REGULAR ARTICLE

blood advances

Measurable residual disease monitoring in AML with *FLT3*-ITD treated with intensive chemotherapy plus midostaurin

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Key Points

- *FLT3*-ITD MRD identifies patients at high relapse risk after intensive chemotherapy with midostaurin.
- Conversion from *FLT3*-ITD MRD^{neg} to *FLT3*-ITD MRD^{pos} during follow-up was associated with a high relapse rate and inferior outcome.

Measurable residual disease (MRD) monitoring in acute myeloid leukemia (AML) with an *FLT3* internal tandem duplication (*FLT3*-ITD^{pos}) has been hampered by the broad heterogeneity of ITD mutations. Using our recently developed *FLT3*-ITD paired-end next-generation sequencing (NGS)–based MRD assay (limit of detection 10^{-4} to 10^{-5}), we evaluated the prognostic impact of MRD at different time points in 157 patients with *FLT3*-ITD^{pos} AML who were enrolled in the German-Austrian Acute Myeloid Leukemia Study Group 16-10 trial and who were treated with a combination of intensive chemotherapy and midostaurin, followed by midostaurin maintenance. MRD negativity (MRD^{neg}) after 2 cycles of chemotherapy (Cy2), which was observed in 111 of 142 (78%) patients, was predictive of superior 4-year rates of cumulative incidence of relapse (CIR) (4y-CIR; 26% vs 46%; P = .001) and overall survival (OS) (4y-OS; 70% vs 44%; P = .012). This survival advantage was also seen among patients who underwent allogeneic hematopoietic-cell transplantation during first complete remission (4y-CIR, 14% vs 39%; P = .001; 4y-OS, 71% vs 49%; P = .029). Multivariate models for CIR and OS after Cy2 revealed *FLT3*-ITD MRD^{neg} as the only consistent favorable variable for CIR (hazard ratio [HR], 0.29; P = .006) and OS (HR, 0.39; P = .018). During follow-up, conversion from MRD^{neg} to MRD positivity (MRD^{pos}) was a

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The full-text version of this article contains a data supplement.

strong, independent factor for inferior CIR (HR, 16.64; $P < .001$) and OS (HR, 4.05; $P < .001$). NGS-based *FLT3*-ITD MRD monitoring identifies patients at high risk for relapse and death following treatment with intensive chemotherapy and midostaurin. Using NGS-based technology.

Introduction

Internal tandem duplications of the *FLT3* gene (*FLT3*-ITD) are found in ~10% to 15% of adult patients with newly diagnosed acute myeloid leukemia (AML).[1-4] ITD mutations have been shown to be associated with poor prognosis because of a high relapse rate, in particular in cases with a high mutant to wild-type allelic ratio (AR; ≥0.5),[5-9] an insertion site located in the beta-1 sheet of tyrosine kinase domain-1,[9-11] and in patients without concomitant mutations in *NPM1*.[7-9,12,13]

In the 2022 European LeukemiaNet (ELN) risk classification, AML with *FLT3*-ITD (without adverse-risk genetic lesions) is now categorized as intermediate risk, irrespective of the AR or concurrent presence of *NPM1* mutation (*NPM1*^{mut}).[14] This revision to the 2017 ELN classification was based on methodologic issues with standardizing the assays for measurement of the *FLT3*-ITD AR, the modifying impact of midostaurin-based therapy on *FLT3*-ITD,[11,13,15] and the increasing role of measurable residual disease (MRD) in treatment decisions.[14,16] MRD monitoring allows response assessment and the early detection of relapse and therefore can be used for treatment decision-making and early intervention. In addition, MRD data have contributed significantly to refining relapse risk. Moreover, MRD monitoring is a powerful tool to assess kinetics and the depth of response during therapy, which is particularly informative for the evaluation of treatment effects within clinical trials that are investigating novel therapies.[14,16,17] Based on these many clinical implications, MRD is currently being considered to serve as a surrogate end point in clinical trials, which may accelerate the approval of new drugs.

The currently most widely used methods for MRD assessment are multiparameter flow cytometry and quantitative polymerase chain reaction (qPCR).[18] When compared with other molecular targets in AML, such as the recurrent gene fusions *RUNX1*::*RUNX1T1*, *CBFB*::*MYH11*, and *PML*::*RARA*, as well as *NPM1*^{mut}, *FLT3*-ITD MRD monitoring using qPCR has been hampered by the heterogeneity of the ITD mutation types that are determined by the broad variety of ITD lengths and insertion sites. Recent advances in next-generation sequencing (NGS) have been shown to overcome these limitations and now offers the opportunity for MRD monitoring in *FLT3*-ITD–positive (*FLT3*-ITD^{pos}) AML.[19-23] The 2021 update consensus document set forth by the ELN MRD Working Party acknowledged these efforts and included technical specifications for NGS-based MRD testing and integrative assessment of MRD, irrespective of technique.[16]

The objective of our study was to prospectively evaluate the prognostic impact of NGS-based MRD monitoring of *FLT3*-ITD in a cohort of 157 patients with *FLT3*-ITD^{pos} AML who received intensive chemotherapy in combination with midostaurin, followed by midostaurin maintenance within the German-Austrian Acute Myeloid Leukemia Study Group 16-10 (AMLSG16-10) treatment trial (ClinicalTrials.gov identifier: NCT01477606).[24,25]

Patients and methods

Patient selection

All patients were enrolled in the AMLSG16-10 treatment trial.[25] Patients were selected based on the following criteria: (1) achievement of complete remission (CR) or CR with incomplete blood count recovery (CRi) after 2 cycles of intensive chemotherapy (Cy2) combined with midostaurin, (2) availability of a diagnostic bone marrow (BM) or peripheral blood (PB) sample, and (3) ≥1 subsequent sample (BM after Cy2 and/or at end of treatment [EOT] and/or BM or PB during 3 to 12 months follow-up [FU]). Following these criteria, 157 patients were evaluated at diagnosis, 142 after Cy2, 116 at EOT, and 148 during the defined FU period (supplemental Figure 1). All patients gave informed consent according to the Declaration of Helsinki. Approval was obtained from the institutional review boards of the participating AMLSG institutions.

Molecular analyses

At the time of diagnosis, mutation analysis for *FLT3*-ITD and *NPM1*, as well as *NPM1*^{mut} MRD assessment, was performed as previously described.[15,17,26]

FLT3-ITD detection by targeted NGS

The NGS libraries were paired-end sequenced on an Illumina NGS platform according to the manufacturer's recommendation (Illumina, San Diego, CA) with minor modifications to our previously described assay that exhibited a variant allele frequency (VAF) sensitivity of 10^{-4} to 10^{-5}.[20] Details on the experimental procedures are available in the supplemental Data. The raw sequencing data were analyzed using the bioinformatics program *getITD*.[20]

Statistical analyses

CR/CRi, partial remission, overall survival (OS), relapse-free survival, and cumulative incidence of relapse (CIR) were defined according to standard criteria.[14] Using landmark analyses, survival times for the time point after Cy2 were calculated from the date of first CR (CR1) or from the date of allogeneic hematopoietic-cell transplantation (HCT) in CR1 and were determined from the date the MRD sample was obtained for the EOT and FU time points. Patients who did not experience the event of interest at the end of FU were censored at the date of last contact. CIR was computed according to the method described by Gray.[27] The median FU for survival was calculated using the reverse Kaplan-Meier estimate.[28] Logistic regression and Cox proportional hazards models were used to identify prognostic variables for CR/CRi and OS.[29] CIR was analyzed using cause-specific Cox models in which death during CR was considered a competing event.

Additional covariates in the multivariate analysis were sex, *FLT3*-ITD AR, and *NPM1* mutation status as dichotomous variables and BM blast count, white blood cell (WBC) count (log_{10} transformed), and age (10-year increase) as continuous variables; HCT during CR1 and MRD status over time were included as time-dependent variables. Missing values for covariates (WBC and BM blasts) were addressed by multiple imputation using chained equations. Mann-Whitney *U* tests were used to compare quantitative variables between patient subgroups; categorical variables were compared by means of Fisher exact tests. Associations between continuous variables were analyzed using the Spearman rank correlation coefficient. Survival distributions were estimated using the Kaplan-Meier method, and differences between groups were analyzed using 2-sided log-rank tests. An effect was considered significant if its *P* value was <5%. The analyses were not adjusted for multiple testing. All statistical analyses were performed using IBM SPSS Statistics 28.0.1.0, statistical software R (version 4.2.2), using the packages survival (version 3.5-0) and cmprsk (version 2.2-11), and/or GraphPad Prism7.

Results

Patient and disease characteristics

Table 1 summarizes the baseline characteristics of the 157 patients with *FLT3*-ITD^{pos} AML and of the 142 patients according to the *FLT3*-ITD MRD status after Cy2.

Paired BM and PB analysis

To determine tissue-dependency on VAF, we compared 29 paired PB and BM samples at diagnosis (n = 10) and after Cy2 (n = 19). Although the median *FLT3*-ITD VAF was slightly higher in BM than in PB (29.24% vs 23.59%; *P* = .065) at diagnosis, the median VAF (BM, 0.11% vs PB, 0.03%; *P* < .001) and MRD negativity (MRD^{neg}; BM, 0/19 [0%] vs PB, 7/19 [37%]; *P* = .008) differed significantly after Cy2 (supplemental Figure 2), clearly indicating the higher sensitivity in BM. Therefore, subsequently, only BM samples were selected for MRD assessment after Cy2 and at EOT. PB samples were analyzed only at diagnosis (n = 37) and during FU (n = 29).

NGS-based assessment of *FLT3*-ITD at diagnosis

A total of 465 ITDs were identified in 157 patients with *FLT3*-ITD^{pos} AML at diagnosis. Of the 465 ITDs, the median ITD length was 51 nucleotides (range, 9-285) and the median ITD VAF was 0.312% (0.006-92.26) (supplemental Figure 3). In total, 108 patients (69%) exhibited >1 ITD (median, 2; range, 1-16). The median total ITD VAF per patient (determined as sum of individual ITD VAFs) was 31.54% (0.46-92.26). Total ITD VAF per patient was correlated positively with higher WBC count (Rho, 0.287; *P* < .001), BM blast count (Rho, 0.196; *P* = .020), PB blast count (Rho, 0.264; *P* = .001), and lactate dehydrogenase level (Rho = 0.316; *P* < .001) and correlated inversely with the number of ITD clones (Rho, −0.226; *P* = .004). There was no correlation with age, sex, or *NPM1* mutation status. The NGS-based calculated ITD AR ($\sum$VAF/[100 − $\sum$VAF]) correlated positively with AR as determined by a GeneScan analysis (Rho, 0.855; *P* < .001; supplemental Figure 4).

At diagnosis, *FLT3*-ITD VAF a as log_2 transformed continuous variable did not have an impact on OS (hazard ratio [HR], 1.15; 95% confidence interval [CI], 0.92-1.42; *P* = .218) or CIR (HR, 0.95; 95% CI, 0.78-1.17; *P* = .646).

Prognostic impact of *FLT3*-ITD MRD during therapy

The median FU time of the 157 patients with *FLT3*-ITD^{pos} AML was 47.4 months (95% CI, 40.9-53.9); 59 of the 157 (37.6%) patients died. The median OS and 2-year OS rate were 68.9 months (95% CI, 51.5% to not applicable) and 0.72 (95% CI, 0.64-0.79), respectively. Allogeneic HCT during CR1 was performed in 122 of 157 (78%) patients and was performed at any time during the disease course in 135 of 157 (86%) patients.

Impact of FLT3-ITD MRD after Cy2. All *FLT3*-ITDs identified after Cy2 were already detectable at diagnosis. In relation to diagnosis, the median log_{10} reduction in the total *FLT3*-ITD VAF was 4.6 (range, 0.42-5.17; *P* < .001); *FLT3*-ITD MRD^{neg}, defined as undetectable *FLT3*-ITD, was achieved in 111 of 142 (78%) patients (Figure 1A). Patients with *FLT3*-ITD MRD^{neg} and MRD positivity (MRD^{pos}) differed significantly in terms of concurrent *NPM1*^{mut} and the ELN 2017 risk classification (Figure 1B; Table 1). The only favorable factor for achievement of *FLT3*-ITD MRD^{neg} after Cy2 was concurrent *NPM1*^{mut} (odds ratio [OR], 10.45; 95% CI, 3.40-32.07; *P* < .001); adverse factors were WBC count (OR for 10-fold increase, 0.31; 95% CI, 0.10-0.92; *P* = .035) and the administration of a second induction cycle (administered in 37 patients who achieved partial remission only after the first induction; OR, 0.19; 95% CI, 0.06-0.58; *P* = .004; supplemental Table 1).

A higher log_{10} reduction in *FLT3*-ITD VAF was significantly associated with a lower CIR rate (HR for 10-fold better VAF reduction, 0.56; 95% CI, 0.43-0.71; *P* < .001) and improved OS (HR, 0.75; 95% CI, 0.60-0.93; *P* = .010; supplemental Table 2).

We next examined the prognostic impact of MRD^{neg}, which was achieved in 111 of 142 (78%) patients. In the univariate analysis, achieving MRD^{neg} was predictive of a superior 4-year CIR rate (4y-CIR; 26% vs 46% for MRD^{pos}; HR, 0.33; 95% CI, 0.17-0.64; *P* = .001) and 4-year OS (4y-OS; 70% vs 44%; HR, 0.47; 95% CI, 0.26-0.85; *P* = .012) (Figure 2A-B; supplemental Table 2). Figure 3 illustrates the course of events for every individual patient according to *FLT3*-ITD MRD status after Cy2. Of note, for patients with *FLT3*-ITD MRD^{pos} status, the risk for relapse correlated with the MRD burden, in particular ≥0.1%, the threshold provisionally used to define NGS-MRD test positivity according to the 2021 ELN MRD Working Party (Figure 4; supplemental Table 2). We additionally evaluated the impact of a ≥3 log_{10} reduction in *FLT3*-ITD VAF (MR$^{3.0}$). Achieving MR$^{3.0}$ after Cy2 was associated with a lower CIR rate (4y-CIR, 30% vs 54%; HR, 0.31; 95% CI, 0.16-0.62; *P* < .001) but not with improved OS (4y-OS, 68% vs 45%; HR, 0.58; 95% CI, 0.30-1.10; *P* = .097) (supplemental Figure 5; supplemental Table 2).

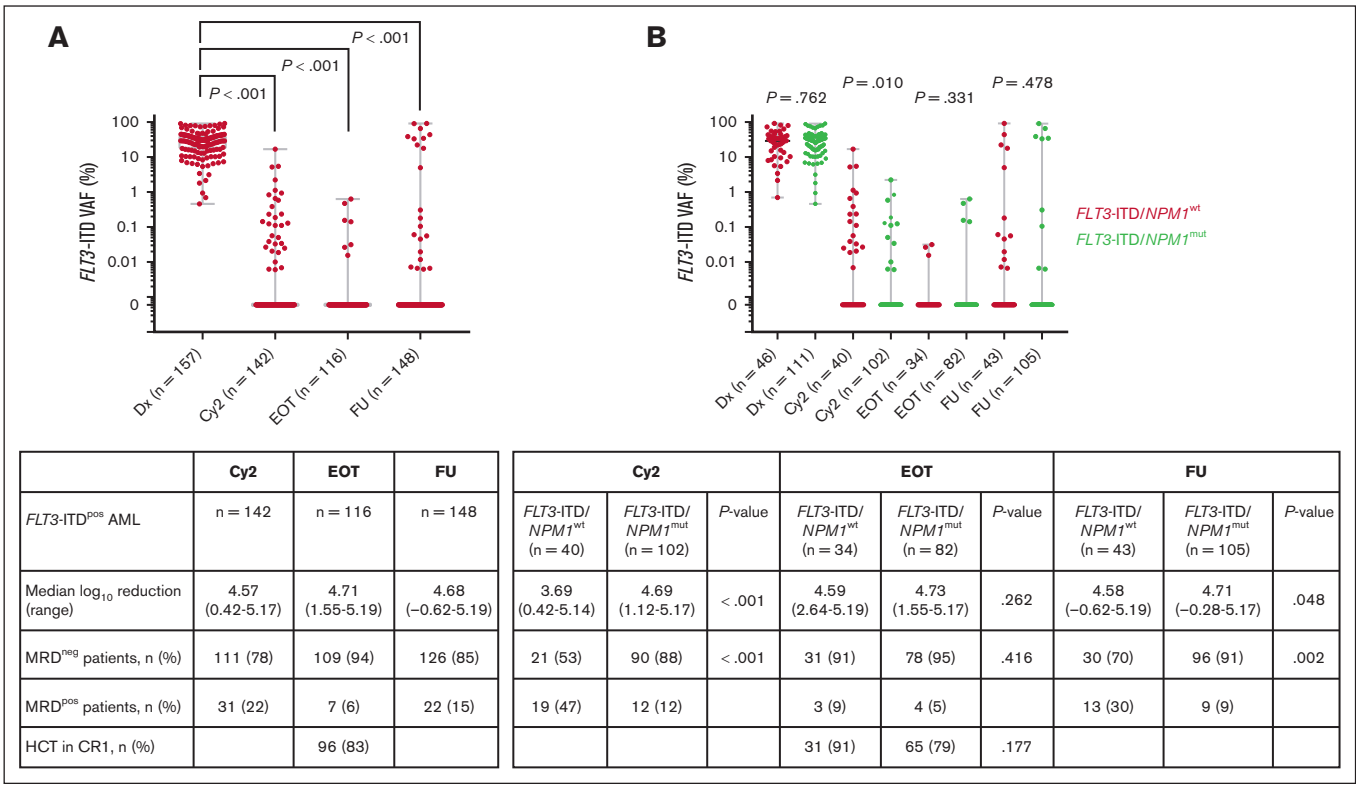
Next, we performed a multivariate Cox regression analysis in different models that included the *FLT3*-ITD MRD log_{10} reduction, an *FLT3*-ITD VAF <0.1%, achievement of MR$^{3.0}$, or *FLT3*-ITD MRD^{neg} status. In all models, a higher log_{10} reduction, FLT3-ITD VAF <0.1%, achievement of MR$^{3.0}$, and MRD^{neg} were the only consistent favorable variables for risk of relapse and OS with the exception of MR$^{3.0}$ for OS. *NPM1*^{mut} and HCT during CR1 were favorable factors only for CIR (Table 2; supplemental Table 3).

Table 1. Characteristics of the 157 patients with *FLT3*-ITD-positive AML according to MRD status after Cy2 (n = 142)

Clinical and genetic variables	All patients (N = 157)	MRD^{neg} Cy2 (n = 111)	MRD^{pos} Cy2 (n = 31)	*P* value
Age (y), median (range)	54 (20-70)	54 (20-70)	51 (25-70)	.229
Sex, n (%)				
Male	69 (44)	45 (40)	17 (55)	.219
Female	88 (56)	66 (60)	14 (45)	
WBC (10^9/L)				
Median (range)	51.8 (0.5-356.4)	46.3 (0.5-356.4)	67.8 (1.1-279.6)	.056
Missing	2	1	0	
Hemoglobin (g/dL)				
Median (range)	9.3 (4.1-15.0)	9.2 (4.1-15.0)	9.6 (5.6-13.8)	.553
Missing	2	1	0	
Platelets (10^9/L)				
Median (range)	60 (5-352)	56 (5-352)	59 (13-148)	.547
Missing	2	1	0	
BM blasts (%)				
Median (range)	81 (0-100)	85 (0-100)	83 (20-100)	.564
Missing	18	13	5	
PB blasts (%)				
Median (range)	46 (0-99)	42 (0-99)	53 (0-98)	.364
Missing	10	8	0	
AML type, n (%)				
De novo	142 (90)	100 (90)	29 (94)	.388
Secondary	8 (5)	6 (5)	0	
Therapy-related	7 (5)	5 (5)	2 (6)	
ELN 2017 risk classification, n (%)				
Favorable	31 (20)	22 (20)	4 (13)	<.001
Intermediate	100 (64)	79 (72)	15 (48)	
Adverse	25 (16)	9 (8)	12 (39)	
Missing	1	1	0	
***FLT3*-ITD AR, n (%)**				
Low (<0.5)	67 (43)	47 (42)	12 (39)	.837
High (≥0.5)	90 (57)	64 (58)	19 (61)	
***FLT3*-TKD, n (%)**				
Yes	6 (4)	2 (2)	3 (10)	.069
No	151 (96)	109 (98)	28 (90)	
Mutated *NPM1*, n (%)				
Yes	111 (71)	90 (81)	12 (39)	<.001
No	46 (29)	21 (19)	19 (61)	

TKD, tyrosine kinase domain.

Impact of FLT3-ITD MRD before allogeneic HCT. Of the 142 patients assessed for *FLT3*-ITD MRD after Cy2, 107 patients underwent HCT during CR1 with 84 patients undergoing HCT immediately after Cy2 and 23 patients after receiving additional consolidation therapy with high-dose cytarabine. In total, 81 of 107 patients (76%) were *FLT3*-ITD MRD^{neg} before HCT; the median time from MRD assessment after Cy2 and HCT was 30 days (range, 7-88). *FLT3*-ITD MRD^{pos} before HCT was associated with an increased risk for relapse (4y-CIR, 39% vs 14%; HR, 4.90; 95% CI, 1.87-12.83; $P = .001$) and inferior outcome (4y-OS, 49% vs 71%; HR, 2.17; 95% CI, 1.06-4.43; $P = .029$) (Figure 2C-D). As shown in supplemental Figure 6, patients with *FLT3*-ITD MRD^{pos} status before HCT had a comparable outcome as those who were *FLT3*-ITD MRD^{neg} after Cy2 and were treated with conventional consolidation. Of the 26 patients with *FLT3*-ITD MRD^{pos} status before HCT, 21 patients were also eligible at EOT, and 15 (71%) patients achieved *FLT3*-ITD MRD^{neg} after HCT.



	Cy2	EOT	FU
FLT3-ITD^{pos} AML	n = 142	n = 116	n = 148
Median log_{10} reduction (range)	4.57 (0.42-5.17)	4.71 (1.55-5.19)	4.68 (−0.62-5.19)
MRD^{neg} patients, n (%)	111 (78)	109 (94)	126 (85)
MRD^{pos} patients, n (%)	31 (22)	7 (6)	22 (15)
HCT in CR1, n (%)		96 (83)	

Cy2			EOT			FU		
FLT3-ITD/*NPM1*^{wt} (n = 40)	*FLT3*-ITD/*NPM1*^{mut} (n = 102)	*P*-value	*FLT3*-ITD/*NPM1*^{wt} (n = 34)	*FLT3*-ITD/*NPM1*^{mut} (n = 82)	*P*-value	*FLT3*-ITD/*NPM1*^{wt} (n = 43)	*FLT3*-ITD/*NPM1*^{mut} (n = 105)	*P*-value
3.69 (0.42-5.14)	4.69 (1.12-5.17)	< .001	4.59 (2.64-5.19)	4.73 (1.55-5.17)	.262	4.58 (−0.62-5.19)	4.71 (−0.28-5.17)	.048
21 (53)	90 (88)	< .001	31 (91)	78 (95)	.416	30 (70)	96 (91)	.002
19 (47)	12 (12)		3 (9)	4 (5)		13 (30)	9 (9)	
			31 (91)	65 (79)	.177			

Figure 1. Kinetics of *FLT3*-ITD VAF during treatment and early FU. (A) For the entire cohort of 157 *FLT3*-ITD^{pos}, ITD VAF significantly decreased during therapy and during early FU. *FLT3*-ITD MRD^{neg} was achieved in 78% after Cy2, 94% at EOT, and 85% during FU. (B) Patients separated according to *NPM1*^{mut} status, demonstrating a significantly higher VAF reduction after Cy2 and higher frequencies of MRD^{neg} for concurrent *NPM1*^{mut} patients. Dx, diagnosis; wt, wild-type.

Impact of FLT3-ITD MRD at EOT. All *FLT3*-ITDs detected at EOT were already detectable at diagnosis. When compared with diagnosis, the median log_{10} reduction in the total *FLT3*-ITD VAF was 4.7 (range, 1.55-5.19; $P < .001$); *FLT3*-ITD MRD^{neg} status was achieved in 109 of 116 (94%) patients (Figure 1A). In the Cox regression analysis, MRD^{neg} status at EOT was significantly associated with improved OS and trended toward a reduced risk for relapse, likely because of the high rate of patients with *FLT3*-ITD MRD^{neg} statusat EOT (Table 2).

FLT3-ITD MRD monitoring during FU

To assess the risk for relapse after completion of intensive therapy, we analyzed MRD at least at 1 time point between 3 and 12 months after EOT during FU in 148 patients (BM, n = 119; PB, n = 29). Of these 148 patients, 117 (79%) had started maintenance with midostaurin with a median of 9 cycles (range, 1-12). Overall, 22 (15%) patients were *FLT3*-ITD MRD^{pos} during FU (all *FLT3*-ITDs were detected at diagnosis; Figure 3C). *FLT3*-ITD MRD persistence, defined as MRD^{pos} at all time points (range, 3-4) assessed, was detected in 6 patients and all relapsed; 16 patients (including 9 under maintenance) converted from *FLT3*-ITD MRD^{neg} to MRD^{pos} (median VAF, 0.058%; range, 0.006%-91.965%) within 5.78 months (median time from last MRD^{neg} to first MRD^{pos} sample; range, 2.07-10.0 months). Of the 16 patients, 13 (81%) (including 7 after HCT) relapsed within a median time of 7 days (measured from time point of MRD conversion in FU [MRD^{conv_FU}] to hematologic relapse; range, 0-197 days), translating into a significantly increased relapse risk (2y-CIR, 81% vs 16%; HR, 11.00; 95% CI, 5.44-22.28; $P < .001$) and inferior OS (2y-OS, 31% vs 80%; HR, 4.31; 95% CI, 2.22-8.38; $P < .001$) (supplemental Figure 7). Three of the 16 converted patients remained in continuous remission after allogeneic HCT, and all 3 exhibited low *FLT3*-ITD MRD levels (<0.008%); 2 of them received maintenance with midostaurin, and all 3 became MRD negative later on.

To analyze the impact of the *FLT3*-ITD MRD status over time, we performed 2 Cox regression models with MRD status as a time-dependent covariate. One model considered the impact of MRD^{pos} at any time point (after Cy2, at EOT, and/or during FU) and the second addressed MRD conversion (MRD^{conv}, MRD^{neg} to MRD^{pos}) after achievement of MRD^{neg} after Cy2. In both models, MRD^{pos} was the only consistent unfavorable variable for risk of relapse and OS (Table 3).

Impact of concurrent *NPM1* mutation on *FLT3*-ITD MRD

In total, 111 of the 157 (71%) patients had concomitant *NPM1*^{mut}. Concurrent *NPM1*^{mut} favorably impacted the log_{10} VAF reduction (median, 4.7 vs 3.7 for *NPM1*^{wt}; $P < .001$) and the achievement of *FLT3*-ITD MRD^{neg} status (88% vs 53%; $P < .001$) after Cy2 (Figure 1B). This translated into a lower CIR rate (4y-CIR *FLT3*-ITD MRD^{neg}/*NPM1*^{mut} vs *FLT3*-ITD MRD^{neg}/*NPM1*^{WT}, 19% vs 68%; HR, 0.23; 95% CI, 0.10-0.50; $P < .001$) and a trend toward improved OS (4y-OS, 72% vs 59%; HR, 0.52; 95% CI, 0.25-1.09; $P = .071$; supplemental Figure 8). No additional benefit was observed for *NPM1*^{mut} at

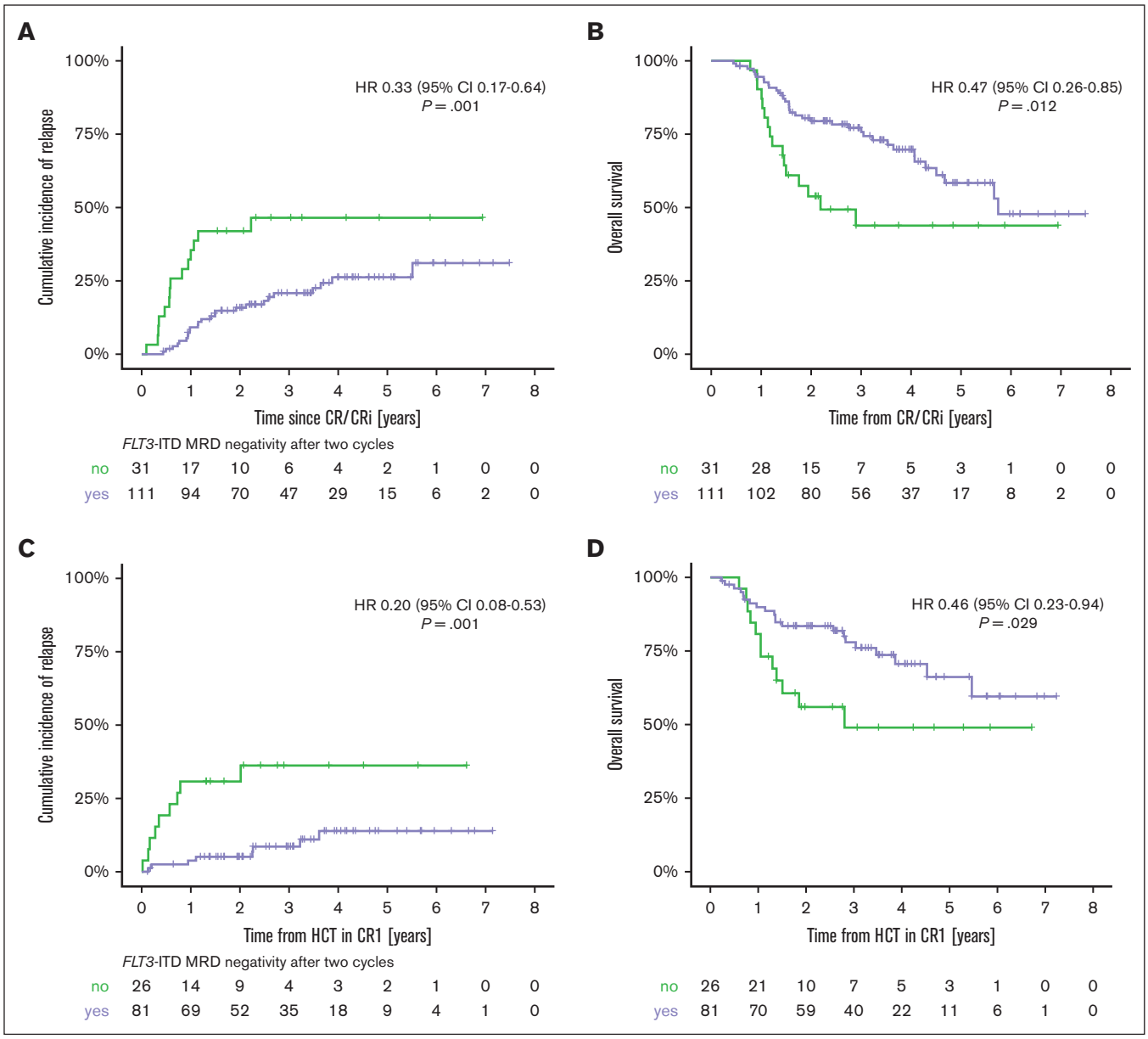


Figure 2. Prognostic impact of *FLT3*-ITD MRD^{neg} after Cy2. (A) Marginal Aalen-Johansen plot of CIR and (B) marginal Kaplan-Meier plot of OS for the 142 *FLT3*-ITD^{pos} patients during CR according to *FLT3*-ITD MRD status. (C) CIR and (D) OS according to *FLT3*-ITD MRD status before HCT in CR1. HRs and 95% CIs are given for *FLT3*-ITD MRD^{neg} status.

EOT, but *NPM1*^{mut} was associated with *FLT3*-ITD MRD^{neg} during FU (91% vs 70%; $P = .002$; Figure 1B).

Comparative analysis of *FLT3*-ITD and *NPM1*^{mut} MRD assessment

According to the ELN MRD Working Party, in *NPM1*^{mut} AML, MRD should be assessed, preferentially in PB, after Cy2 and in BM at EOT and during FU. In line with this recommendation, we correlated NGS-based *FLT3*-ITD MRD assessed in BM with qPCR-based *NPM1*^{mut} MRD assessed in PB after Cy2 (Figure 5A). Of the 82 eligible patients, 41 (50%) were *FLT3*-ITD MRD^{neg}/*NPM1*^{mut} MRD^{neg}, 33 (40%) were *FLT3*-ITD MRD^{neg}/*NPM1*^{mut} MRD^{pos}, 7 (9%) were *FLT3*-ITD MRD^{pos}/*NPM1*^{mut} MRD^{pos}, and 1 (1%) was *FLT3*-ITD MRD^{pos}/*NPM1*^{mut} MRD^{neg}. It should be noted that an *FLT3*-ITD MRD^{neg} status was associated with a lower relapse risk and improved outcome irrespective of *NPM1*^{mut} MRD status (Figure 5B-C). Because of the small number of events in this subgroup (relapses, n = 13; deaths, n = 14) Cox regression analysis on relapse-free survival was performed and confirmed the beneficial impact of *FLT3*-ITD MRD^{neg} status regardless of the *NPM1*^{mut} MRD status (supplemental Table 4).

At EOT and during FU, an *FLT3*-ITD MRD^{neg} status was more frequent than an *NPM1*^{mut} MRD^{neg} status. In addition, all 3 patients with *FLT3*-ITD MRD^{pos} statuswere also *NPM1*^{mut} MRD^{pos} at EOT.

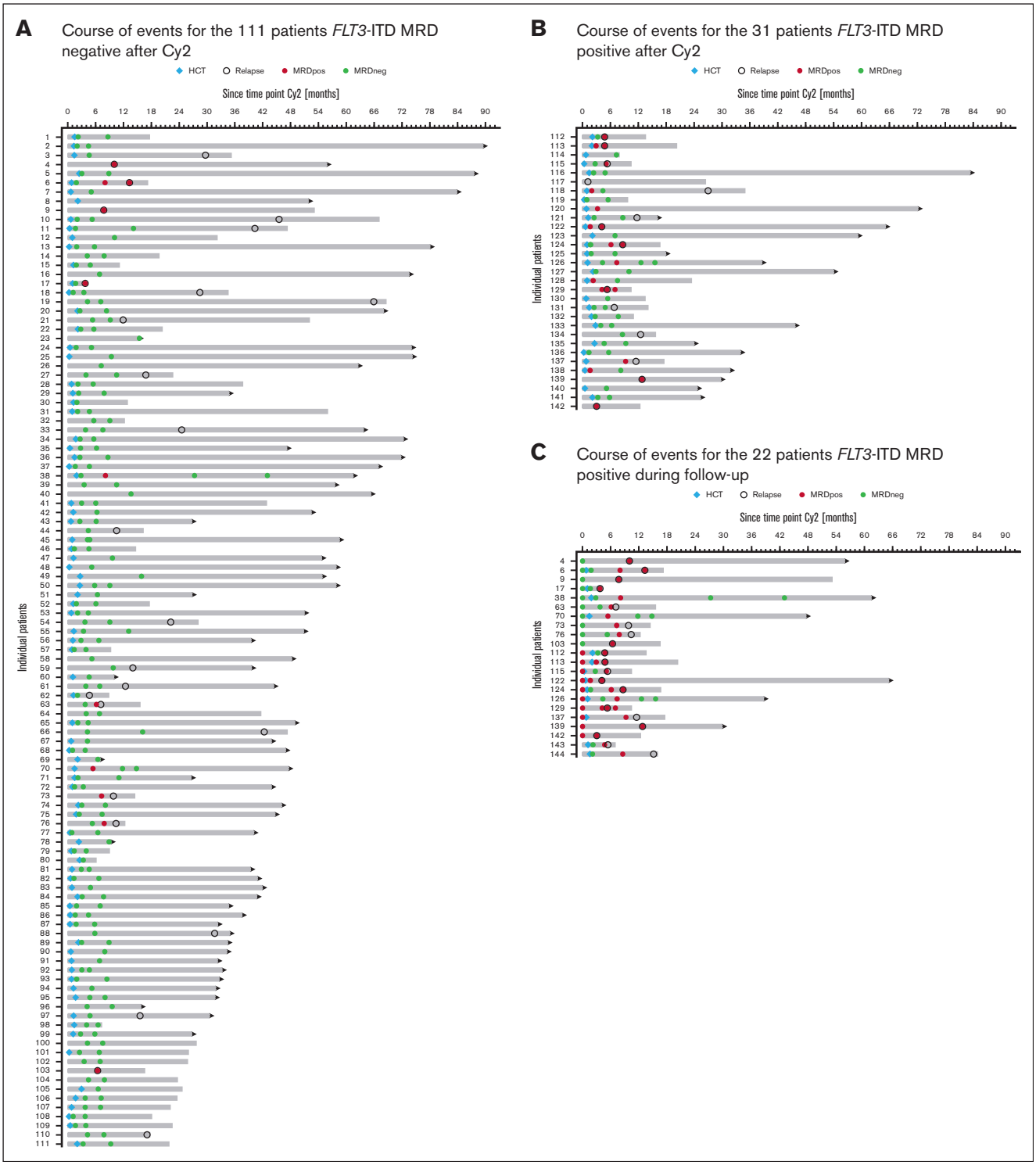
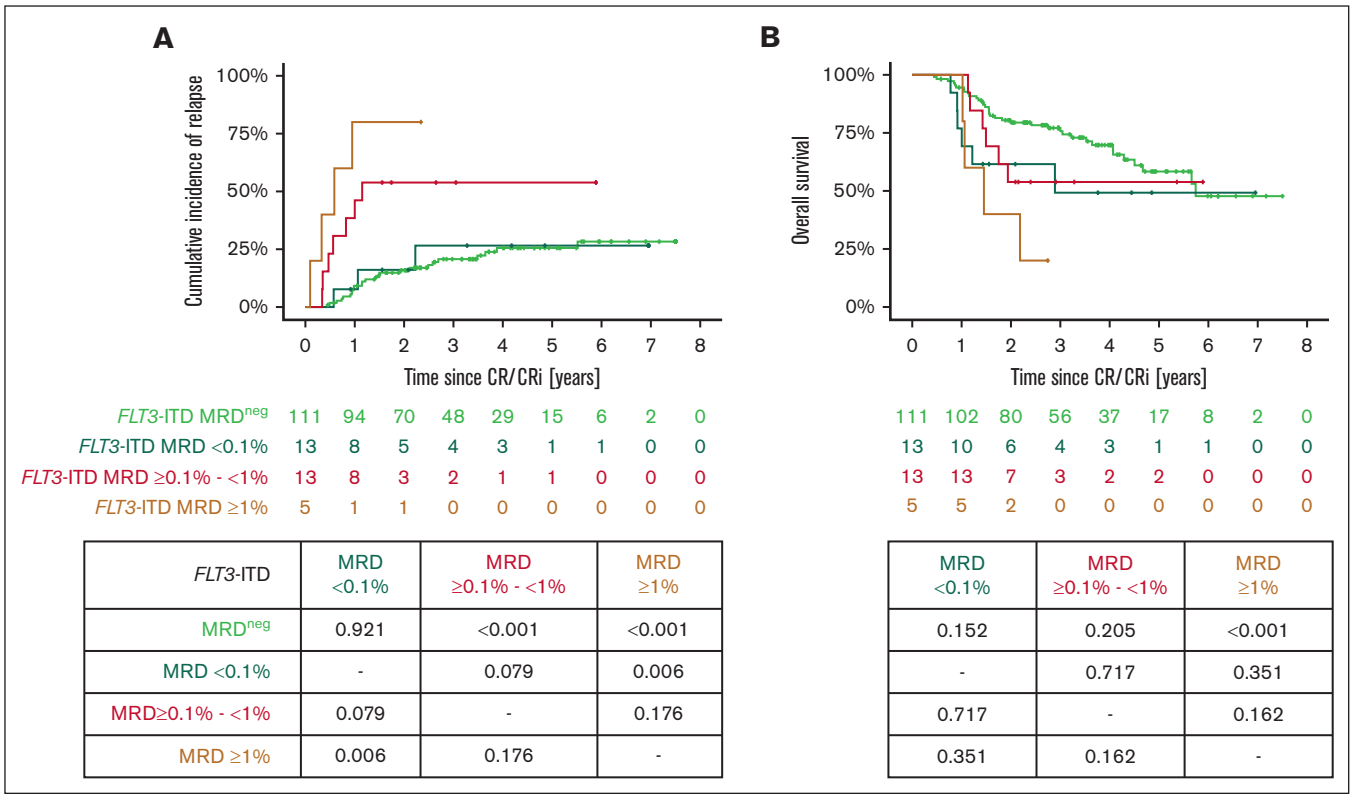


Figure 3. Individual disease and treatment course after Cy2 according to *FLT3*-ITD MRD status. (A) Course of events for the 111 patients who were *FLT3*-ITD MRD negative after Cy2 and (B) for the 31 patients who were *FLT3*-ITD MRD positive after Cy2. (C) Course of events for the 22 patients who were *FLT3*-ITD MRD positive during FU.



FLT3-ITD MRD^{neg}	111	94	70	48	29	15	6	2	0
FLT3-ITD MRD <0.1%	13	8	5	4	3	1	1	0	0
FLT3-ITD MRD ≥0.1% - <1%	13	8	3	2	1	1	0	0	0
FLT3-ITD MRD ≥1%	5	1	1	0	0	0	0	0	0

FLT3-ITD	MRD <0.1%	MRD ≥0.1% - <1%	MRD ≥1%
MRD^{neg}	0.921	<0.001	<0.001
MRD <0.1%	-	0.079	0.006
MRD≥0.1% - <1%	0.079	-	0.176
MRD ≥1%	0.006	0.176	-

111	102	80	56	37	17	8	2	0
13	10	6	4	3	1	1	0	0
13	13	7	3	2	2	0	0	0
5	5	2	0	0	0	0	0	0

MRD <0.1%	MRD ≥0.1% - <1%	MRD ≥1%
0.152	0.205	<0.001
-	0.717	0.351
0.717	-	0.162
0.351	0.162	-

Figure 4. Outcome according to *FLT3*-ITD MRD cutoffs after Cy2. CIR (A) and OS (B) according to various *FLT3*-ITD MRD cutoffs after Cy2. Results of pairwise comparisons are provided below the x-axis.

Furthermore, of the 30 patients with *NPM1*^{mut} MRD^{pos}, 26 exhibited *NPM1*^{mut} MRD at low level provisionally defined as <2%. Similar findings were also observed during FU. The proportion of *FLT3*-ITD MRD^{neg}/*NPM1*^{mut} MRD^{neg} increased from 50% after Cy2 to 68% during FU (Figure 5).

Discussion

In this study, we performed *FLT3*-ITD MRD monitoring in 157 adult patients with *FLT3*-ITD^{pos} AML using a highly sensitive NGS-based assay as previously reported by us.[20] The data revealed that *FLT3*-ITD MRD was a highly significant risk factor for relapse and OS and even outperformed known risk factors, such as *NPM1* mutational status and *FLT3*-ITD AR.

The particular strengths of our study are twofold. First, sequential biosampling was done prospectively within a controlled clinical trial at defined time points, that is, after Cy2, at the EOT, and during FU. Second, all patients received intensive chemotherapy in combination with the FLT3 inhibitor midostaurin, which has improved outcomes and is now considered standard of care in patients with *FLT3*-ITD^{pos} AML.[15,25] Recently, addition of the second-generation FLT3 inhibitor quizartinib to intensive chemotherapy has also been approved for the treatment of patients with newly diagnosed *FLT3*-ITD^{pos} AML.

Three recently published studies reported on the clinically relevant impact of *FLT3*-ITD MRD monitoring using NGS-based assays.[21-23] In contrast with our study, these analyses were restricted to a single time point (after 1 or 2 induction cycles) and were performed in heterogeneous patient cohorts, particularly with respect to treatment with an additional FLT3 inhibitor.[21-23] Furthermore, 2 of these studies evaluated the impact of *FLT3*-ITD MRD specifically before HCT; the Pre-MEASURE study analyzed the PB of 608 patients with *FLT3*-ITD^{pos} AML before HCT during CR1[23] and the study by Loo et al and Dillon et al analyzed samples from 104 patients with *FLT3*-ITD^{pos} AML, irrespective of CR1, CR2, or molecular relapse.[22] In the HOVON study, 93 of 161 (58%) intensively treated patients underwent HCT during CR1.[21] Regarding concurrent *NPM1*^{mut}, the prevalence of 75% in the study by Loo et al and Dillon et al was comparable with ours (71%) and lower than that in the HOVON (57%) and Pre-MEASURE studies (58%).

After 2 cycles of intensive chemotherapy is the first time point at which MRD assessment in the BM is considered to be clinically relevant.[16] In our study, a higher reduction in *FLT3*-ITD VAF and achieving *FLT3*-ITD MRD^{neg} after Cy2 in the BM were statistically significant favorable prognostic factors in terms of both relapse risk and OS and therefore enable a refined risk assessment of patients in hematologic response. A concurrent *NPM1* mutation and HCT during CR1 were additional significant favorable factors for CIR, whereas *FLT3*-ITD MRD^{neg} was the only significant factor for OS. Concurrent *NPM1*^{mut} correlated with a deeper molecular response, as reflected in a better *FLT3*-ITD VAF reduction and a significantly higher rate of *FLT3*-ITD MRD^{neg} after Cy2.

Table 2. Multivariate analyses used to determine the prognostic significance of *FLT3*-ITD MRD^{neg} at various landmarks

	CIR		OS	
Clinical and genetic variables	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Landmark		Cy2 (n = 142)		
Age (10 y-increase)	0.95 (0.71-1.27)	.727	1.42 (1.07-1.89)	.017
Female	0.61 (0.32-1.15)	.123	0.63 (0.36-1.09)	.097
WBC (log_{10})	1.18 (0.69-2.05)	.534	0.68 (0.38-1.23)	.200
BM blasts	1.00 (0.98-1.02)	.890	1.00 (0.99-1.02)	.684
NPM1^{mut}	0.31 (0.14-0.68)	.005	0.85 (0.45-1.59)	.599
FLT3-ITD^{high}	1.02 (0.52-2.01)	.948	1.12 (0.61-2.07)	.711
HCT in CR1*	0.13 (0.05-0.30)	<.001	0.64 (0.37-1.12)	.117
FLT3-ITD MRD^{neg}	0.29 (0.13-0.69)	.006	0.39 (0.18-0.85)	.018
Landmark		EOT (n = 116)		
Age (10 y-increase)	0.98 (0.71-1.36)	.900	1.73 (1.27-2.35)	<.001
Female	0.61 (0.30-1.25)	.175	0.64 (0.36-1.14)	.127
WBC (log_{10})	1.10 (0.61-2.00)	.743	0.82 (0.49-1.37)	.448
BM blasts	1.00 (0.98-1.02)	.955	0.99 (0.98-1.01)	.354
NPM1^{mut}	0.32 (0.14-0.76)	.009	0.82 (0.42-1.60)	.568
FLT3-ITD^{high}	1.03 (0.45-2.36)	.948	0.96 (0.50-1.85)	.901
HCT in CR1*	0.19 (0.08-0.44)	<.001	0.59 (0.30-1.17)	.130
FLT3-ITD MRD^{neg}	0.34 (0.09-1.20)	.093	0.30 (0.09-0.98)	.046
Landmark		FU (3-12 mo) (n = 148)		
Age (10 y-increase)	1.03 (0.80-1.35)	.826	1.24 (0-95-1.629)	.109
Female	0.48 (0.24-0.95)	.035	0.62 (0.35-1.11)	.107
WBC (log_{10})	0.74 (0.40-1.354)	.321	0.69 (0.40-1.19)	.180
BM blasts	1.00 (0.99-1.02)	.749	1.00 (0.99-1.02)	.626
NPM1^{mut}	0.53 (0.25-1.12)	.094	0.95 (0.47-1.90)	.879
FLT3-ITD^{high}	1.12 (0.52-2.39)	.774	0.99 (0.51-1.93)	.9477
HCT in CR1	0.12 (0.05-0.32)	<.001	0.81 (0.40-1.62)	.541
FLT3-ITD MRD^{neg}	1		1	
FLT3-ITD MRD^{conv_FU}	16.64 (6.52-42.48)	<.001	4.05 (1.78-9.18)	<.001
FLT3-ITD MRD^{pers}	51.98 (14.75-183.15)	<.001	3.69 (1.26-10.82)	.017

FLT3-ITD^{high}, *FLT3*-internal tandem duplication with AR ≥0.5; MRD^{conv_FU}, conversion from MRD negative at the last previously assessed time point to MRD positive at the time point FU; MRD^{pers}, persistent MRD^{pos}.

*As time-dependent variable.

Our observation that *FLT3*-ITD MRD^{neg} status after Cy2 is of high prognostic relevance is in line with the data recently published by the HOVON group.[21] In their study, achievement of MRD^{neg} after 2 induction cycles identified patients with a significantly lower 4y-CIR rate (33% vs 75%; $P < .001$) and improved OS (4y-OS, 57% vs 31%; $P < .001$). Similar results were reported for the QuANTUM-first study that showed that patients who achieved CR/composite CR after Cy2 and MRD levels $<10^{-4}$ had a significantly improved OS when compared with patients above that MRD cutoff.[30] The current ELN Working Party on MRD proposed a cutoff of 0.1% for NGS-based MRD assessment but also stated that this cutoff is provisional and not based on robust data. As shown in the 3 published studies and this study, the evaluation of different cutoffs is limited by the small patient numbers that underline the current uncertainty regarding thresholds and highlight the need for future studies (eg, meta-analysis) to define this.[16,21-23] For MRD-based prognostication and prediction and for comparability between different study populations and techniques, the value of cutoffs beyond MRD^{neg}, such as the log reduction in the transcript level between diagnosis and after induction, are under evaluation in clinical trials, as are other well-established MRD targets.[16]

In *FLT3*-ITD^{pos} AML, concurrent *NPM1*^{mut} has been shown to be a favorable prognostic factor for all survival endpoints.[7,8,12,13,22] Consistent with the HOVON data, concurrent *NPM1*^{mut} was associated with a significantly higher percentage of *FLT3*-ITD MRD^{neg} after Cy2. Although, in the HOVON study, identical relapse rates were observed for the entire *FLT3*-ITD MRD^{neg} cohort and the *FLT3*-ITD MRD^{neg}/*NPM1*^{mut} cohort (4y-CIR 33% each), we found a lower CIR rate for *FLT3*-ITD MRD^{neg}/*NPM1*^{mut} patients than for the entire cohort of patients with *FLT3*-ITD MRD^{neg} (4y-CIR, 19% vs 29%), underlining the favorable effect of a concurrent *NPM1*^{mut} in terms of achieving a deeper molecular response.

Table 3. Multivariate analyses used to determine the prognostic significance of *FLT3*-ITD MRD status over time

	CIR		OS	
Clinical and genetic variables	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Patients	N = 157			
Events	n = 35		n = 59	
Age (10 y-increase)	0.99 (0.96-1.02)	.644	1.03 (1.01-1.06)	.016
Female	0.36 (0.17-0.76)	.007	0.57 (0.33-0.97)	.038
WBC (log_{10})	0.91 (0.48-1.74)	.779	0.67 (0.40-1.12)	.129
BM blasts	1.00 (0.98-1.01)	.769	1.00 (0.99-1.01)	.882
NPM1^{mut}	0.34 (0.16-0.71)	.004	0.90 (0.49-1.65)	.727
FLT3-ITD^{high}	1.16 (0.51-2.64)	.715	1.28 (0.69-2.36)	.437
HCT in CR1	0.17 (0.08-0.38)	<.001	0.95 (0.50-1.82)	.885
FLT3-ITD MRD^{pos}*	6.83 (2.72-17.13)	<.001	3.32 (1.69-6.54)	<.001
Patients	n = 110			
Events	n = 21		n = 36	
Age (10 y-increase)	0.99 (0.95-1.03)	.597	1.03 (0.99-1.07)	.106
Female	0.34 (0.13-0.89)	.028	0.48 (0.24-0.95)	.034
WBC (log_{10})	0.82 (0.36-1.87)	.646	0.56 (0.29-1.09)	.087
BM blasts	0.99 (0.97-1.02)	.603	1.00 (0.99-1.02)	.630
NPM1^{mut}	0.42 (0.13-1.28)	.126	1.00 (0.39-2.58)	.998
FLT3-ITD^{high}	1.52 (0.50-4.66)	.464	1.30 (0.57-2.93)	.534
HCT in CR1	0.09 (0.03-0.27)	<.001	1.30 (0.59-2.86)	.508
FLT3-ITD MRD^{conv}*	9.94 (2.24-44.22)	.003	2.83 (0.98-8.18)	.055

FLT3-ITD^{high}, *FLT3*-internal tandem duplication with AR ≥0.5; MRD^{conv}, conversion from MRD negative after Cy2 of intensive chemotherapy to MRD positive over time; MRD^{pos}, MRD positive at any time point.
*As time-dependent variable.

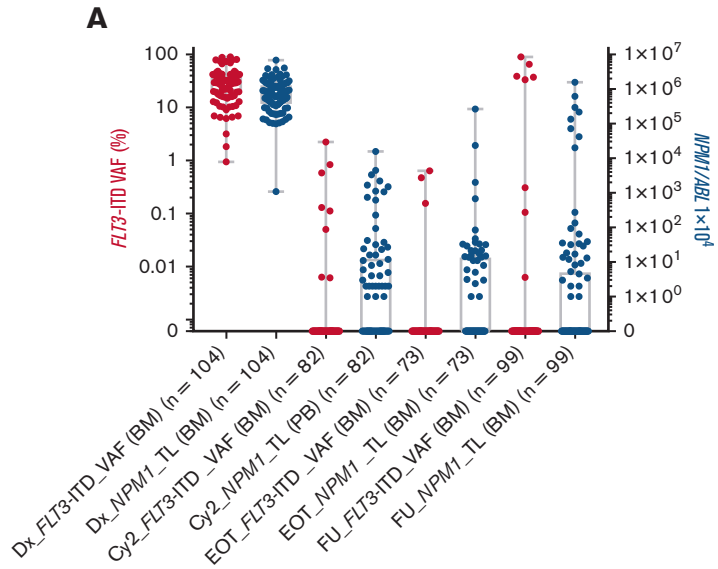
Allogeneic HCT has been shown to improve the outcome of patients with *FLT3*-ITD^{pos} AML.[8,9,13,14] In our study, HCT during CR1 was an independent favorable factor for CIR at all time points, demonstrating that HCT during CR1 is an important pillar for treatment of *FLT3*-ITD^{pos} AML. Of note, *FLT3*-ITD MRD status before HCT provided an additional prognostic impact. An *FLT3*-ITD MRD^{neg} status before HCT was associated with a lower risk for relapse and superior outcome (Figure 2). In line with the recently published studies that demonstrated the prognostic impact of *FLT3*-ITD MRD^{neg} status before HCT and considering previous studies on various molecular MRD markers, these data underline the prognostic value of molecular remission before HCT.[21-23,31-34] Moreover, recent results from the MORPHO study demonstrated that *FLT3*-ITD MRD^{neg} before and after HCT was associated with improved outcomes and only patients who were MRD positive significantly benefited from maintenance treatment with gilteritinib.[35]

The proportion of patients who achieved *FLT3*-ITD MRD^{neg} status after Cy2/before HCT varied slightly across the published cohorts (63%,[22] 71%,[21] and 86%[23]) and our cohorts (78%). Differences might be explained by patient characteristics and the tissues assessed, the various prevalence rates of concurrent *NPM1*^{mut}, and/or the additional treatment with midostaurin in our cohort.

To our knowledge, this was the first study to evaluate the impact of *FLT3*-ITD MRD status over time and MRD conversion during early FU. In patients with *FLT3*-ITD MRD conversion (MRD^{neg} to MRD^{pos}) during FU, we observed subsequent relapses in 13 of 16 (81%) patients, which translated into a significantly increased relapse risk. However, the *FLT3*-ITD MRD status was not systematically evaluated during the FU period, and *FLT3*-ITD MRD monitoring for relapse surveillance needs to be confirmed in additional studies. The 3 patients who converted to MRD^{pos} during FU but who remained in continuous remission exhibited very low VAF levels. This reflects the clinical challenge to interpret MRD^{pos} status during FU and underscores the indispensability to discriminate molecular persistence at (very) low VAF level from molecular progression and molecular relapse by analyzing a second sample as recommended by the ELN MRD Working Party.[16]

In line with the 2 previous studies, combined *FLT3*-ITD and *NPM1*^{mut} MRD positivity was associated with a poor outcome.[21,22] Favorable outcomes were observed for patients with *FLT3*-ITD MRD^{neg} status irrespective of the *NPM1*^{mut} MRD status. This implies that within *FLT3*-ITD^{pos}/*NPM1*^{mut} AML, the *FLT3*-ITD MRD status may further refine the individual prognosis by better discrimination of patients at high and low risk for relapse than when using *NPM1*^{mut} MRD detection alone.

Considering the different assays used for *FLT3*-ITD MRD monitoring, an international consensus on standardizing the approach is needed, which is also currently discussed in the ELN MRD Working Party.



	Cy2 (n = 82)		EOT (n = 73)		FU (n = 99)	
	FLT3-ITD MRD	*NPM1* MRD	*FLT3*-ITD MRD	*NPM1* MRD	*FLT3*-ITD MRD	*NPM1* MRD
Median $\log_{10}$ reduction (range)	4.70 (1.12-5.17)	5.94 (1.67-7.56)	4.73 (1.55-5.17)	6.31 (0.71-7.84)	4.71 (−0.28-5.17)	6.55 (−0.47-7.84)
MRD^{neg} patients, n (%)	74 (90)	42 (51)	70 (96)	43 (59)	91 (92)	67 (68)
MRD^{pos} patients, n (%)	8 (10)	40 (49)	3 (4)	30 (41)	8 (8)	32 (32)
FLT3-ITD MRD^{neg}/*NPM1*^{mut} MRD^{neg} pts, n (%)	41 (50)		43 (59)		67 (68)	

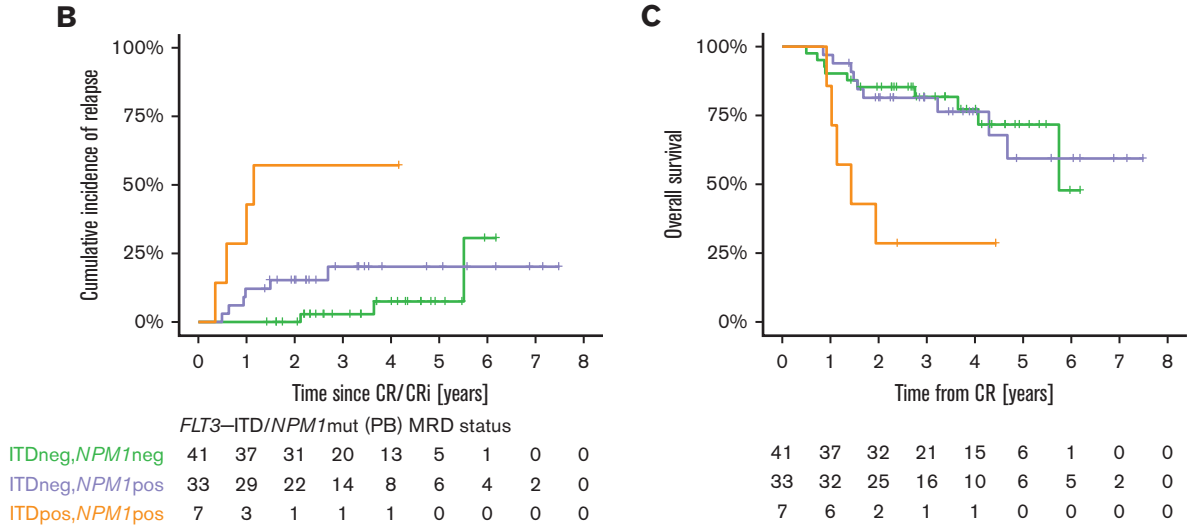


Figure 5. Paired *FLT3*-ITD and *NPM1*^{mut} MRD status. (A) MRD kinetics of *FLT3*-ITD and *NPM1*^{mut} and corresponding frequencies of MRD^{neg} status of respective pts during therapy and early FU. (B) CIR and (C) OS according to *FLT3*-ITD MRD and *NPM1*^{mut} MRD status after Cy2 (1 pt with *FLT3*-ITD MRD^{pos}/*NPM1*^{mut} MRD^{neg} was excluded). Dx, diagnosis; pts, patients.

In summary, beyond the known risk factors, NGS-based *FLT3*-ITD MRD monitoring allows for the identification of patients at high risk for relapse and death. Currently, randomized studies that are comparing midostaurin and second-generation FLT3 inhibitors are ongoing (eg, NCT04027309). *FLT3*-ITD MRD analysis will be informative in assessing whether these more selective inhibitors will increase the depth of molecular remission and if these deeper responses are associated with an improved outcome.

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Authorship

Contribution: F.G.R., L.B., H.D., and K.D. designed the study; S.C., S.S., T.J.L., A.C., V.I.G., A.M., S.A., and F. Theis performed the experiments and validated the data; F.G.R., J.K., E.P., and A.B. performed the statistical analyses; F.G.R., D.W., F.S., A.S., W.F., H.R.S., G.W., H.S., T.S., K.S.G., M.W.M.K., M.L., R.F.S., F. Thol, M.H., A.G., H.D., and K.D. collected, assembled, analyzed and interpreted data; F.G.R., H.D., and K.D. wrote the first draft of the manuscript; and all authors undertook manuscript writing, editing and approval, revised the manuscript, and reviewed and approved the final version.

Conflict-of-interest disclosure: F.G.R. reports receiving honoraria from and serving as a consultant for Jazz Pharmaceuticals, Novartis, and Bristol Myers Squibb (BMS)/Celgene, and receiving travel support from Jazz Pharmaceuticals. L.B. reports receiving honoraria from AbbVie, Amgen, Astellas, BMS/Celgene, Daiichi Sankyo, Gilead, Janssen, Jazz Pharmaceuticals, Menarini, Novartis, Pfizer, Roche, and Sanofi, and receiving research support from Bayer and Jazz Pharmaceuticals. V.I.G. reports serving in an advisory role for Jazz Pharmaceuticals, AbbVie, and Boehringer Ingelheim; serving on the speakers' bureau of Pfizer, Janssen, and AbbVie; and receiving travel support from AbbVie. F.S. reports receiving honoraria from and serving as a consultant for AOP Orphan Pharmaceuticals, MorphoSys, BMS/Celgene, Incyte, Novartis, and Pfizer. W.F. reports receiving personal fees and nonfinancial support from AbbVie; receiving grants, personal fees, and nonfinancial support from Amgen and Pfizer; receiving personal fees from Jazz Pharmaceuticals, Celgene, MorphoSys, Incyte, Stemline Therapeutics, Clinigen, Daiichi Sankyo, Otsuka, and Servier outside the submitted work; receiving research support from Apis; filing a patent with Amgen; and receiving support for medical writing for Amgen, Pfizer, and AbbVie. H.S. reports receiving honoraria from AbbVie, Amgen, AstraZeneca, BMS/Celgene, Genzyme, GlaxoSmithKline, Janssen, Oncopeptides, Pfizer, Roche, Sanofi, Stemline Therapeutics, and Takeda, and travel expenses from Amgen, BMS/Celgene, Janssen, and Sanofi. K.S.G. reports serving in an advisory role for BMS, Jazz Pharmaceuticals, Pfizer, and AbbVie. M.W.M.K. reports receiving honoraria from and serving as a consultant for Pfizer, Kura Oncology, Jazz Pharmaceuticals, BMS/Celgene, and AbbVie, and serving on the speakers bureau of Gilead. M.L. reports serving in an advisory role for AbbVie, Astex Pharmaceuticals, Imago BioSciences, Janssen, Otsuka, and Syros, and receiving research support from Janssen and Cheplapharm. R.F.S. reports serving in an advisory role or as a consultant for Daiichi Sankyo, Pfizer, Astellas, and Novartis; receiving research funding from PharmaMar, AstraZeneca, Pfizer, Roche, Boehringer Ingelheim, Daiichi Sankyo, and Recordati; and receiving funding for travel, accommodation, and expenses from Daiichi Sankyo. F. Thol reports serving in an advisory role for Novartis, BMS, AbbVie, Menarini, and Rigel. M.H. reports receiving honoraria from Certara, Jazz Pharmaceuticals, Janssen, Novartis, and Sobi; serving as a paid consultant for AbbVie, Amgen, BMS/Celgene, Glycostem, Delbert Lab, Pfizer, Pinotbio, and Servier; and receiving research funding to his institution from AbbVie, Agios, Astellas, BMS/Celgene, Glycostem, Jazz Pharmaceuticals, Karyopharm, Loxo Oncology, and Pinotbio. H.D. reports serving in an advisory role for AbbVie, Agios, Amgen, Astellas, AstraZeneca, Berlin-Chemie, BMS/Celgene, Daiichi Sankyo, GEMoaB, Gilead, Janssen, Jazz Pharmaceuticals, Novartis, Servier, Stemline Therapeutics, and Syndax, and receiving research funding from AbbVie, Agios, Amgen, Astellas, BMS/Celgene, Jazz Pharmaceuticals, Kronos Bio, Novartis, and Pfizer. K.D. reports serving in an advisory role for Amgen, BMS/Celgene, Daiichi Sankyo, Janssen, Jazz Pharmaceuticals, Novartis, and Roche, and receiving research funding from Agios, Astex, Astellas, BMS/Celgene, and Novartis. The remaining authors declare no competing financial interests.

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