Next-generation sequencing-based measurable residual disease detection predicts outcomes in patients with acute myeloid leukemia undergoing allogeneic stem cell transplantation

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Abstract

Sensitive, scalable and affordable measurable residual disease (MRD) assays are needed to guide treatment decisions in acute myeloid leukemia (AML), particularly around allogeneic hematopoietic stem cell transplantation (allo-SCT). Next-generation sequencing (NGS)-based MRD assays offer broad applicability and high sensitivity, but remain too costly for routine use in resource-limited environments. We developed a cost-efficient, sensitive NGS MRD assay utilizing single-molecule molecular inversion probes (smMIP) targeting 92 genomic loci in 33 AML driver genes and applied it to 93 AML patients in remission prior to allo-SCT. MRD positivity, defined as the presence of ≥1 non-DTA (*DNMT3A, TET2, ASXL1*) gene variant with ≥0.5% variant allele frequency (VAF), was associated with significantly shorter post-transplantation overall survival (OS; *P*<0.001). In multivariable analysis, NGS-based MRD detection remained an independent predictor of inferior OS (hazard ratio=4.58; *P*=0.002). Conditioning intensity did not associate with outcome of MRD-positive patients in this retrospective cohort. Mutations at diagnosis and pre-transplantation showed variable concordance across genes, and consistently lower VAF at the later time point. In three patients, multiple low-VAF clustered variants in *RUNX1* and *TET2* were detected pre-transplantation, potentially indicating treatment-induced mutagenesis. These findings demonstrate that a broadly applicable, smMIP-based NGS MRD assay can provide clinically relevant risk stratification before allo-SCT in AML, while its low library preparation costs of approximately €8 per sample may facilitate wide implementation in routine practice and allow more patients to receive MRD-directed therapeutic interventions.

Introduction

Measurable residual disease (MRD), i.e., the persistence of detectable leukemia cells in patients with acute myeloid leukemia (AML) in complete remission (CR), predicts increased cumulative incidence of relapse (CIR) and shorter overall survival (OS).[1] MRD detection assays have therefore emerged as critical tools for assessing treatment response, predicting outcomes and guiding therapy in AML, and are specifically relevant for the decision to pursue allogeneic stem cell transplantation (allo-SCT).[2-4] Peri-transplantation MRD positivity predicts inferior outcomes after allo-SCT, and emerging data suggest that MRD-positive (MRD pos) patients may benefit from intensified, MRD-directed strategies before or after transplant.[5-7]

Nevertheless, reliable MRD assessment remains challenging, as currently available methods vary in sensitivity, applicability and standardization. Multiparameter flow cy-

tometry and quantitative polymerase chain reaction (qPCR) are the most well-established methods for assessing MRD and provide important prognostic information, but each has limitations.[2,4] While flow cytometry can be applied to most patients, it is often less sensitive than qPCR, difficult to standardize across laboratories, and (for leukemia-associated immunophenotype-based approaches) requires a pre-treatment specimen.[4] qPCR offers superior sensitivity if recurrent molecular alterations, such as *NPM1* mutations or gene fusions, are found at initial diagnosis (Dx), but covering multiple or rare targets can be complex and time consuming.[8-10] Next-generation sequencing (NGS)-based assays have therefore emerged as an attractive alternative, enabling parallel, sensitive interrogation of many leukemia-associated genetic regions in a large proportion of AML patients.[11,12]

Use of unique molecular identifiers (UMI) and computational error correction further improves discrimination of low-frequency true variants from NGS artifacts, allowing detection of single nucleotide variants and short insertions/deletions at sensitivities down to 10^{-5} while maintaining broad applicability.[13] However, limited availability, relatively high costs, and the challenge to distinguish true MRD from non-leukemic clonal hematopoiesis (CH) still impede routine clinical implementation of NGS-based MRD assays.

Accurate interpretation of NGS-based MRD requires discrimination between persistent leukemia-specific mutations and CH-related variants. CH is an age-associated clonal expansion of hematopoietic cells without overt hematological malignancy that increases the risk of progression to AML and can precede diagnosis of hematological malignancy by years.[14,15] Importantly, pre-leukemic CH clones can persist in AML patients achieving CR.[16] Persistence of the most common CH-related mutations in *DNMT3A, TET2*, and *ASXL1* ("DTA" mutations) during CR generally does not adversely affect outcome, but significance of other CH-associated variants is less clear.[12,17-19] Recently, the European LeukemiaNet (ELN)-DAVID MRD working party updated their recommendations to not only exclude DTA-variants but all age-related CH-variants as markers of MRD before allo-SCT, underscoring the need to distinguish CH from residual leukemia when interpreting molecular MRD.[4,20]

Here, we report a novel, cost efficient and highly sensitive NGS-based MRD assay utilizing single-molecule molecular inversion probes (smMIP) to target genetic regions recurrently mutated in AML. smMIP represent a probe-based targeted sequencing approach in which each captured DNA molecule is tagged with an UMI, enabling consensus-based error correction while maintaining highly multiplexed target enrichment and broad, high-throughput genomic coverage.[21] This approach was applied to a cohort of patients undergoing allo-SCT to evaluate its prognostic value.

Patients and methods

Study population

We used samples from 93 consecutively enrolled adult AML patients with adequate material who underwent allo-SCT at our institution between 2003 and 2022. Patients were in first or subsequent morphologic remission (CR; CR with incomplete hematologic recovery [CRi] or morphologic leukemia-free state [MLFS]) and had a pre-transplant bone marrow sample obtained within 28 days prior to allo-SCT. Median follow-up for survivors was 35 months. All patients signed written informed consent to participate in biosampling and data analysis in accordance with the Declaration of Helsinki. The study was approved by the institutional ethics committee (reference number 363/16-ek).

Analysis of genomic variants via next-generation sequencing

A smMIP-based assay was used to screen genomic DNA from bone marrow for genetic mutations, as previously described,[22] with modifications detailed below and in the Online Supplementary Appendix. In contrast to amplicon-based targeted sequencing, this approach combines probe-based capture with integrated molecular barcoding. Compared to our previous panel, additional smMIP were designed using MIPgen[23] to expand coverage to further genes (*BCOR, CHEK2, ETNK1, EZH2, GATA2, GNAS, NF1, SETBP1, ZBTB33*), while ensuring coverage of both DNA strands of all target regions with a minimum probe overlap of five bases (*Online Supplementary Table S1*). The extended assay covers 92 regions across 33 AML- and CH-associated genes (approximately 25 kb; *Online Supplementary Table S2*). Target regions were selected to capture at least one informative variant in ≥95% of patients in a previously published, intensively treated AML cohort.[24] All samples were sequenced and analyzed in a single batch. Median sequencing depth per base was 24,467x before and 402x after UMI deduplication. Unique coverage ≥100x was achieved for 79.5% of target bases, and ≥200x for 63.4%. Based on these metrics, a variant allele frequency (VAF) threshold of ≥ 0.5% was set for variant calling.

Statistical analysis

Clinical and molecular data were analyzed to evaluate associations between MRD status and outcomes. Patients with detectable non-DTA mutations (MRD pos) were compared to those with no or DTA-only mutations (MRD-negative [MRD neg]) using the Wilcoxon rank-sum test for continuous and Fisher's exact test for categorical variables. Median VAF of variants detected exclusively at Dx or pre-transplant (pre-Tx), as well as concordantly or discordantly mutated genes, were compared using the Wilcoxon rank-sum test. When appropriate, *P* values were adjusted for multiple testing using the Benjamini-Hochberg method, with q values <0.05 considered statistically significant. OS was analyzed using Kaplan-Meier estimates and log-rank tests; CIR and non-relapse mortality (NRM) were estimated in competing risk analyses using the Aalen-Johansen method and com-

pared between groups with Gray's test. A multivariable Cox proportional hazards regression model was fitted including variables associated with OS at an univariable $P \leq 0.10$; stepwise backward variable selection (fastbw function, rms package[25]) was used to identify factors significantly associated with OS. The proportional hazards assumption was assessed using Schoenfeld residuals. The model was internally validated using bootstrap resampling (B=1,000) with backward selection repeated within each bootstrap sample. Statistical analyses were performed using R (version 4.4.1[26]); color palettes were optimized for color vision deficiency.

Results

Cohort characteristics and mutational profile

A total of 93 patients with AML in morphologic remission prior to allo-SCT were included in this retrospective study (37 female, 56 male; Table 1). The median age was 59 years (range, 14-75) at Dx and 60 years (range, 21-75) at allo-SCT.

Table 1. Characteristics of 93 patients included in this study, according to measurable residual disease status.

Variable	Overall	MRD neg	MRD pos	*P*
N of patients	93	48	45	-
Age at diagnosis, years, median (range)	59 (14-75)	59 (14-74)	60 (32-75)	0.3[1]
Age at transplantation, years, median (range)	60 (21-75)	59 (21-74)	60 (32-75)	0.3[1]
Sex female, N (%)	37 (40)	18 (38)	19 (42)	0.7[2]
AML type, N (%)				0.9[2]
de novo AML	65 (70)	33 (69)	32 (71)	
sAML	18 (19)	9 (19)	9 (20)	
tAML	10 (11)	6 (13)	4 (8.9)	
Remission status, N (%)				0.044[2]
CR1	70 (75)	40 (83)	30 (67)	
CRi1	6 (6.5)	1 (2.1)	5 (11)	
MLFS1	2 (2.2)	0 (0)	2 (4.4)	
CR2	12 (13)	7 (15)	5 (11)	
CRi2	3 (3.2)	0 (0)	3 (6.7)	
Blood counts at diagnosis, median (range)				
White blood cell count, $\times 10^9$/L	4.1 (0.7-385.0)	3.9 (0.7-98.0)	5.1 (0.9-385.0)	0.6[1]
Blasts in PB, %	26 (0-97)	26 (0-88)	31 (1-97)	0.8[1]
Blasts in BM, %	54 (8-93)	56 (20-92)	52 (8-93)	0.9[1]
Hemoglobin, g/dL	8.5 (4.0-13.3)	8.4 (4.0-13.2)	8.7 (5.5-13.3)	0.8[1]
Platelets, $\times 10^9$/L	60 (9-517)	56 (9-501)	60 (13-517)	0.3[1]
ELN 2022 classification, N (%)				0.5[2]
Favorable	10 (14)	7 (19)	3 (8.8)	
Intermediate	30 (42)	15 (41)	15 (44)	
Adverse	31 (44)	15 (41)	16 (47)	
Unknown	22	11	11	
Conditioning intensity, N (%)				0.019[2]
MAC	26 (28)	18 (38)	8 (18)	
RIC	21 (23)	13 (27)	8 (18)	
NMA	46 (49)	17 (35)	29 (64)	
Acute GVHD grade, N (%)				0.4[2]
Absent - 0	59 (69)	33 (75)	26 (62)	
Limited - 1/2	18 (21)	7 (16)	11 (26)	
Extensive - 3/4	9 (10)	4 (9.1)	5 (12)	
Unknown	7	4	3	
Chronic GVHD grade, N (%)				0.2[2]
Absent - 0	27 (38)	18 (47)	9 (27)	
Limited - 1	15 (21)	6 (16)	9 (27)	
Extensive - 2	29 (41)	14 (37)	15 (45)	
Unknown	22	10	12	

[1]Wilcoxon rank-sum test; [2]Fisher's exact test. AML: acute myeloid leukemia; BM: bone marrow; CR: complete remission; Cri: complete remission with incomplete count recovery; ELN: European LeukemiaNet; GVHD: graft-*versus*-host disease; MAC: myeloablative conditioning; MLFS: morphologic leukemia-free state; MRD: measurable residual disease; NMA: non-myeloablative conditioning; PB: peripheral blood; RIC: reduced-intensity conditioning; sAML: secondary AML; tAML: therapy-related AML.

Ninety-one patients received intensive induction therapy, two patients received lower-intensity regimens, reflecting the fact that all transplantations were performed in 2022 or earlier (*Online Supplementary Appendix; Online Supplementary Table S3*). According to ELN 2022, baseline risk was favorable for ten, intermediate for 30, and adverse for 31 patients; risk could not be assigned for 22 because of incomplete baseline genetics. Most patients underwent allo-SCT in first complete remission (CR/CRi; N=76) or morphologic leukemia-free state (MLFS1, N=2), and 15 were transplanted in second remission. Conditioning regimens comprised myeloablative (MAC, N=26), reduced-intensity (RIC, N=21), and non-myeloablative (NMA, N=46) protocols.

Targeted sequencing of pre-Tx bone marrow samples detected 238 variants in 73 of 93 patients (78%; *Online Supplementary Table S4*). The most frequently mutated genes were *TET2* (75 variants in 38 patients), *DNMT3A* (48 in 36), *PPM1D* (20 in 14), *IDH2* (12 in 11) and *ASXL1* (8 in 8; Figure 1). Among patients with variants, the median number of mutations per patient was 2 (range, 1-21), with a median VAF of 1.9% and a mean VAF of 10.8% (*Online Supplementary Figure S1*). Overall, 131 variants affected the CH-related "DTA" genes (*DNMT3A, TET2, ASXL1*); 28 patients (30%) had only DTA variants, whereas 45 (48%) had at least one non-DTA gene mutation.

Concordance of mutations between diagnosis and pre-transplantation

NGS data from both Dx and pre-Tx was available for 46 patients, covering 24 shared genomic regions. Sequencing at Dx had been performed using several different assays (detailed in the *Online Supplementary Appendix*), which varied in sensitivity and locus coverage. Of 111 variants identified at Dx, 39 were also detected pre-Tx (*Online Supplementary Tables S4* and *S5*). Because multiple distinct variants within the same gene were frequently observed, mutation concordance was assessed on a per-gene basis: genes were classified as concordantly mutated when identical mutations were present at both time points, discordantly mutated when ≥1 mutation was lost or newly acquired, and non-informative when not covered at Dx (Figure 2A). Overall, 17 of 46 patients carried at least one concordantly mutated gene. Among 133 mutated genes in this analysis across all 46 patients, 60 were mutated at Dx only, 33 at pre-Tx only, and 29 were concordantly and 11 discordantly mutated (Figure 2B). Most genes mutated at Dx only were non-DTA genes (53/60), whereas discordant status was largely observed in DTA genes (10/11; Figure 2C). Pre-Tx only and concordantly mutated status were observed at comparable frequency in DTA and non-DTA genes. Considering mutation status at either time point, *TET2* (N=23/46) was most frequently mutated, followed by *DNMT3A* (N=18), *IDH2* (N=13) and *NPM1* (N=8; Figure 2D). Several AML-associated genes including *NPM1* (N=8), *FLT3* (N=7), *NRAS* (N=4) and *BCOR* (N=4) were mutated at Dx only, whereas *IDH2* (7/13) and *SRSF2* were concordantly mutated in roughly half of cases, and WT1 (4/5) was predominantly mutated pre-Tx. In contrast, mutations in DTA genes *TET2* (N=12) and *DNMT3A* (N=5) were frequently pre-Tx only or discordantly mutated (N=5, both). Across all genes, VAF were significantly lower pre-Tx than at Dx, regardless of whether they were detected in genes mutated at a single time point only (median, 30.7% *vs*. 1.5%; q<0.0001), concordantly mutated (median, 45.1% *vs*. 19.6%;

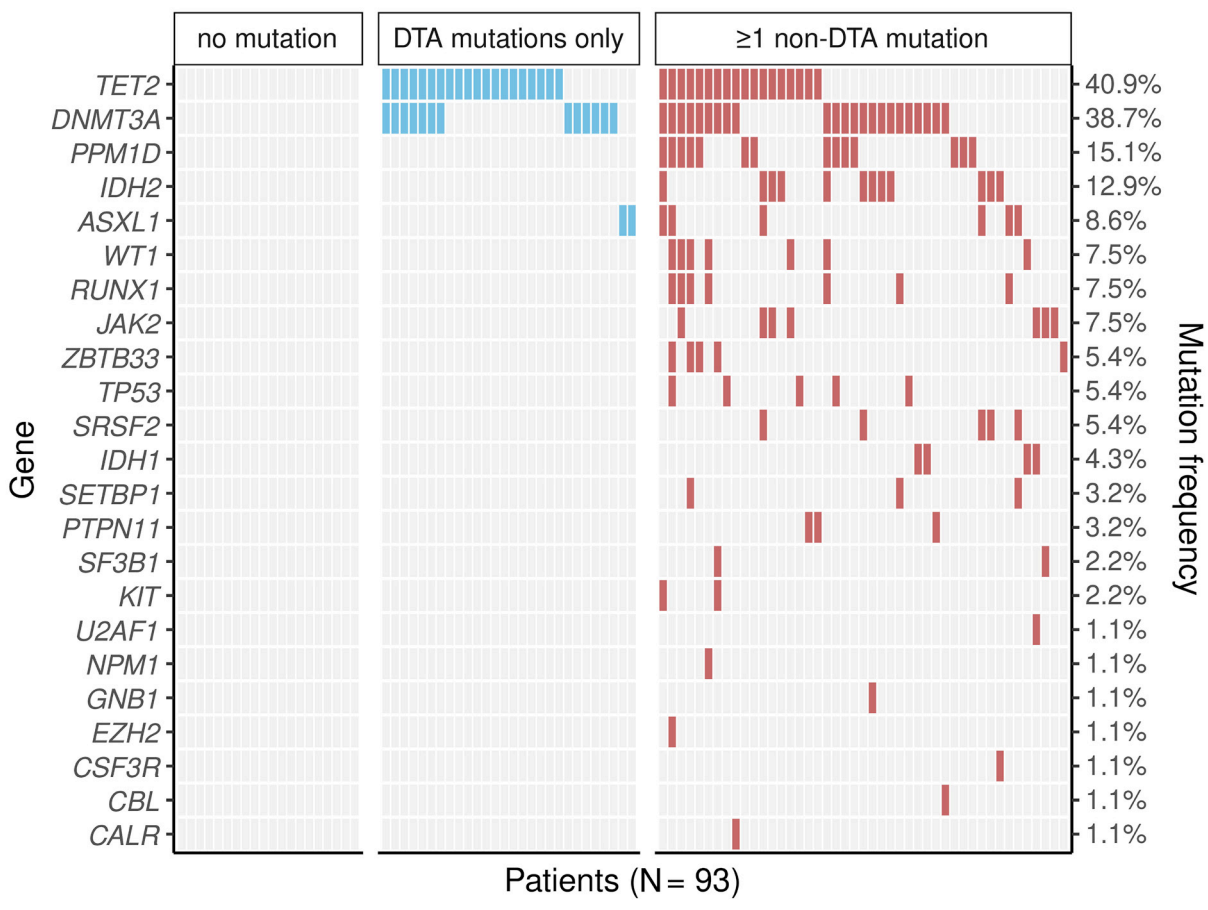


Figure 1. Oncoplot depicting mutations across all 93 patients stratified by DTA mutation status. Each column represents 1 patient, while each row represents a specific gene. Percentages on the right axis display the frequency of mutated genes in the cohort. DTA: *DNMT3A/TET2/ASXL1*.

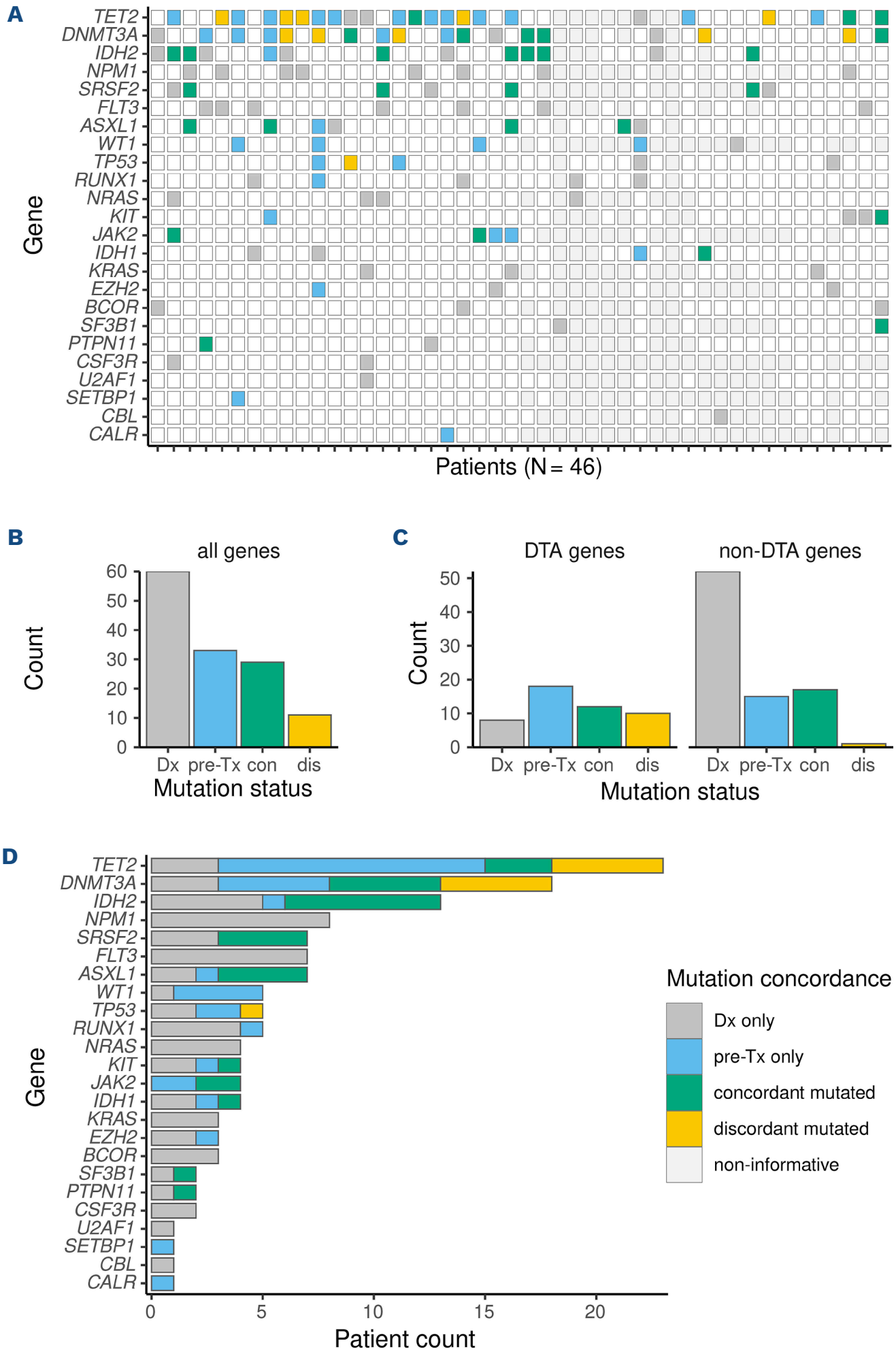


Figure 2. Overview of mutation concordance on a per-gene level between diagnosis and pre-transplantation time points. Data is shown for 46 patients with next-generation sequencing (NGS) data available for both time points. Coloring indicates concordance status. Genes with identical mutations at both time points (irrespective of variant allel frequencies [VAF]) are labeled “concordantly mutated”. Genes with differing mutations are labeled “discordantly mutated” (dis), reflecting loss of detectable mutations from diagnosis (Dx) to pre-transplantation (pre-Tx) and/or emergence of additional mutations at pre-Tx. NGS data at diagnosis were obtained using different sequencing assays, and genes not covered by NGS at Dx are labeled “non-informative”. (A) Mutation concordance per gene (rows) and patient (columns). (B) Bar plots summarizing frequency of concordance statuses across all genes. (C) Bar plots comparing frequency of concordance status across DTA (*DNMT3A/TET2/ASXL1*) and non-DTA genes. (D) Stacked barplots displaying frequency of concordance status per gene. con: concordant mutated.

q<0.001) or discordantly mutated (median, 41.8% *vs.* 1.9%; q<0.001). Similar patterns were observed when analyzing DTA and non-DTA genes separately (*Online Supplementary Figure S2*).

Measurable residual disease status predicts clinical outcomes

Next, we analyzed impact of mutations in pre-Tx samples on post-transplant outcomes. Patients were grouped as having no detectable mutations, DTA-only mutations, or ≥1 non-DTA mutation. OS differed significantly across these three groups (*P*=0.002), but survival was similar in patients without mutations and those with DTA-only variant (*P*=0.9). In contrast, patients with ≥1 non-DTA mutation had significantly inferior OS compared to both mutation-negative (*P*=0.02) and DTA-only patients (*P*=0.005). CIR did not differ significantly across all three groups, but was higher in those with ≥1 non-DTA mutation (*P*=0.24; *Online Supplementary Figure S3*).

These findings supported a binary MRD definition, classifying patients with ≥1 non-DTA mutation as MRD pos, and those with no or DTA-only variants as MRD neg. By this definition, 45 patients (48%) were MRD pos pre-Tx. Baseline characteristics including age, sex, AML subtype, baseline ELN 2022 risk, and conditioning intensity, were comparable between both groups (Table 1). Median VAF were similar for mutations found in MRD pos and MRD neg patients (1.95% *vs.* 1.9%; *P*=0.39).

During follow-up, 28 patients (30%) relapsed and 26 (28%) died (12 due to relapse, 11 from transplant-related causes and 3 from other causes). MRD pos patients had significantly worse OS (5-year OS, 45% *vs.* 89%; *P*=0.0047), but CIR (5-year CIR, 47% *vs.* 20%; *P*=0.10) and non-relapse mortality (NRM; 5-year rates, 17% vs. 6.4%; *P*= 0.20;) did not differ significantly between both groups (Figure 3). Stratifying MRD pos patients by maximum VAF (<5% *vs.* ≥5%) did not improve risk assessment: both subgroups had inferior OS with MRD neg patients (5-year OS, 46% and 44%, respectively, *vs.* 89%), with no difference between low- and high-VAF groups (*P*=0.8; *Online Supplementary Figure S4*).

Impact of conditioning intensity and measurable residual disease status on outcomes

Patients receiving MAC were younger than those receiving RIC/NMA (median, 48 *vs.* 62 years; *P*<0.001). When outcomes were analyzed by conditioning intensity, MRD pos patients had numerically inferior OS after both MAC and RIC/NMA,

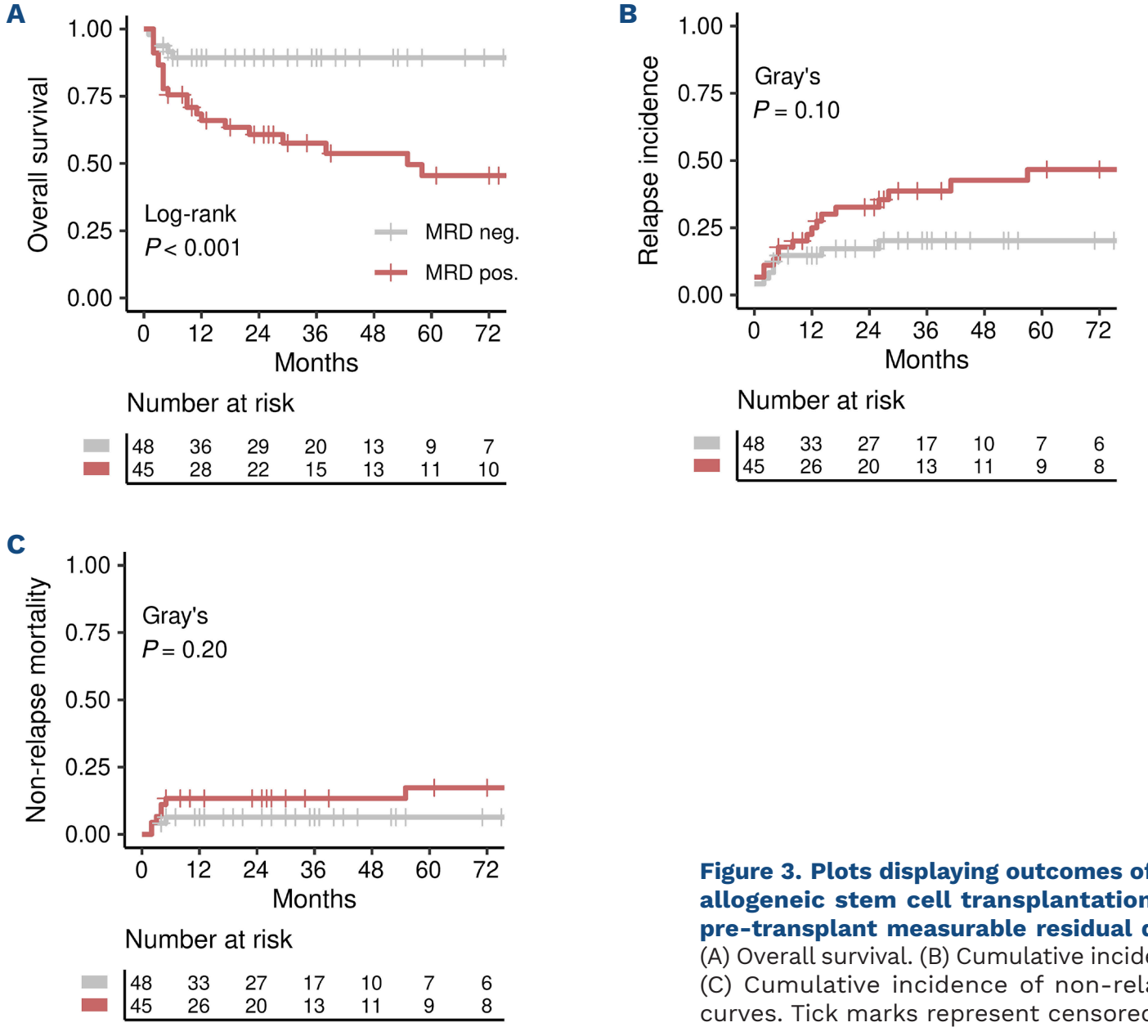


Figure 3. Plots displaying outcomes of patients after allogeneic stem cell transplantation, stratified by pre-transplant measurable residual disease status. (A) Overall survival. (B) Cumulative incidence of relapse. (C) Cumulative incidence of non-relapse mortality curves. Tick marks represent censored observations.

reaching significance in the larger RIC/NMA subgroup (N=67; 5-year OS, 44% *vs*. 87%; *P*=0.004) but not in the smaller MAC subgroup (N=26; 5-year OS, 38% *vs*. 94%; *P*=0.20; Figure 4A, B). MRD pos patients also tended to have higher CIR in both MAC (5-year CIR, 65% *vs*. 14%; *P*=0.089) and RIC/NMA groups (5-year CIR, 45% *vs*. 24%; *P*=0.39; Figure 4C, D), without significant differences in NRM (5-year NRM; MAC, 0% *vs*. 6.3%; *P*=0.48; RIC/NMA, 21% *vs*. 6.7%; *P*=0.19; Figure 4E, F). We did not detect significant differences in OS or CIR among MRD pos patients according to conditioning intensity, and a test for interaction between MRD status and conditioning intensity in a Cox proportional hazards model was non-significant. However, this analysis was limited by the small number of MAC-treated patients (N=26; of whom 8 were MRD pos; *Online Supplemerntary Figure S5*).

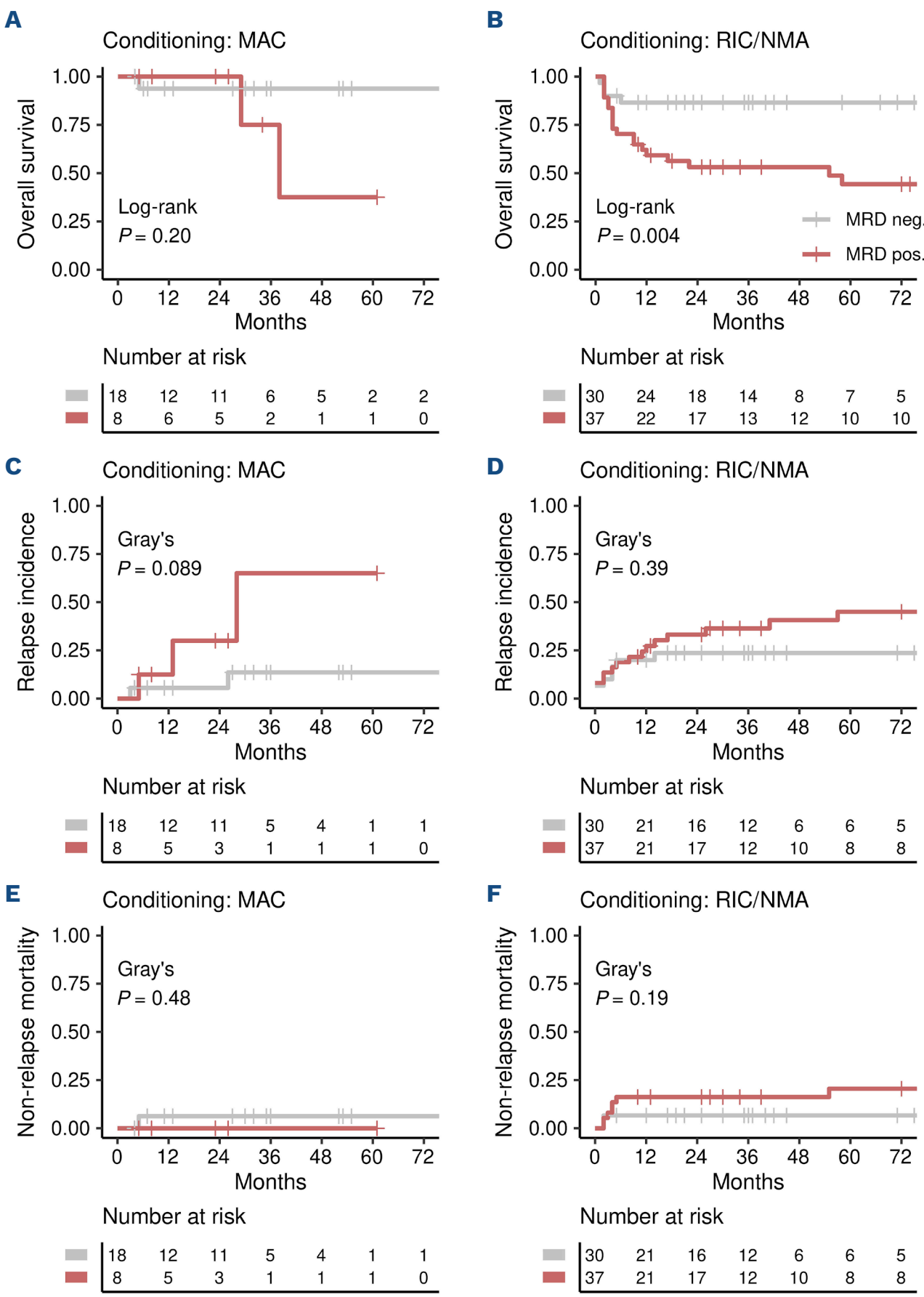


Figure 4. Plots displaying outcomes curves of patients after allogeneic stem cell transplantation, stratified by conditioning regimen. (A, B) Overall survival. (C, D) Cumulative incidence of relapse. (E, F) Cumulative incidence of non-relapse mortality. Plots in the left column show patients treated by myeloablative conditioning (MAC), plots in the right column patients treated by reduced-intensity conditioning (RIC) or non-myeloablative conditioning (NMA). Tick marks represent censored observations.

Multivariable analysis of survival after allogeneic stem cell transplantation

To assess whether MRD status independently associated with survival, we performed uni- and multivariable Cox regression analyses. In univariable analysis, age, sex, MRD status, AML type and conditioning regimen were associated with OS ($P \leq 0.1$; *Online Supplementary Figure S6*). Favorable ELN 2022 risk (N=10) yielded unstable hazard ratio estimates because no deaths occurred in this subgroup, although Kaplan-Meier curves showed the expected trend towards inferior OS in intermediate- and adverse-risk patients (*Online Supplementary Figure S7*). Variables associated with OS in univariable analysis were entered into a multivariable model using stepwise backward selection, retaining age and sex as forced covariates. In the final model, MRD status and sex remained independent predictors of OS: MRD positivity was associated with shorter OS (hazard ratio [HR]=4.58; 95% confidence interval [CI]: 1.72-12.21; P=0.002), whereas female sex showed an association with improved survival (HR=0.42; 95% CI: 0.18-0.99; P=0.046; Figure 5). The proportional hazards assumption was met for the global model (P=0.063); a borderline violation for sex (P=0.045) did not affect the HR for MRD status in a sensitivity analysis using sex as stratification variable (HR=4.59). In bootstrap internal validation, MRD status was retained in 87.5% of samples, compared with 32.4% for ELN 2022 risk, 31.9% for AML type, and 23.9% for conditioning regimen. The bootstrap validation yielded an optimism-corrected C-index of 0.65, indicating a moderate discriminative ability after correction for in-sample optimism.[27] The calibration slope was 0.62, suggesting that hazard ratios may be overestimated and would shrink in independent datasets.

Clustered variants in *RUNX1* and *TET2* in pre-transplantation bone marrow specimens

During variant review, we observed an unusual pattern of clustered nucleotide changes in pre-Tx samples from three patients, all of whom had received high-dose cytarabine during induction therapy. In patient 279, 12 *RUNX1* G>A transitions clustered within 42.3 kb, predominantly between the Runt and central domains or within the inhibition domain. Patient 280 harbored 12 *TET2* mutations across 41.5 kb (10 C>T/G>A transitions, one G>T transversion and 1 frameshift deletion), and patient 417 had 10 *TET2* C>T/G>A transitions across 42.1 kb (*Online Supplementary Figure S8*). Most *TET2* mutations were located outside the Oxygenase domain. Except for the *TET2* frameshift deletion (VAF 30%), VAF ranged from 0.6% to 6.7%, and all resulted in missense or nonsense protein changes. Read quality, coverage and strand distribution were consistent with other variants in the dataset.

Discussion

In this single-center study of 93 adult AML patients in morphologic remission before allo-SCT, detection of AML-associated mutations by a broadly applicable, smMIP-based NGS assay was associated with poorer post-transplantation outcomes in terms of both OS and, in trend, CIR. MRD positivity remained independently associated with shorter OS in multivariable analysis after adjustment for age and sex, and was a stronger prognostic factor than baseline ELN 2022 genetic risk. Importantly, this assay can be implemented at low per-sample library preparation costs of about €8 plus standard sequencing (2x150-bp paired-end sequencing, 2

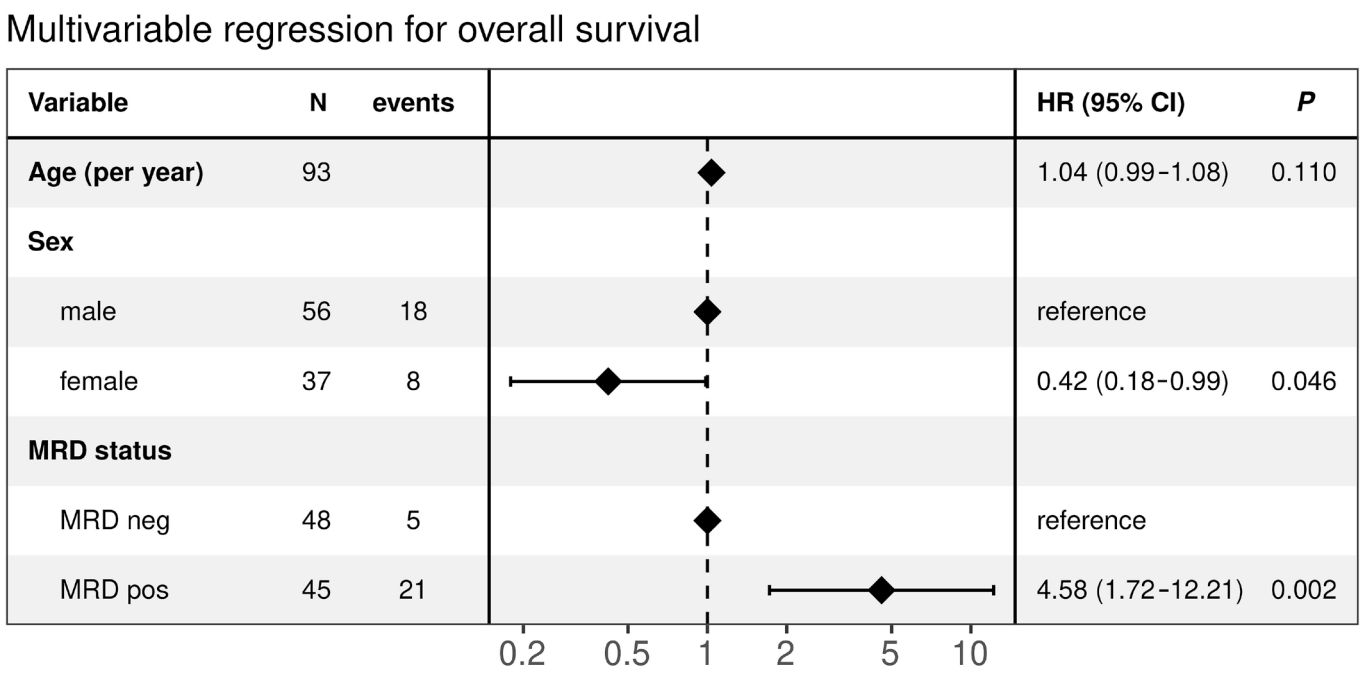


Figure 5. Multivariable Cox proportional hazards regression analyses for overall survival after allogeneic stem cell transplantation. Variables associated with overall survival (OS) in univariable analysis ($P \leq 0.1$; *Online Supplementary Figure S6*) were included and refined using stepwise backward selection based on model Akaike information criterion (AIC), with patient age and sex retained as forced co-variates. CI: confidence interval; HR: hazard ratio; MRD: measurable residual disease; MRD neg: MRD negative; MRD pos: MRD positive.

million read-pairs per sample; *Online Supplementary Table S6*), supporting feasibility in routine practice, including in resource-limited settings. Our findings are consistent with previous studies linking molecular MRD before allo-SCT to adverse outcomes in AML patients.[1,28]

Flow cytometry and qPCR-based assays remain the most widely used MRD methods, with NGS emerging as an additional genetics-based option in recent years. Although NGS is generally less sensitive than optimized qPCR-based approaches, it enables standardized analysis of multiple genomic regions in parallel. We used a smMIP-based NGS panel, a probe-based targeted sequencing approach in which individual captured DNA molecules are labeled with UMI, enabling consensus-based computational error correction. In contrast to conventional amplicon-based assays, smMIP do not rely on multiplex PCR for each target region. Compared to broader hybrid-capture workflows, they provide a more modular und streamlined approach for focused, highly multiplexed sequencing panels. This design supports broad applicability across genetically heterogeneous AML patients while keeping library preparation requirements low. The panel in our study covered recurrently mutated regions in 33 AML- and CH-related genes, minimizing the need for patient-specific assays. Such broad genomic coverage is clinically relevant given the dynamic mutational profile of AML and the potential for clonal shifts over time.[29]

Variants found in blood or bone marrow during CR generally fall into three categories: persisting pre-leukemic CH clones that may or may not be ancestral to the AML clone, mutations in residual AML clones (true MRD), and newly arising clones with uncertain leukemogenic potential.[30] DTA mutations frequently reflect persisting age-related CH and, consistent with ELN MRD recommendations, isolated DTA variants were not prognostic in our cohort.[14,17,31,32] However, other genes, such as *SRSF2*,[33] may also be mutated in non-leukemic clones, and some leukemia-specific mutations can be lost during therapy. For example, mutations in the well-established MRD marker gene *NPM1* can be lost at relapse in a subset of patients.[34,35] These complexities can reduce the predictive power of assays that focus on single mutations and underscore the value of panels that capture a broader spectrum of potential MRD markers. In our paired Dx/pre-Tx analysis, mutational concordance varied by gene, with emerging mutations detected both in CH-related and AML-associated genes. VAF were consistently lower pre-Tx, reflecting both clonal dynamics and the higher analytical sensitivity of the smMIP assay compared with molecular testing done at initial diagnosis.

The limit of detection of our assay (VAF threshold ≥0.5%) is substantially higher than that of some ultra-sensitive NGS MRD panels, but nevertheless, it detected non-DTA variants in almost half of patients and clearly stratified post-transplantation risk. Our platform is therefore not intended to replace highly optimized single-target PCR-based assays or dedicated ultra-deep NGS MRD panels in informative patients, but rather to provide a broadly applicable and resource-efficient alternative for mutation-bases MRD assessment across a large proportion of AML cases. Reproducibility across sequencing runs was not systemically assessed in this study and should be addressed in future validation work.

For many centers, particularly those with constrained resources, the trade-off between ultra-high sensitivity and feasibility is crucial. In addition, higher analytical sensitivity does not necessarily result in stronger prognostic power.[9,36] Our smMIP-based approach combines broad genomic coverage with low library preparation costs of about €8 per sample and only moderate sequencing requirements, which may facilitate routine clinical MRD monitoring and large-scale studies even in resource-limited settings. However, these costs reflect reagent and consumable expenses of an in-house workflow not including costs for personnel, instrumentation, assay development or bioinformatic analysis and are therefore not directly comparable to the full cost structure of commercial assays.

DTA mutations were common in our cohort, as expected in an older AML population.[14,31] Consistent with ELN MRD guidance, isolated DTA were not associated with outcome, supporting their interpretation as CH rather than persisting AML in most pre-Tx samples.[17,32] Nonetheless, prior work from our group and others has reported worse survival when (DTA) mutations persist after induction therapy and suggested partial mitigation of relapse risk by allo-SCT.[19,37] Post-transplantation MRD studies have also linked detection of non-DTA mutations to worse outcome, and have indicated that re-emerging DTA clones may mark residual recipient hematopoiesis and associated with shorter event-free survival, inferior OS and higher CIR.[38,39] Taken together, these data suggest that the prognostic impact of DTA mutations is context-dependent, differing between post-induction, pre-transplant and post-transplant timepoints.

In our pre-Tx setting, patients with non-DTA mutations had significantly worse OS than those without mutations or with DTA-only variants, supporting a binary MRD definition based on presence of ≥1 non-DTA mutation. MRD positivity was associated with inferior OS and a trend towards higher CIR, while non-relapse mortality was not affected. Further stratification of MRD pos patients by maximum VAF (<5% *vs*. ≥5%) did not improve risk prediction; both subgroups had significantly worse OS than MRD neg patients, arguing against excluding variants above a certain VAF threshold, such as 5%.[5] At the same time, not all patients classified as MRD pos subsequently relapsed, which may in part reflect misclassification of CH-associated mutations beyond canonical DTA genes as residual AML.

Previous studies found that myeloablative conditioning resulted in superior OS in MRD pos patients and reduced relapse rates, compared to lower-intensity conditioning.[32,40] In our cohort, MRD pos patients had inferior OS after both

MAC and RIC/NMA, with significant differences only in the larger RIC/NMA group. We did not observe a clear survival advantage of MAC in MRD pos patients. Since the MAC subgroup was small, a modifying effect of conditioning intensity on MRD-associated risk nevertheless cannot be excluded.

Consistent with univariable analyses, MRD positivity remained an independent risk factor for inferior OS and outperformed baseline ELN 2022 genetic risk in multivariable Cox regression analysis. We also observed the association between female sex and improved OS, as reported previously.[41,42] The multivariable model was developed in a relatively small cohort with only 26 events, raising concerns about overfitting and the stability of effect estimates. However, bootstrap internal validation confirmed the independent prognostic impact of MRD positivity but indicated a moderate degree of overfitting, resulting in potentially exaggerated hazard ratios. Conversely, possible misclassification of CH-associated non-DTA mutations as MRD may have reduced the specificity of our binary MRD definition, thereby attenuating the observed association between MRD positivity and outcome. These limitations inherent to our dataset underscore the need for external validation in a larger cohort.

The clustered low-VAF *RUNX1* and *TET2* mutations observed in three patients, all previously exposed to high-dose cytarabine, are intriguing. No similar clusters have been observed in a cohort of 373 AML long-term survivors analyzed for CH variants with a similar smMIP-based assay in our laboratory, as well as in hundreds of so far unpublished analyzed samples, arguing against a technical artifact.[22] Their mutational spectrum and distribution resemble published patterns of cytarabine-associated mutagenesis centered on specific sequence motifs,[43] raising the possibility of therapy-induced mutations. Given the low VAF, absence of orthogonal validation and small number of cases, these findings should be interpreted with caution, and their clinical relevance remains uncertain.

Our study has limitations, including its single-center design, modest sample size, and heterogenous genetic work-up, which may reduce the generalizability of our findings. Further validation and technical refinement of the consistency with the rest of the manuscript are warranted; for example, smMIP perform poorly in GC-rich regions of the genome such as the *CEBPA*, but Nicotinamide adenine dinucleotide (NAD)-supplemented chemistries may improve coverage in future iterations.[44,45]

Despite these limitations, the current assay's sensitivity of ≥0.5% VAF and its broad coverage of relevant genomic targets successfully stratified post-transplant risk and is predicted to detect at least one informative variant in >95% of intensively treated AML patients based on published cohorts.[24] This broad applicability reduces reliance on patient-specific assays, although direct comparisons with ultra-sensitive single-target methods in informative patients are still needed. Integrating NGS-based MRD with clinical risk factors and baseline genetics may further refine risk-adapted strategies, and prospective trials are required to determine whether interventions that convert MRD pos to MRD neg status before allo-SCT can improve outcomes. Given its low per-sample library preparation cost and moderate sequencing requirements, our smMIP-based assay may provide a practical platform for such studies and enable wider implementation of MRD-guided care in routine clinical practice.

Disclosures

No conflicts of interest to disclose.

Contributions

SMK and TH performed data analyses and drafted the manuscript. SMK also developed the analysis software and prepared the figures. TH performed laboratory analysis. SMK, AW, SS, and KHM conceived the study. LB, DBa, DBr, JU, GNF, VV, UP, SS, and MJ provided patient samples and clinical resources. UP and KHM obtained funding. MJ and KHM supervised the study. All authors critically revised the manuscript and approved the final version.

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Data-sharing statement

The datasets generated and/or analyzed during the current study can be made available from the corresponding author on reasonable request due to ethical/privacy reasons.

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