 inhibitor midostaurin to intensive induction and consolidation chemotherapy, followed by a 1-year midostaurin maintenance therapy, improved overall survival of patients 18 to 59 years of age with newly diagnosed FLT3-mutated AML [17]. Midostaurin showed consistent effects across all FLT3 mutation strata, TKD, and ITD independent of the AR [17]. In addition, in the AMLSG 16–10 trial midostaurin significantly improved outcome also in older patients 60 to 70 years in age in comparison to a historical control cohort [18].

We previously reported on the impact of NPM1/FLT3-ITD genotypes as defined by 2017 ELN risk categories of all FLT3-TKD mutations in patients of the RATIFY trial [12, 19]. The objective of the current study was to comprehensively profile the mutational landscape of FLT3-mutated AML in patients treated within the RATIFY trial using a high-throughput targeted sequencing (HTS) approach, and to correlate genetic patterns with clinical outcomes.

**PATIENTS AND METHODS**

**Patients**

Overall, 717 patients with AML and FLT3 mutation were enrolled in the CALGB 10603/RATIFY trial [17]. HTS was performed on 475 patients (n = 409 bone marrow, n = 66 peripheral blood) who gave informed consent for further molecular studies and for whom DNA was available (Supplementary Table S1). Patient characteristics are provided in Table 1. FLT3 mutation status was available for all 475 pts (TKD: n = 116; 24%; ITD: n = 335; 71%; TKD + ITD: n = 24; 5%) and information on the AR (0.05–0.5; n = 126, ≥0.5; n = 232) for all but one patient with ITD. For analyses, patients (n = 24) harboring both FLT3-TKD and -ITD mutations were assigned to the ITD group based on the prognosis-defining features of ITD compared to TKD mutations.

In the RATIFY trial, patients 18 to 59 years of age were randomly assigned to receive standard chemotherapy plus either midostaurin or placebo. Randomization was stratified according to the subtype of FLT3 mutation; TKD or ITD with either a low (0.05 to 0.7) or a high AR (>0.7). For the latter, a threshold of 0.5 (low, 0.05 to <0.5; high, ≥0.5) was chosen as this cutoff has been demonstrated to better discriminate clinical outcome and was adopted in the 2017 ELN risk classification [15]. 253 (53%) of 475 patients were randomized to the midostaurin arm and 222 patients to the placebo arm. 128 (27%) of the patients underwent allogeneic hematopoietic cell transplantation (HCT) in first complete remission (CR).

The analysis was approved by the institutional review board at each participating center. Written consent was given by all patients for treatment, genetic analysis, and biobanking according to the Declaration of Helsinki.

**Gene mutation analyses**

HTS was performed on the entire coding region of 262 genes involved in hematologic malignancies including 20 kinases targeted by midostaurin [20]. A comprehensive list of all genes is included in the Supplementary Appendix A (Supplementary Tables S2, S3). Library preparation was performed using SureSelectXT from Agilent Technologies (Santa Clara, CA, USA). Paired-end sequencing (read length: 2 × 100 base pairs) was carried out on a HiSeq 2000 platform (Illumina, San Diego, CA, USA). All sequencing data were analyzed using an in-house computational pipeline [21]. Detailed information on the mutation calling and data curation workflow is provided in the Supplementary Information. FLT3 mutations were assessed by GeneScan-based fragment-length analysis as previously described [17].

**Statistical analyses**

Statistical analyses were performed using R, version 3.6.3. Fisher’s exact test and chi-squared test were employed for two-group comparisons of categorical variables and the Wilcoxon rank-sum test for continuous variables. The Kaplan–Meier method was used to estimate the distributions of time-to-event endpoints (OS) and overall survival (OS). Associations between clinical and genetic variables and time-to-event endpoints were analyzed using log rank test and random survival forests.

Additionally, for the analysis of genomic classes log rank tests and multivariable Cox proportional hazards regression models were used, for which the proportional hazards assumption was checked via graphical methods. Covariates age, gender, ECOG performance status (0 vs. 1), WBC count on log2 scale, treatment arm, allogeneic HCT in first remission (as time-dependent variable), FLT3-mutation status as well the genomic classes (NPM1, chromatin-spliceosome, no class, TP53-aneuploidy, and core-binding factor (CBF) AML) were introduced into the multivariable regression model.

An effect was considered significant if P ≤ 0.05. As the proportion of missing values in covariates used for modeling was very low (1 missing observation in FLT3 allelic ratio, 3 missings in white blood cell count [WBC]), single imputation was employed using chained equations as implemented in the mice package [22]. Clinical endpoints were assessed as defined by the 2017 ELN recommendations [15, 17].

Correction for multiple testing was implemented via the Bonferroni–Holm and the Benjamini–Hochberg method, depending on the number of tests [23, 24]. An effect was considered significant if the adjusted P ≤ 0.05. Mutual exclusivity and co-occurrence of genomic mutations were tested via independence tests while accounting for varying mutation rates via the method proposed by Canisius et al. and implemented in the Rediscover R package [25].

Random survival forests were implemented using the R package randomForestSRC to assess possible interactions of clinical and genetic variables [26]. One-hundred forests with distinct seeds were fitted on the imputed dataset hereby growing 1000 trees for each forest. Tree nodes were split using log rank statistics. The impact of a variable was ranked by the variable importance (VIMP) based on the prediction error calculated using Harrell’s C-index [27]. The assessment of pairwise interactions between variables was based on the comparison of the joint (“paired”) VIMP to the sum of their individual VIMPs (called “additive” importance); A large positive or negative difference between “additive” and “paired” importance indicates a possibly relevant association.

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**Table 1.** Baseline characteristics of 475 patients with FLT3-mutation.

|              | Placebo (N = 222) | Midostaurin (N = 253) | Overall (N = 475) |
|--------------|-------------------|----------------------|------------------|
| **Age at registration (years)** |                   |                      |                  |
| Mean (SD)    | 46.0 (11.3)       | 45.2 (10.6)          | 45.5 (10.9)      |
| Median [Min, Max] | 49.3 [18.0, 59.9] | 47.1 [19.0, 59.8]    | 47.9 [18.0, 59.9]|
| **Sex**      |                   |                      |                  |
| Male         | 90 (40.5%)        | 118 (46.6%)          | 208 (43.8%)      |
| Female       | 132 (59.5%)       | 135 (53.4%)          | 267 (56.2%)      |
| **FLT3 mutation type** |               |                      |                  |
| TKD          | 54 (24.3%)        | 62 (24.3%)           | 116 (24.4%)      |
| ITD < 0.5 allelic ratio | 61 (27.5%)       | 65 (25.7%)           | 126 (26.5%)      |
| ITD ≥ 0.5 allelic ratio | 106 (47.7%)     | 126 (49.8%)          | 232 (48.8%)      |
| Missing      | 1 (0.5%)          | 0 (0%)               | 1 (0.2%)         |
| **WBC count at baseline (10E9/L)** |               |                      |                  |
| Mean (SD)    | 54.1 (58.8)       | 48.7 (46.6)          | 51.2 (52.7)      |
| Median [Min, Max] | 33.1 [0.8, 330]  | 36.4 [0.6, 304]      | 35.4 [0.6, 330]  |
| Missing      | 1 (0.5%)          | 2 (0.8%)             | 3 (0.6%)         |
| **ECOG performance status** |               |                      |                  |
| 0–1          | 193 (86.9%)       | 229 (90.5%)          | 422 (88.8%)      |
| 2            | 29 (13.1%)        | 24 (9.5%)            | 53 (11.2%)       |
| **Treatment** |                   |                      |                  |
| Placebo      | 222 (100%)        | 0 (0%)               | 222 (46.7%)      |
| Midostaurin  | 0 (0%)            | 253 (100%)           | 253 (53.3%)      |

ECOG Eastern Cooperative Oncology Group, WBC white blood cell count, SD standard deviation.
RESULTS
Mutational landscape of FLT3-mutated AML

High-throughput sequencing of the 475 FLT3-mutated AML yielded a mean on-target sequencing depth of ~975×. Overall, we found 1888 mutations with a variant allele fraction of equal to or greater than 3% in 200 of the 262 genes. At least one mutation in addition to FLT3-ITD or -TKD was identified in 463 of 475 (97.5%) patients. The overall median number of additional mutations per patient was 3 and—when stratified by mutation type—4 in the TKD vs. 3 in the ITD group. The majority of mutations were missense mutations (49%) and indels (40%). 26 (5.5%) of patients had non-canonical FLT3 mutations outside of TKD and ITD mutational hotspots, including 14 with mutations in exon 16 (codon 663–680), that were missed by GeneScan-based fragment-length analysis. 4 of 14 were concurrent with ITDlow and 10 of them with TKD mutations. Overall, the most frequently affected gene was TET2 (55%, 12%), followed by IDH1 (39%, 8%), IDH2 (38, 8%), ASXL1 (38, 8%), DNMT3A (28, 6%), and RUNX1 (26, 5%), and SMC3 (24, 5%) (Figs. 1, 2; Table S4).

Frequency of gene mutations by FLT3 mutation type. N Ras (28, 24% vs. 25, 7%, p < 0.001), SMC1A (12, 10% vs. 16, 4%, p = 0.02), and KIT (5, 4% vs. 3, 1%, p = 0.02) mutations occurred significantly more often in TKD than ITD groups, respectively, whereas WT1 (15, 13% vs. 84, 24%, p = 0.018) was more frequently co-mutated in the ITD group (Fig. 2A, Table S4).

Frequency of mutations by functional classes (signaling, methylation, chromatin, cohesion, transcription, tumor suppressor, splicing, other). In 29% (138) of cases we identified mutations affecting other signaling molecules, most commonly in NRAS (53, 11%), PTPN11 (45, 10%), KRAS (13, 3%), and KIT (8, 2%) (categorization in Table S5). These additional signaling mutations more often co-occurred in the FLT3-TKD than -ITD group (50, 43% vs. 90, 25%, p = 0.001). In general, mutations altering DNA methylation pathways were common (total: 264, 56%; DNMT3A: 187, 39%, TET2: 55, 12%, IDH1: 39, 8%, IDH2R140: 34, 7%). Other frequently altered functional groups of genes involved chromatin modification (ASXL1: 38, 8%, KMT2D: 18, 4%) and the cohesion complex (SMC1A: 28, 6%, SMC3: 24, 5%, RAD21: 23, 5%, STAG2: 18, 4%). Among genes regulating transcription, we found recurrent loss-of-function mutations in NFEB2 (13, 3%) and codon 73–78 hotspot mutations in MYC (15, 3%), as well as aberrations of WT1 (100, 21%), RUNX1 (53, 11%), CEBPA (26, 5%), BCOR (20, 4%), BCORL1 (16, 3%), ZBTB7A (15, 3%), and GATA2 (14, 3%). Mutations in TP53 (4, 0.8%) and in genes encoding for splicing factors (SF3B1: 9, 1.9%, SRSF2: 1, 1%, U2AF1: 4, 0.8%, ZRSR2: 1, 0.2%) were rare reflecting the de novo disease onset in the vast majority of cases (455, 96%) and the younger age of the patients.

The top 12 most frequently mutated genes were tested for mutual exclusivity (66 possible combinations) resulting in 21 significant pairs before adjustment for multiple testing and 5 significant pairs after FDR adjustment: NPM1-RUNX1 (p < 0.001), DNMT3A-WT1 (p < 0.001), DNMT3A-RUNX1 (p = 0.003), NPM1-WT1 (p = 0.008) and IDH2-TET2 (p = 0.021). Similarly, all combinations were tested for co-occurrence resulting in 2 significant pairs before adjustment for multiple testing: NPM1-DNMT3A and IDH1-PTPN11, which were not significant after FDR adjustment (Fig. S1).

Classification of cases according to genomic AML classes. Using the mutational and cytogenetic data, we assigned cases to the genomic AML classes as previously defined (Fig. 1; Tables S6, 7) [1]. This was possible for 451 of 475 patients, in which the complete...
Mutational and cytogenetic dataset was available or at least one class-defining aberration was present. The majority of cases fell into three classes: NPM1 (287, 60%), chromatin-spliceosome (68, 14%), and no class (60, 13%). All other AML classes were less frequent: TP53-aneuyploidy (18, 4%), inv(16) (10, 2%), t(8;21) (8, 2%), t(11q23)x (8, 2%), t(6;9) (4, 1%), and CEBPAbiallelic (3, <1%). Due to these low sample sizes, we restricted further statistical analyses to the following genomic classes: NPM1, chromatin-spliceosome, no class, TP53-aneuyploidy, and the core-binding factor (CBF) AML class [inv(16) and t(8;21)], thereby excluding a total of 24 patients in the smaller categories resulting in a total of 451 patients for analysis.

Mutations of midostaurin kinase genes. In addition, we screened 20 kinase genes (Table S3) known to be pharmacologically targeted by midostaurin. Pretreatment mutations in the midostaurin kinase were rare events with only 40 mutations in 35 of 475 patients (7%). The most frequently affected genes were KIT (8, 2%), NTRK3 (4, 0.8%), and MAP3K11 (4, 0.8%) (Fig. 2B).

Clinical impact of molecular landscape in FLT3-mutated AML
Prognostic and predictive impact of concurrent gene mutations. We first performed exploratory univariable analysis for genes that were mutated in more than 5% of 475 cases. Five of twelve mutations showed an association with outcome: mutations in ASXL1 (p = 0.0125; hazard ratio (HR): 1.67, 95% confidence interval [95%CI]: 1.11–2.52) and WT1 (p < 0.001; HR: 1.83, 95%CI: 1.38–2.43) were associated with a significantly shortened OS (Table S8 and Figs. S2, 3), whereas NPM1 (p < 0.001; HR: 0.6, 95%CI: 0.46–0.77) and in trend NRAS (p = 0.097; HR: 0.68, 95%CI: 0.43–1.08) mutations conferred a favorable prognosis. After adjusting for multiple testing via Bonferroni-Holm procedure, only NPM1 and WT1 mutations retained significance at the 5% level. With respect to EFS, WT1 (p = 0.018; HR: 1.36, 95%CI: 1.05–1.75), and in trend SMC1A (p = 0.075; HR: 0.64, 95%CI: 0.38–1.05) conferred poor prognosis in univariate analysis, whereas only NPM1 (p < 0.001; HR: 0.6, 95%CI: 0.48–0.74) prolonged survival, both in univariate analysis and after adjustment for multiple testing (Table S8, and Figs. S2, 3).

To assess prognostic effects of both clinical features and genetic variables we fitted random survival forests. To quantify the random variability, a total of 100 forests with different seeds were trained hereby constructing 1000 trees for each forest. The impact of a variable was measured using the variable importance (VIMP) in each forest which reflects the loss in the model’s prediction accuracy after permutation of each variable/feature. The higher the VIMP, the higher the prognostic and/or predictive value of the variable. With respect to OS, highest VIMP was found for WT1 and NPM1 mutations, followed by WBC count, FLT3 mutation type, treatment (midostaurin vs. placebo), ASXL1 mutation, and ECOG performance status (0–1 vs. 2) in descending order (Fig. 3A). With regard to EFS, random forest indicated a higher VIMP only for NPM1, WBC count, sex, and FLT3 mutation status (Fig. 3B). The unfavorable impact of WT1 mutations on OS was predominantly observed across the intermediate and adverse ELN 2017 groups (Figs. S4, S5).

To assess the potential impact of specific gene–gene or gene-clinical variable interactions, resulting random forests were also used to identify relevant pairwise interactions among all paired combinations of mutations, midostaurin treatment, and gender. With regard to OS, the most striking interactions were observed for WT1–NPM1 and WT1 treatment (Figs. 3C, S6). For the ten most relevant interactions we created Kaplan–Meier estimators for further illustration (Figs. 4, S7, S8). There were two important observations: first, the prognostic influence of some gene mutations was severely dependent on the mutational background: As aforementioned, outcome of patients harboring NPM1 mutations was particularly favorable, whereas WT1 mutations were linked to poor prognosis. In case of mutational co-occurrence, the presence of NPM1 seemed to extensively abrogate the negative impact of WT1 (Fig. 4A). Patients who had both NPM1 and SMC1A mutations had a particularly good outcome, out-reaching the prognosis of patients with the NPM1mut/SMC1Awt genotype. Yet, in the absence of NPM1 aberrations, all 5 patients with SMC1A-mutated AML died within 3 years (Fig. 4B). Second, specific genotypes in FLT3-mutated AML seemed to benefit particularly from therapy with midostaurin, in particular those patients with concurrent WT1 mutation. In contrast, WT1-mutated patients randomized to the placebo arm had a very poor prognosis suggesting a particular benefit of midostaurin in the presence of WT1-mutations (Fig. 4C).

Of note; in line with previous data, FLT3 was frequently co-mutated with DNMT3A and NPM1 (n = 157; 33%). [1, 28–30] In our cohort, no relevant interaction could be observed for the combined FLT3-DNMT3A-NPM1 genotype with regard to clinical outcome (OS, EFS) (Figs. 3C, D, S9).

Fig. 2 Mutational landscape of FLT3-mutated acute myeloid leukemia. A Mutational landscape of 475 patients stratified according to the FLT3 mutation type. B Incidence of mutations in the midostaurin kinase in the entire cohort of 475 patients.
Prognostic impact of FLT3 mutations by AML genomic class. In 4S1 of 475 cases that could be subclassified into genomic classes, we found significant differences in long-term survival among the five genomic AML classes (OS: p < 0.001, EFS: p < 0.0001) (Fig. S10, Tables 9, 10). Estimated 4-year-survival rates were highest for CBF-AML (OS: 72%; EFS: 50%) followed by NPM1 classes (OS: 57%; EFS: 38%), whereas patients of the chromatin-spliceosome (OS: 33%; EFS: 16%), TP53-aneploidy (OS: 35%; EFS: 6%), and no class (OS: 37%; EFS: 10%) groups had an adverse OS and EFS. Although patients of the 'no class' group were younger, they had a higher frequency of FLT3-ITD in, in particular with a high ITD AR, WT1 co-mutations, and less often a normal karyotype, possibly explaining the poorer outcome (Fig. 1, Table S7).

Next, we developed a prognostic proportional hazard model including the 5 genomic AML classes, as well as the covariates age, gender, ECOG performance status (0–1 vs. 2), log2 WBC count, FLT3-status (TKD, ITDlow, ITDhigh), treatment (midostaurin, placebo), and allogeneic HCT in first CR1 as a time-dependent variable. In line with univariate analyses, we observed poorer OS and EFS in the TP53-aneploidy, chromatin-spliceosome and no class genomic groups compared to the reference NPM1 group (Fig. 5). CBF-AML patients seemed to have a more favorable outcome than the NPM1 reference group, although not statistically significant likely due to the small sample size. With regard to the FLT3-status, patients with FLT3-ITDhigh had inferior outcome compared to the FLT3-TKD mutated group. In addition, higher WBC counts conferred poor OS and EFS: A doubling was associated with almost 10% higher risk of an event or death. Treatment with midostaurin and allogeneic HCT in CR1 both had a positive impact on OS, midostaurin in addition on EFS (Fig. 5).

Finally, we assessed whether the genomic classification had a predictive impact on the hazard of death after midostaurin treatment. Treatment with midostaurin prolonged OS in the NPM1, chromatin-spliceosome and in the no class groups (Table 2). This was not the case for TP53-aneploidy and CBF-AML classes (Table 2, Figs. S11, 12). However, data need to be interpreted with caution due to the limited sample size. With regard to OS and EFS, similar treatment effects of midostaurin were observed across all FLT3 mutation strata substantiating the therapeutic benefit irrespective of the FLT3 mutation type (Table S11; Fig. S13).

DISCUSSION
In this study we characterized the mutational landscape and its clinical significance in 475 patients with FLT3-mutated AML enrolled on the pivotal CALGB 10603/RATIFY trial evaluating intensive chemotherapy with or without midostaurin.

The most frequent gene mutations co-occurring with FLT3 mutations were, in order of decreasing frequency, mutations in NPM1, DNMT3A, WT1, TET2, NRAS, RUNX1, TP53, IDH1, ASXL1, IDH2, SMC1A, CEBPA, and SMC3 genes. Compared to the general AML population, mutations in WT1 were overrepresented which is in line with previous studies showing this particular co-mutation pattern [31, 32]. In contrast, mutations in TP53, and in genes encoding for splicing factors (SF3B1, SRSF2, U2AF1, ZRSR2) were less common, likely reflecting differences in disease biology—e.g., high frequency of normal karyotypes and NPM1 mutations generally observed in FLT3-mutated AML—and age distribution (median age 47.9 years) compared to the median age of the general AML population of about 70 years [33].

To assess prognostic and potentially predictive effects of gene mutations in the context of clinical covariates, we fitted random forests. Here, the highest prognostic effect was found for WT1 and NPM1 mutations, followed by WBC count, FLT3 mutation type (ITD vs. TKD mutations), treatment (midostaurin vs. placebo), ASXL1 mutation, and ECOG performance status. The prognostic effect of NPM1 mutations, especially in the context of concurrent FLT3-ITD, is well established and reflected in the 2017 ELN risk stratification [15]. The results from this study provide additional novel and clinically relevant data, in that mutations in WT1 and ASXL1 have independent prognostic value in the context of AML with FLT3 mutations. In previous studies in pediatric AML and in

Prognostic survival forest model for impact of clinical and/or genetic variables as well as pairwise interactions on prognosis. Prognostic and potential predictive impact of 18 most relevant clinical and/or genetic variables (A, B) and pairwise interactions (C, D) in 475 patients on overall and event-free survival using random survival forests. The prognostic impact of a variable is measured via VIMP (variable importance). Higher VIMP values indicate that a variable may have prognostic impact on the survival endpoint. EFS event-free survival, OS overall survival.

Fig. 3
The three most interesting interactions regarding overall survival as selected by random survival forests. NMP1:WT1 (A), NPM1:SMC1A (B) and WT1:treatment (C). Kaplan–Meier plots illustrating the influence of the clinical and/or genetic variables and their combination on overall survival: first variable (left) and the second variable (middle) as well as the combination of the two (right).
cytogenetically-normal adult AML, the presence of WT1 mutations has been linked to primary resistance to therapy and shortened survival [34–37]. Here, the unfavorable effect was most pronounced in the intermediate and adverse ELN risk group, whereas the effect in the favorable-risk group was largely mitigated by the concurrent presence of NPM1 mutation. WT1 is a co-transcriptional factor involved in epigenetic regulation [38]. Several groups have reported that WT1 physically interacts with TET2 to modulate 5-hydroxymethylation of cytosine, a pathway commonly dysregulated in AML [39, 40]. Interestingly, in a recent study Pronier et al. have demonstrated that WT1 haploinsufficiency cooperates with FLT3-ITD to induce leukemogenesis in vivo [41].

Extending on the random forest model, when analyzing pairwise interactions the most striking interactions were observed for WT1:NPM1 and WT1:treatment. The presence of NPM1 mutations seemed to largely abrogate the negative impact of WT1 mutations. A similar observation has previously been made for other NPM1 co-mutation patterns, e.g., NPM1:FLT3-TKD, in that NPM1 mutation abrogates the negative prognostic effect of a FLT3-TKD mutation [19, 42–44]. With regard to treatment effects, patients with FLT3:WT1 co-mutations appeared to benefit from the addition of midostaurin; overall, patients with FLT3:WT1 co-mutations had poor outcomes, however, patients with FLT3:WT1 co-mutations on the midostaurin arm had similar long-term survival compared to patients with the FLT3mut/WT1wt genotype treated on the placebo arm. Another new finding relates to the FLT3:NPM1:SMC1A gene–gene interaction. Patients who had co-mutation of both NPM1 and SMC1A genes had a particularly good outcome, outreaching the prognosis of patients with the FLT3mut/NPM1mut/SMC1Awt genotype. Yet, in the absence of NPM1 mutations all patients harboring a SMC1A mutation died. Little is known about the biological basis of SMC1A mutations in the context of NPM1 and FLT3 mutations. However, AML with concurrent cohesin and NPM1 gene mutations define a specific cluster within the group of NPM1 mutated AML, that is reflected by a distinct metabolic gene signature [45]. Whether or not these features render the NPM1mut/SMC1Amut genotype more susceptible to treatment with chemotherapy ± midostaurin remains to be determined. Another similar gene–gene interaction with a cohesin complex gene was previously found with the interaction of NPM1 with RAD21 mutations, i.e., AML with the NPM1mut/RAD21mut

![Fig. 5 Prognostic impact of genomic AML classes and clinical characteristics in FLT3-mutated AML.](https://example.com/fig5.png)

Cox proportional hazard model for prognostic impact of genomic AML classes and clinical features on (A) overall and (B) event-free survival in the cohort of 451 of 475 patients, in which subcategorization into genomic AML classes was possible. A hazard ratio of >1 indicates a higher and a hazard ratio of <1 a lower hazard of death or event, respectively. The NPM1 class was used as reference group, allogeneic HCT in first remission as time-dependent variable. ECOG Eastern Cooperative Oncology Group, HCT hematopoietic cell transplantation, WBC white blood cell count.
genotype have been shown to be associated with high response rates and favorable long-term outcome [1]. Although these findings are very interesting and provide basis for further studies, they have to be interpreted cautiously due to small numbers. In line with previous reports, the \( \text{FLT3:DNMT3A:NPM1} \) genotype represented a common co-mutational pattern in our study. Data on the impact of \( \text{FLT3:DNMT3A:NPM1} \) genotypes are still discussed controversially—with some publications showing evidence of poor outcome whereas others do not [1, 28–30]. Differences may be explained by variations in cohort size and features (e.g., age distribution), treatment, and genotypes (\( \text{DNMT3A} \) total vs. \( \text{DNMT3A} \) non-R882 vs. R882, \( \text{FLT3} \) total vs. \( \text{FLT3-ITD} \)). In our study the \( \text{FLT3:DNMT3A:NPM1} \) genotype did not impact clinical outcome. However, the analysis might be hampered by the size and heterogeneity of this specific subpopulation.

Based on the co-mutational and cytogenetic data we categorized the majority of the cases into four of the recently defined genomic AML classes [1] and evaluated whether the underlying class impacted response to therapy with midostaurin. A strong beneficial effect was seen for midostaurin treatment in the \( \text{NPM1} \), chromatin-spliceosome and to a lesser extent “no class” groups. No impact of midostaurin was found for CBF- and \( \text{TP53} \)-aneuploidy groups which is likely attributable to the small group sizes. Overall, these results suggest a broad beneficial effect of midostaurin across various genetic backgrounds.

In conclusion, our targeted gene sequencing study in more than 450 patients with \( \text{FLT3} \) mutation entered on the randomized CALGB 10603/RATIFY trial provides important and novel insights into the genomic background of \( \text{FLT3} \)-mutated AML, on prognostic impact of mutations co-occurring with \( \text{FLT3} \) mutations as well as specific gene–gene interactions, and about possible treatment effects of midostaurin. Despite some limitations (e.g., the study was not powered for genetic subgroup analysis and results are of exploratory nature) our study provides the basis for future studies and validation of the findings in an independent dataset. Here, randomized trials comparing midostaurin with next-

### Table 2.

| Variable | Overall survival | Event-free survival |
|----------|-----------------|---------------------|
|          | HR [CI. 95]     | p value             | HR [CI. 95]     | p value             |
| Age      | 1.01 [0.99; 1.02] | 0.357               | 1.00 [0.99; 1.01] | 0.841               |
| Female   |                 |                     |                   |                     |
| Sex      | Male Reference  |                     |                   |                     |
| FLT3 mutation type | Reference |                     |                   |                     |
| ITD      | 1.15 [0.75; 1.76] | 0.533               | 1.36 [0.96; 1.93] | 0.085               |
| ECOG     | 1.27 [0.84; 1.91] | 0.261               | 0.90 [0.62; 1.31] | 0.595               |
| Log2WBC  | 1.08 [1.00; 1.17] | 0.046               | 1.08 [1.01; 1.16] | 0.018               |
| Allogeneic HCT in CR1 | 0.59 [0.41; 0.84] | 0.004               | 0.71 [0.48; 1.04] | 0.077               |
| NPM1     | Midostaurin vs. Placebo | 0.65 [0.45; 0.93] | 0.018 | 0.76 [0.57; 1.03] | 0.077 |
| CBF      | Midostaurin vs. Placebo | 1.37 [0.23; 8.23] | 0.729 | 0.88 [0.24; 3.29] | 0.853 |
| TP53-auneuploidy | Midostaurin vs. Placebo | 1.11 [0.32; 3.86] | 0.866 | 0.39 [0.15; 1.02] | 0.055 |
| Chromatin-Spliceosome | Midostaurin vs. Placebo | 0.52 [0.29; 0.92] | 0.026 | 0.48 [0.28; 0.82] | 0.007 |
| No Class | Midostaurin vs. Placebo | 0.55 [0.28; 1.07] | 0.079 | 0.64 [0.36; 1.13] | 0.123 |
| Placebo  | CBF vs. NPM1     | 0.46 [0.11; 1.92] | 0.289 | 0.77 [0.31; 1.91] | 0.569 |
| TP53-auneuploidy vs. NPM1 | 1.42 [0.51; 3.93] | 0.499 | 5.40 [2.58; 11.29] | <0.001 |
| Chromatin-Spliceosome vs. NPM1 | 2.52 [1.58; 4.03] | <0.001 | 2.38 [1.55; 3.66] | <0.001 |
| No Class vs. NPM1 | 2.31 [1.31; 4.05] | 0.004 | 2.71 [1.66; 4.44] | <0.001 |
| Midostaurin | CBF vs. NPM1 | 0.99 [0.31; 3.20] | 0.985 | 0.89 [0.32; 2.44] | 0.816 |
| TP53-auneuploidy vs. NPM1 | 2.45 [1.11; 5.42] | 0.027 | 2.74 [1.37; 5.47] | 0.004 |
| Chromatin-Spliceosome vs. NPM1 | 2.02 [1.22; 3.34] | 0.006 | 1.49 [0.96; 2.32] | 0.073 |
| No Class vs. NPM1 | 1.96 [1.14; 3.36] | 0.014 | 2.28 [1.48; 3.51] | <0.001 |

A hazard ratio of >1 indicates a higher and a hazard ratio of <1 a lower risk of death or event, respectively. CBF Core-binding factor AML, CI. 95% confidence interval, CR1 first complete remission, ECOG Eastern Cooperative Oncology Group, HR hazard ratio, HCT hematopoietic cell transplantation, WBC white blood cell count.
generation FLT3 inhibitors in patients with FLT3 mutations (e.g., NCT04027309) are currently underway and likely will provide additional valuable data with regard to the underlying genetic landscape and its clinical implications.

**DATA AVAILABILITY**
A Supplementary Appendix accompanies this paper on the Leukemia website. Mutational data are available as.xlsx file as part of the Supplementary Information. Raw data were generated at the University of Ulm and are available on request from the corresponding author (KD).

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AUTHOR CONTRIBUTIONS
NJ and EJ designed study, performed molecular analyses, analyzed data, and wrote the paper. MS, JK, and AB performed statistical analyses and wrote the paper. LB, RAL, TO, SA, TWP, JMB, FRA, BCM, MST, GE, MH, AG, CP, IG, CT, and RMS collected, assembled, analyzed, interpreted data, and reviewed the paper. CDB designed study, collected, assembled, analyzed, and interpreted data. HD and KD designed study, collected, assembled, analyzed, and interpreted data, and wrote the paper.

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