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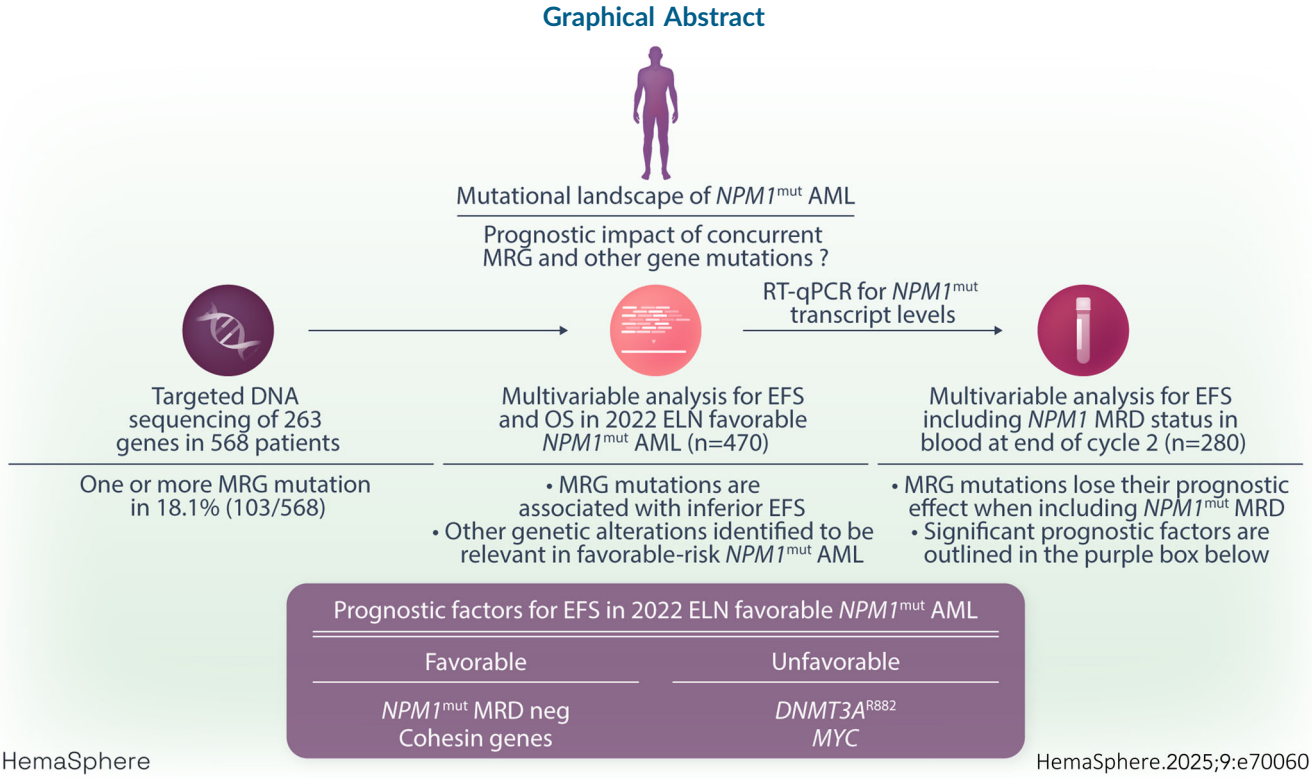
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ARTICLE

Impact of myelodysplasia-related and additional gene mutations in intensively treated patients with *NPM1*-mutated AML

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Graphical Abstract



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Impact of myelodysplasia-related and additional gene mutations in intensively treated patients with *NPM1*-mutated AML

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ABSTRACT

This study aimed to evaluate the impact of the myelodysplasia-related gene (MRG) as well as additional gene mutations on outcomes in intensively treated patients with *NPM1*-mutated (*NPM1*^{mut}) AML. Targeted DNA sequencing of 263 genes was performed in 568 *NPM1*^{mut} AML patients (median age: 59 years) entered into the prospective AMLSG 09-09 treatment trial. Most commonly co-mutated genes were *DNMT3A* (49.8%), *FLT3*-TKD (25.9%), *PTPN11* (24.8%), *NRAS* (22.7%), *TET2* (21.7%), *IDH2* (21.3%), *IDH1* (18%), and *FLT3*-ITD (17.3%). MRG mutations were identified in 18.1% of cases (18–60 years: 9.8%; >60 years: 28.7%). When focusing on the 470 patients with 2022 ELN favorable-risk *NPM1*^{mut} AML, multivariable analysis for event-free survival (EFS) identified age ($p < 0.001$), *DNMT3A*^{R882} ($p < 0.001$), *IDH1* ($p = 0.007$), and MRG mutations ($p = 0.03$) as unfavorable factors, cohesin gene co-mutations ($p = 0.001$) and treatment with gemtuzumab ozogamicin ($p = 0.007$) as favorable factors. Restricting the analysis to a subset of CR/CRi patients with available data on *NPM1*^{mut} measurable residual disease (MRD) status in blood post cycle 2 in the model, MRG mutations lost their significant effect, whereas *DNMT3A*^{R882}, *MYC*, and cohesin gene mutations retained the adverse and favorable effects. For OS, age ($p < 0.001$), *DNMT3A*^{R882} ($p = 0.042$), *IDH1* ($p = 0.045$), and *KRAS* (0.003) mutations were unfavorable factors, sole favorable factor was *IDH2* co-mutation ($p = 0.037$). In 2022 ELN favorable-risk *NPM1*^{mut} AML, MRG mutations are associated with inferior EFS; however, this effect is no longer present when considering *NPM1*^{mut} MRD status post cycle 2; *DNMT3A*^{R882} and *MYC* mutations remained adverse, and cohesin gene mutations favorable prognostic factors independent of the *NPM1*^{mut} MRD status.

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INTRODUCTION

Based on its distinct clinical and biological features, AML with *Nucleophosmin1* mutation (*NPM1*^{mut}) represents a defined disease entity in the 2022 International Consensus Classification (ICC) of Myeloid Neoplasms and Acute Leukemias as well as 5th edition of the World Health Organization (WHO) Classification of Haematolymphoid Tumours.[1,2] *NPM1*^{mut} AML most commonly presents *de novo* and in general is associated with a more favorable prognosis.[3,4] *NPM1*^{mut} AML is frequently associated with mutations in signaling (e.g., *FLT3*, *NRAS*, *PTPN11*), DNA methylation (e.g., *DNMT3A*, *TET2*, *IDH1*, *IDH2*), and cohesin complex genes (e.g., *RAD21*, *STAG2*, *SMC3*).[4]

In 2022, a new disease entity was introduced in the AML classifications defined by the presence of one or two mutations in *ASXL1*, *BCOR*, *EZH2*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, and *ZRSR2* genes and occurring outside the category of AML with recurrent genetic abnormalities. This gene signature has been shown to have a high specificity for secondary AML evolving from a preceding hematologic neoplasm.[3,5] In the ICC, these cases are categorized as "AML with myelodysplasia-related gene (MRG) mutations,"[1] in the WHO classification as "AML, myelodysplasia-related" (AML-MR; *RUNX1* not included in the WHO signature).[2] In intensively treated patients, the presence of MRG mutations has been associated with inferior outcomes. As a consequence, in the 2022 European LeukemiaNet (ELN) risk classification, these mutations—if occurring outside ELN favorable-risk AML—were included in the adverse-risk group.[6]

In 2022 ELN favorable-risk AML that includes *NPM1*^{mut} AML without *FLT3*-ITD, the prognostic impact of MRG co-mutations is largely unknown. *NPM1*^{mut} AML may exhibit features of myelodysplasia, both morphologically and cytogenetically. In fact, in the 2008 WHO classification, *NPM1*^{mut} AML with multilineage dysplasia was classified as AML with myelodysplasia-related changes (AML-MRC).[7] However, subsequent studies showed that these morphologic features do not impact prognosis.[8,9] All subsequent classifications now consider *NPM1*^{mut} AML as an entity irrespective of the presence or absence of morphologic dysplasia. However, *NPM1*^{mut}/FLT3-ITD$^{neg/low}$ AML exhibiting adverse-risk cytogenetic abnormalities, occurring in rare cases (3.6%), have been shown to confer poor survival,[10] and *NPM1*^{mut} AML with adverse-risk cytogenetic abnormalities is now classified as adverse-risk in the 2022 ELN risk classification.[6]

Due to the lack of data, in the 2022 ELN recommendations, MRG mutations were not considered for risk assignment in favorable-risk AML.[6] Recent studies analyzed the impact of MRG mutations in *NPM1*^{mut} AML, which yielded contradictory results.[11–15]

In this study, we aimed to investigate the clinical impact of baseline MRG as well as additional gene mutations using targeted sequencing in a large cohort of *NPM1*^{mut} AML. All patients were treated in the prospective AMLSG 09-09 clinical trial in which patients were assigned to intensive chemotherapy plus all-*trans* retinoic acid with or without gemtuzumab ozogamicin (GO).[16,17] For a more comprehensive risk assessment, we also included data from prospective measurable residual disease (MRD) monitoring of mutant *NPM1* transcripts by quantitative PCR (qPCR).[18] Finally, we aimed to validate the data in an independent cohort of 435 *NPM1*^{mut} AML.[3]

MATERIALS AND METHODS

Patients

In total, 568 patients with *NPM1*^{mut} AML (median age: 59 years) representing 97% (568/588) of the clinical trial population were included in this study. Patient and disease characteristics of the 568 patients are given in Supporting Information S1: Table S1. All patients were enrolled in the randomized open-label phase 3 AMLSG 09-09 trial (NCT00893399). In this trial, patients were assigned to intensive chemotherapy plus all-*trans* retinoic acid with or without GO; the results of the clinical trial were previously reported.[16,17] MRD data from peripheral blood performed by qPCR at the end of cycle 2 were available in 330/568 (58%) cases and $n = 280/470$ (60%) of the 2022 ELN favorable-risk group.[18]

For confirmation of results, we used an independent cohort of 435 intensively treated patients with *NPM1*^{mut} AML (median age: 50 years) from a previous targeted sequencing study.[3] Patient and disease characteristics are given in Supporting Information S1: Table S1b.

The study was approved by the institutional review board at each participating center. Written consent was given by all patients according to the Declaration of Helsinki.

Gene mutation analysis

Targeted sequencing (mean read depth: 1857x [536-2586]) was performed in diagnostic samples on the entire coding region of 263 genes (panel size: 1.381 Mbp) associated with hematologic disorders (Supporting Information S1: Table S2). Using the SureSelectXT HS Kit from Agilent Technologies, libraries were prepared from 100 ng DNA and sequenced (read length: 2 × 150 base pairs) on a NextSeq. 500 platform (Illumina). Sequencing data were analyzed using an in-house computational pipeline,[19] with the difference that Mutect2[20] was used for variant calling and Ensembl Variant Effect Predictor (VEP)[21] for variant prediction. Manual evaluation of variants to exclude artifacts, polymorphisms and (likely) benign variants was carried out as described.[19] The analysis of internal tandem duplications in the *FLT3* gene was performed using GeneScan-based fragment length analysis.[22]

Statistical analyses

The Kaplan-Meier method was used for the estimation of survival curves. Differences between survival curves were tested by log-rank tests. Pairwise odds ratios for mutation associations (mutual exclusivity and co-occurrence) found in ≥3% of cases were calculated for all patients. Hypothesis testing via Fisher's exact test was applied with discrete Benjamini-Hochberg (step-down) correction for multiple testing.[23] Study endpoints are event-free survival (EFS; defined as in ELN 2022 criteria) and overall survival (OS; defined as the time from randomization until death from any cause).[6,17] Prognostic factors for EFS and OS were identified using a multivariate Cox regression model. The model included age, sex, AML type (*de novo* vs. secondary or therapy-related AML), white blood cell counts (log10-transformed), lactate dehydrogenase (LDH) serum levels, treatment arm (with or without GO), allogeneic transplantation in first CR (time-dependent), and gene mutations with a frequency of ≥3%. Missing values were observed in white blood cell counts (3/568, 0.05%) and LDH (2/568, 0.03%), and the corresponding four patients were excluded in multivariable models. Landmark analyses were conducted for the landmark timepoint "end of induction cycle 2" to evaluate the impact of MRD status on EFS and OS. All analyses were conducted in R version 4.3, packages survival (version 3.5), and DiscreteFDR (version 1.3.6) among others.

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RESULTS

Genomic landscape of co-mutations in AML with mutated *NPM1*

In total, $n = 3058$ variants (variant allele frequency of ≥1%) were identified in 195/263 genes. The median number of co-mutations was 4 (range 0–11). The most commonly co-mutated genes were *DNMT3A* (49.8%; *DNMT3A*^{R882}, 28.0%; *DNMT3A*^{nonR882}, 21.8%) *FLT3* tyrosine kinase domain (TKD) (25.9%), *PTPN11* (24.8%), *NRAS* (22.7%), *TET2* (21.7%), *IDH2* (21.3%, $n = 121$; *IDH2*^{R140}, $n = 118$; *IDH2*^{R170}; $n = 2$; *IDH2*^{R179}, $n = 1$), *IDH1* (18%), and *FLT3*-ITD (17.3%) (Figure 1 and Supporting Information S1: Table S3). AML with concurrent *FLT3*-ITD (17%) were underrepresented due to the fact that a clinical trial for *FLT3*^{mut} AML (AMLSG 16-10) was active in parallel.[24] One or more MRG mutations (*ASXL1*, *BCOR*, *EZH2*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, and *ZRSR2*) were present in 103 (18.1%) cases (Figure 1 and Supporting Information S1: Table S3). Age-related differences in frequencies were found for *DNMT3A*^{R882} hotspot mutations (36.6% vs. 17.1% in younger and older patients, respectively; no difference for *DNMT3A*^{nonR882} mutations: 21.8% and 21.9%), *PTPN11* (30% vs. 18.3%), *NRAS* (29% vs. 14.7%), *TET2* (13.9% vs. 31.5%), and MRG (9.8% vs. 28.7%) mutations (Supporting Information S1: Table S3). Figure 2 shows a heatmap of co-mutations grouped by functional classes (DNA methylation, signaling, cohesin genes, transcriptional factors) and MRG mutations.

MRGs mutated were *SRSF2* (7.7%), *STAG2* (4.9%), *ASXL1* (2.5%), *EZH2* (1.2%), *U2AF1* (1.2%), *ZRSR2* (1.1%), *SF3B1* (1.1%), *RUNX1* (0.7%), and *BCOR* (0.4%) (Supporting Information S1: Table S3); 89 (86%) patients had one, 13 (13%) patients two, and one patient >2 MRG mutations. Variant allele frequencies (VAF) were 0.43 (range, 0.11–0.50) for *SRSF2*, 0.44 (range, 0.08–0.98) for *STAG2*, and 0.41 (range, 0.07–0.47) for *ASXL1*. Based on the VAF, MRG mutations represented the dominant clone over *NPM1* mutations in 76% of cases; they were co-dominant in 16%, and in only 8% of cases the *NPM1* clone was dominant (Supporting Information S1: Figure S1).

Patients with MRG mutations were significantly older, more frequently male, and had lower white blood cell and platelet counts; with regard to the co-mutational pattern, *NPM1*^{mut}/MRG^{pos} AML less frequently had activating signaling gene mutations (*FLT3*-ITD, *PTPN11*, *NRAS*), less frequently *DNMT3A* mutations (in particular *DNMT3A*^{R882}), and more frequently *TET2* and cohesin gene mutations (Table 1).

Patterns of mutational cooperativity and mutual exclusivity

After correction for multiple testing, the most significant mutational cooperativities were found for *NFE2*:*STAG2* ($p < 0.001$), *IDH2*:*SRSF2* ($p < 0.001$), *CEBPA*:*TET2* ($p < 0.001$), *DNMT3A*^{R882}:*NRAS* ($p = 0.002$), *NRAS*:*PTPN11* ($p = 0.004$), and *KRAS*:*NRAS* ($p = 0.013$). Significant mutual exclusivities were found for *DNMT3A*^{nonR882}:*DNMT3A*^{R882} ($p < 0.001$), *IDH2*:*TET2* ($p < 0.001$), *IDH1*:*TET2* ($p < 0.001$), *DNMT3A*^{R882}:*SRSF2* ($p < 0.001$), *FLT3*-ITD:*KRAS* ($p = 0.002$), *FLT3*-ITD:*NRAS* ($p = 0.004$), and *IDH2*:*STAG2* ($p = 0.014$) mutations (Figure S2). While *FLT3*-ITD showed mutual exclusivity with other signaling mutations (*FLT3*-TKD, *NRAS*, *KRAS*, and *PTPN11*), *NRAS*, *KRAS*, and *PTPN11* were frequently co-mutated. The patterns of cooperativity and mutual exclusivity were similar between younger and older patients.

Outcome analysis of the entire cohort

With a median follow-up for the survival of 5.3 years (95% CI, 5.1 to 5.6 years), the estimated 5-year EFS and OS rates of the 568 patients were 40.4% and 60.9%, respectively.

Event-free survival

A total of 323 patients experienced an event. In univariable models (Supporting Information S1: Figure S3), the presence of MRG mutations did not impact EFS ($p = 0.334$), neither did any of the three most commonly (≥3%) mutated MRGs *SRSF2* ($p = 0.342$), *STAG2* ($p = 0.327$), and *ASXL1* ($p = 0.359$) as a single marker (Supporting Information S1: Figure S3). Genetic markers associated with inferior EFS were *DNMT3A*^{R882} (<0.001), *FLT3*-ITD (<0.001), and *IDH1* ($p = 0.029$), whereas *IDH2* was associated with improved EFS ($p = 0.045$). The cohesin complex genes *RAD21* ($p = 0.052$), *SMC1A* (0.093), and *SMC3* (0.053) showed a trend toward improved EFS, and when all cohesin genes (*RAD21*, *SMC1A*, *SMC3*, *STAG2*) were grouped the effect was statistically highly significant ($p < 0.001$).

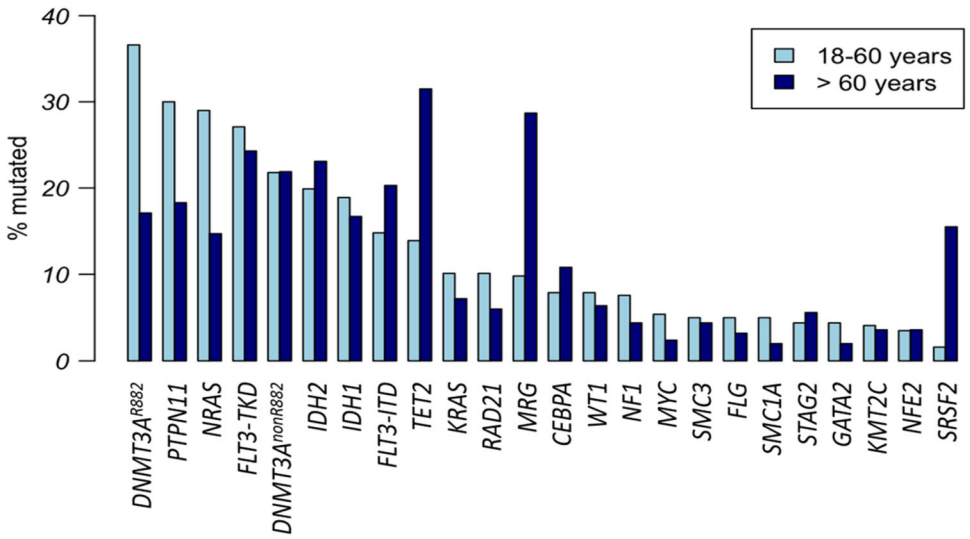


FIGURE 1 **Frequency of co-mutations in 568 *NPM1*^{mut} AML according to age group (18–60 vs. >60 years).** Myelodysplasia-related genes (MRG) include *ASXL1*, *BCOR*, *EZH2*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, and *ZRSR2* genes.

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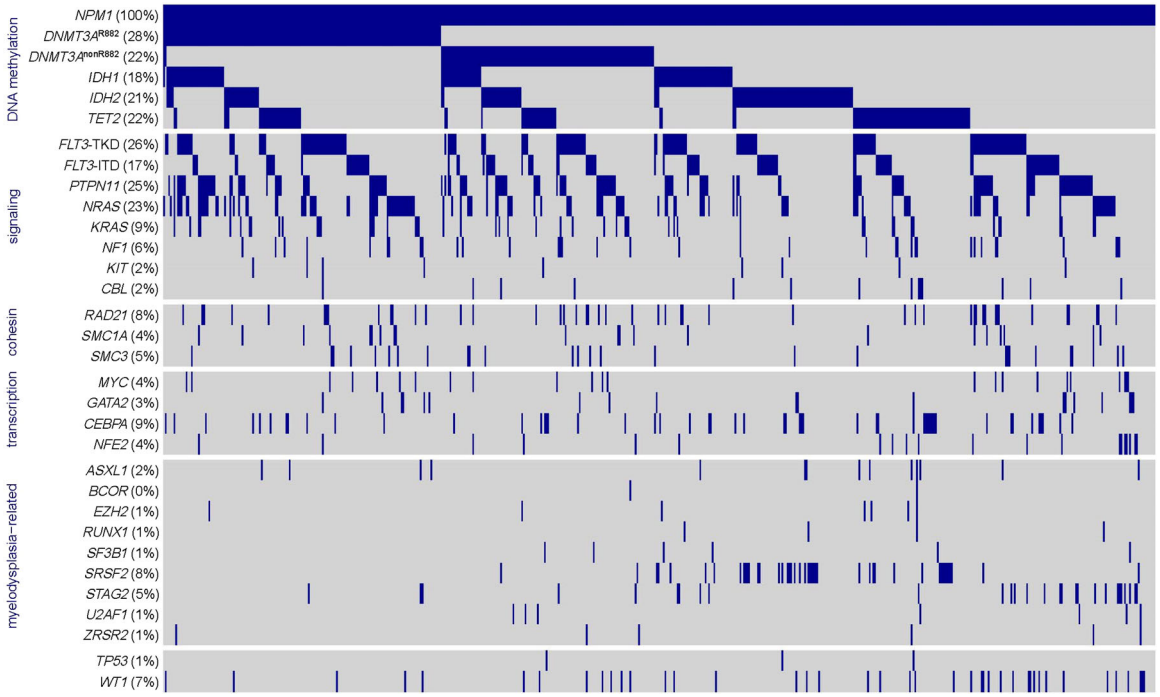


FIGURE 2 **Mutational landscape of 568 *NPM1*^{mut} AML**. Co-mutations are grouped by functional classes (DNA methylation, signaling, cohesin complex genes, transcriptional factors) and myelodysplasia-related genes.

Multivariable analysis identified *DNMT3A*^{R882} ($p < 0.001$), *FLT3*-ITD ($p = 0.004$), *IDH1* ($p = 0.015$), *WT1* ($p = 0.012$), and *MYC* ($p = 0.026$) as unfavorable factors, whereas cohesin complex genes combined (p = 0.002) were a favorable factor (Supporting Information S1: Table S4); MRG mutations were in trend unfavorable ($p = 0.077$). Of the clinical variables, higher age was an unfavorable factor ($p < 0.001$), and treatment with GO had in trend a favorable effect on EFS ($p = 0.059$). Results from multivariable analysis entering all gene mutations (with a frequency of ≥3%) as single variables in the model are given in Supporting Information S1: Table S5.

Overall survival

Results of univariable analyses for OS by selected genetic markers are shown in Supporting Information S1: Figure S5. In multivariable analysis (Supporting Information S1: Table S4), age ($p < 0.001$), *FLT3*-ITD (<0.001), *KRAS* ($p = 0.003$), *DNMT3A*^{R882} ($p = 0.019$), and *MYC* ($p = 0.041$) were unfavorable factors, and *IDH2* was associated with superior OS ($p = 0.020$). MRG mutations had no impact ($p = 0.471$).

Cumulative incidence of relapse

The results of the molecular markers for CIR reflected those of the EFS analysis with the difference that not only *DNMT3A*^{R882} (<0.001) but also *DNMT3A*^{nonR882} ($p = 0.007$) was significantly associated with an increased relapse (Supporting Information S1: Table S6). As previously reported in the clinical trial results,[17] GO significantly reduced the risk of relapse ($p = 0.002$).

Outcome analysis of *NPM1*^{mut} AML of the 2022 ELN favorable-risk group

With 470/568 (83%) patients, 2022 ELN favorable-risk *NPM1*^{mut} AML represented the vast majority of patients.

Event-free survival

In all, 252 patients experienced an event. In univariable models, *DNMT3A*^{R882} (<0.001), *IDH1* ($p = 0.006$) mutations were associated with inferior EFS, cohesin complex gene mutations ($p = 0.002$) with improved EFS; similar to the entire cohort, MRG mutations had no significant impact ($p = 0.11$) (Figures 3 and Supporting Information S1: Figure S4). However, in multivariable analysis, MRG mutations ($p = 0.03$) were a significant adverse factor; in addition to *DNMT3A*^{R882} (<0.001) and *IDH1* ($p = 0.007$), the cohesin complex genes combined ($p = 0.001$) were associated with superior EFS (Table 2). Of the clinical variables, older age was an unfavorable factor ($p < 0.001$), treatment with GO had a favorable effect on EFS ($p = 0.007$); allogeneic hematopoietic cell transplantation (HCT) in first remission had no beneficial effect ($p = 0.487$). To visualize the effect of MRG mutations, we generated predicted survival curves resulting from the Cox model for two exemplary patients, aged 58, female, *de novo* disease, without (Figure 4A) and with (Figure 4B) *DNMT3A*^{R882} co-mutation.

Overall survival

In multivariable analysis (Table 2), age ($p < 0.001$) *DNMT3A*^{R882} ($p = 0.042$), *IDH1* ($p = 0.045$), and *KRAS* ($p = 0.003$), were unfavorable factors, and *IDH2* was associated with superior OS ($p = 0.037$).

Cumulative incidence of relapse

Multivariate analysis for CIR in the favorable-risk group was comparable to the analysis of the entire cohort (Supporting Information S1: Table S6). Adverse factors were age ($p < 0.001$), *DNMT3A*^{R882} ($p < 0.001$), *DNMT3A*^{nonR882} ($p = 0.010$), *IDH1* ($p = 0.008$), and *MYC*

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TABLE 1 Clinical and genetic characteristics of *NPM1*^{mut} AML with or without myelodysplasia-related gene (MRG) mutations.

Variable	N	*NPM1*^{mut}/ MRG^{neg}, *n* = 465	*NPM1*^{mut}/ MRG^{pos}, *n* = 103	*p* Value
Age	568	57 (50, 67)	66 (57, 71)	<0.001
Sex	568			0.004
Male		203 (44%)	61 (59%)	
Female		262 (56%)	42 (41%)	
History of AML	568			0.6
De novo		434 (93%)	94 (91%)	
Secondary		8 (1.7%)	2 (1.9%)	
Therapy-related		23 (4.9%)	7 (6.8%)	
WBC, Giga/L	565	21 (6, 55)	6 (2, 31)	<0.001
Missing		2	1	
Hemoglobin, g/dL	565	9.1 (8.1, 19.5)	9,2 (8.1, 10.6)	0.7
Missing		2	1	
Platelets, Giga/L	564	75 (42, 123)	58 (28, 94)	0.001
Missing		3	1	
Bone marrow blasts, %	547	73 (42, 90)	76 (45, 90)	0.6
Missing		17	4	
LDH, U/L	567	443 (302, 661)	393 (278, 674)	0.2
Missing		1	0	
FLT3-ITD	568	89 (19%)	9 (8.7%)	0.011
FLT3-TKD	568	121 (26%)	26 (25%)	0.9
DNMT3A	568	260 (56%)	21 (20%)	<0.001
DNMT3A^{R882}		151 (32%)	8 (7.8%)	
DNMT3A^{nonR882}		111 (24%)	13 (13%)	
PTPN11	568	124 (27%)	17 (17%)	0.031
NRAS	568	116 (25%)	13 (13%)	0.007
KRAS	568	41 (8.8%)	9 (8.7%)	>0.9
MYC	568	19 (4.1%)	4 (3.9%)	>0.9
TET2	568	93 (20%)	30 (29%)	0.042
IDH1	568	87 (19%)	15 (15%)	0.3
IDH2	568	95 (20%)	26 (25%)	0.3
RAD21	568	43 (9.2%)	4 (3.9%)	0.074
Cohesin genes (all)	568	86 (18%)	33 (32%)	0.002

Abbreviations: ITD, internal tandem duplication; LDH, lactate dehydrogenase; TKD, tyrosine kinase domain; WBC, white blood cell count.

($p = 0.038$), whereas cohesin complex genes ($p = 0.009$) and treatment with GO ($p < 0.001$) were favorable factors.

Outcome (landmark) analysis of *NPM1*^{mut} AML of the 2022 ELN favorable-risk group including *NPM1* MRD status in peripheral blood post cycle 2

MRD data from the AMLSG 09-09 trial were previously published.[18] For this analysis, we included qPCR MRD data from peripheral blood post cycle 2. This time point has previously been shown to be highly prognostic and to represent a clinically relevant decision point.[18,25–27]

When including *NPM1* MRD in blood post cycle 2 in the landmark model for EFS in the subgroup of 2022 ELN favorable-risk patients (restricted to a subset of CR/CRi patients with available MRD information), MRG mutations only showed a trend for inferior outcome ($p = 0.096$); *DNMT3A*^{R882} ($p < 0.001$) and *MYC* (0.021) remained statistically significant unfavorable factors, while cohesin complex gene mutations remained a highly statistically significant favorable factor ($p < 0.001$) (Figure 5).

Outcome analysis of an independent second cohort

Finally, to confirm our findings, in particular for MRG mutations, we analyzed a cohort of 435 intensively treated patients with *NPM1*^{mut} AML from a previous targeted sequencing study, which included all nine MRG;[3] 49 of the 435 *NPM1*^{mut} AML (11.3%) exhibited MRG co-mutations. The frequencies of additional gene mutations are given in Supporting Information S1: Table S3.

In the univariable analysis of the entire cohort of 435 patients, the presence of MRG mutations did not impact EFS ($p = 0.130$) and OS ($p = 0.880$) (Supporting Information S1: Figure S6). However, in multivariable analysis for EFS, MRG mutations had a significant negative prognostic impact on EFS ($p = 0.002$); there was no effect on OS ($p = 0.249$) (Supporting Information S1: Table S7). Additional unfavorable factors for EFS were *DNMT3A* ($p = 0.024$), *IDH1* ($p = 0.014$), and *FLT3*-ITD ($p < 0.001$), while *FLT3*-TKD mutations ($p = 0.022$) and cohesin complex genes ($p = 0.026$) were favorable factors; for OS *DNMT3A* ($p = 0.038$), *IDH1* ($p = 0.011$) and *FLT3*-ITD ($p < 0.001$) were unfavorable genetic factors, whereas cohesin complex genes combined was a favorable factor ($p = 0.039$).

Multivariable analysis of the subset of 265 2022 ELN favorable-risk *NPM1*^{mut} AML patients (61%) identified MRG ($p = 0.016$) and *MYC* mutations ($p = 0.047$) as unfavorable factors for EFS, while allogeneic HCT in the first CR was associated with improved EFS ($p = 0.001$) (Supporting Information S1: Table S8). For OS, only the clinical variables age ($p = 0.044$) and secondary or therapy-related AML ($p = 0.003$) were significant adverse factors while allogeneic HCT in the first CR was a favorable factor ($p = 0.005$). No *NPM1* MRD data were available for this second independent cohort.

In the combined AMLSG 09-09 and independent second cohort, a total of only 18 of 735 (2.45%) ELN favorable-risk patients exhibited two or more MRG mutations precluding a meaningful outcome analysis of cases with one *versus* two or more MRG mutations.

DISCUSSION

In this study, we investigated the impact of MRG and additional gene mutations in 568 *NPM1*^{mut} AML. Particular strengths of our study are, first, all patients were treated in a prospective randomized clinical trial[17]; second, *NPM1* MRD data were available in a subset of patients,[18] and third, we validated findings in an independent cohort from a previous targeted sequencing study that included 435 *NPM1*^{mut} AML.[3]

Consistent with previous studies,[4,15] most commonly co-mutated genes were *DNMT3A*, *FLT3*-TKD, *PTPN11*, *NRAS*, *TET2*, *IDH2*, *IDH1*, and *FLT3*-ITD (Figure 1, Supporting Information S1: Table S3). AML with *NPM1*^{mut}/*FLT3*-ITD were underrepresented in our study, since during the conduct of the AMLSG 09-09 trial many patients with concurrent *FLT3*-ITD were entered in the AMLSG 16-10 trial with midostaurin, which limits the comparability of our data to other studies focusing on all *NPM1*^{mut} AML.[24] One or more MRG mutations were detected in 103 of 568 (18.1%) *NPM1*^{mut} AML, most commonly *SRSF2* and *STAG2*. Patients with MRG co-mutations were older, more frequently male, and had lower WBC and platelet counts; with regard to the co-mutational pattern, *NPM1*^{mut}/MRG^{pos} AML appeared less

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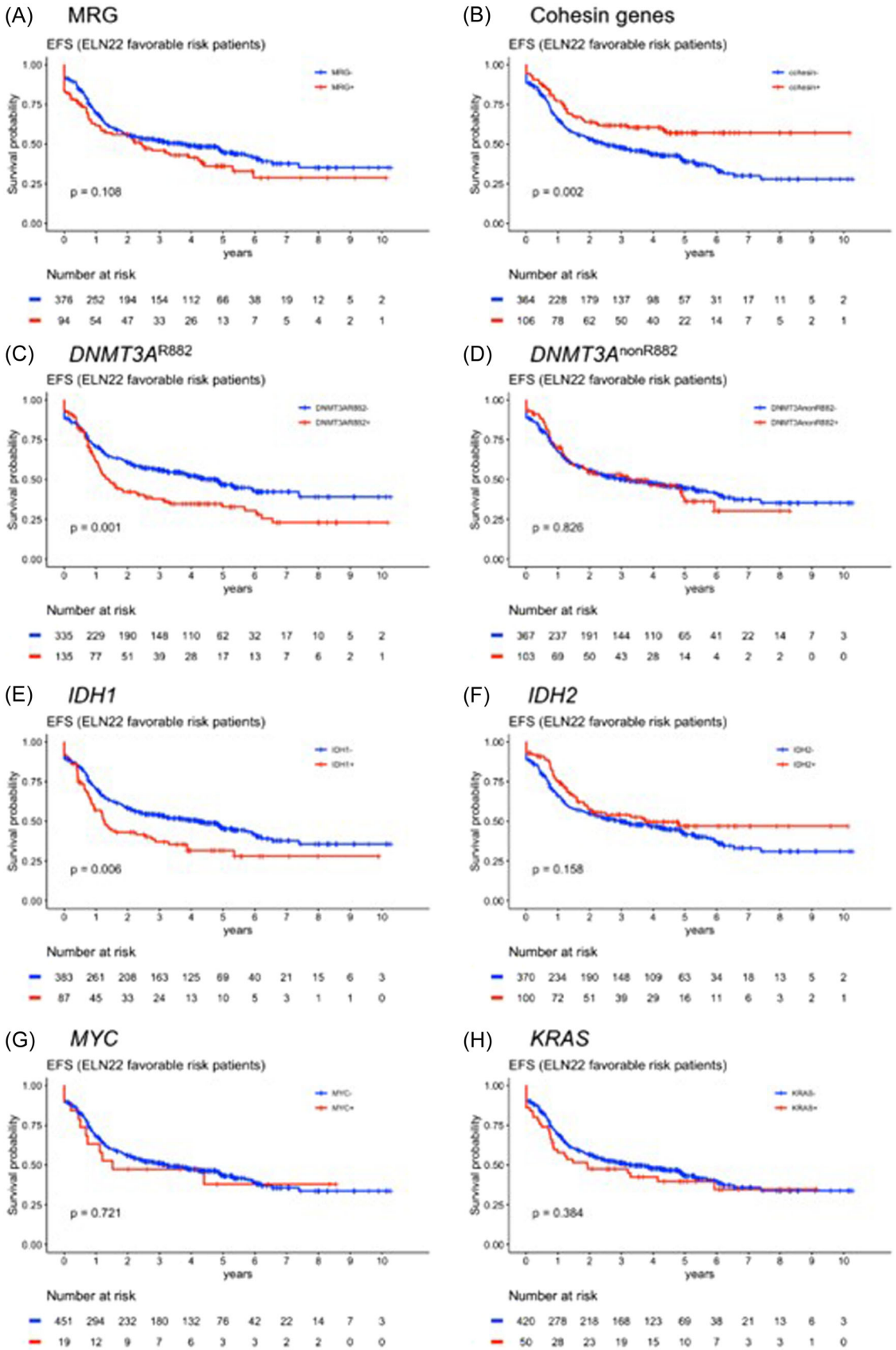


FIGURE 3 **Univariate analysis of event-free survival of 470 patients with 2022 ELN favorable-risk *NPM1*^{mut} AML by co-mutation pattern**. Myelodysplasia-related genes (MRG) **(A)**, cohesin complex genes *(RAD21*, *SMC1A*, *SMC3A*, *STAG2)* **(B)**, *DNMT3A*^{R882} **(C)**, *DNMT3A*^{nonR882} **(D)**, *IDH1* **(E)**, *IDH2* **(F)**, *MYC* **(G)**, and *KRAS* mutations **(H)**.

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TABLE 2 Results of multivariable analysis for event-free and overall survival in the 2022 ELN favorable-risk subset of *NPM1*^{mut} AML ($n = 470$).

Variable	Event-free survival			Overall survival		
	HR	95% CI	*p* Value	HR	95% CI	*p* Value
Treatment arm with GO	0.69	0.52; 0.90	0.007	0.81	0.58; 1.13	0.208
Age (10 years increase)	1.28	1.13; 1.45	<0.001	1.69	1.44; 1.99	<0.001
Male sex	0.91	0.69; 1.20	0.519	0.73	0.52; 1.03	0.070
s-AML/t-AML	1.01	0.57; 1.81	0.965	0.99	0.55; 1.8	0.974
WBC (log10)	1.12	0.88; 1.42	0.362	1.20	0.88; 1.63	0.244
LDH (log10)	1.38	0.79; 2.43	0.258	1.42	0.77; 2.6	0.262
Allogeneic HCT (in first CR)	1.36	0.57; 3.24	0.487	1.54	0.66; 3.64	0.320
CEBPA	0.93	0.61; 1.40	0.713	0.85	0.51; 1.4	0.515
DNMT3A^{nonR882}	1.35	0.96; 1.90	0.083	1.14	0.74; 1.76	0.539
DNMT3A^{R882}	1.91	1.40; 2.62	<0.001	1.54	1.02; 2.34	0.042
FLG	1.74	0.92; 3.27	0.086	1.90	0.84; 4.27	0.121
FLT3-TKD	1.17	0.87; 1.57	0.308	1.13	0.77; 1.64	0.536
GATA2	1.48	0.78; 2.77	0.227	0.74	0.25; 2.17	0.578
IDH1	1.56	1.13; 2.16	0.007	1.50	1.01; 2.23	0.045
IDH2	0.77	0.53; 1.11	0.160	0.61	0.38; 0.97	0.037
KMT2C	0.72	0.35; 1.49	0.378	0.81	0.34; 1.95	0.641
KRAS	1.40	0.90; 2.18	0.130	2.11	1.29; 3.45	0.003
MYC	1.57	0.79; 3.14	0.201	1.39	0.59; 3.29	0.451
NF1	0.69	0.38; 1.25	0.219	0.94	0.44; 1.99	0.866
NFE2	1.05	0.42; 2.62	0.912	0.65	0.2; 2.1	0.473
NRAS	0.90	0.65; 1.23	0.503	0.79	0.53; 1.18	0.251
PTPN11	1.05	0.78; 1.40	0.760	0.85	0.58; 1.25	0.410
TET2	1.02	0.71; 1.47	0.902	1.07	0.68; 1.69	0.770
WT1	1.59	0.97; 2.62	0.067	1.32	0.73; 2.41	0.360
Cohesin complex genes[a]	0.53	0.36; 0.78	0.001	0.65	0.41; 1.05	0.076
Myelodysplasia-related genes[b]	1.50	1.04; 2.16	0.030	1.27	0.8; 2.01	0.308

Abbreviations: CI, confidence interval; CR, complete remission; GO, gemtuzumab ozogamicin; HCT, hematopoietic cell transplantation; HR, hazard ratio; ITD, internal tandem duplication; LDH, lactate dehydrogenase; NA, not applicable; s-AML, secondary AML; t-AML, therapy-related AML; TKD, tyrosine kinase domain; WBC, white blood cells.

All genes mutated in ≥3% of cases were included in the model.

[a]Cohesin complex genes: *RAD21*, *SMC1A*, *SMC3*, *STAG2*.

[b]Myelodysplasia-related genes: *ASXL1*, *BCOR*, *EZH2*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, and *ZRSR2*.

dependent on signaling gene mutations (*FLT3*-ITD, *PTPN11*, *NRAS*), had fewer *DNMT3A* (in particular *DNMT3A*^{R882}), and more frequently *TET2* and cohesin complex gene mutations (Table 1). Based on VAFs, MRG mutations were dominant over *NPM1* mutations in 76% of cases, which is consistent with data from previous studies.[11,12,15] Clinical and disease characteristics are similar to those reported in the previous studies. In all studies,[11–13] patients were older, had lower WBC and/or platelet counts, in three studies MRG^{pos} cases also had fewer *DNMT3A* and *FLT3*-ITD mutations,[11,12,15]

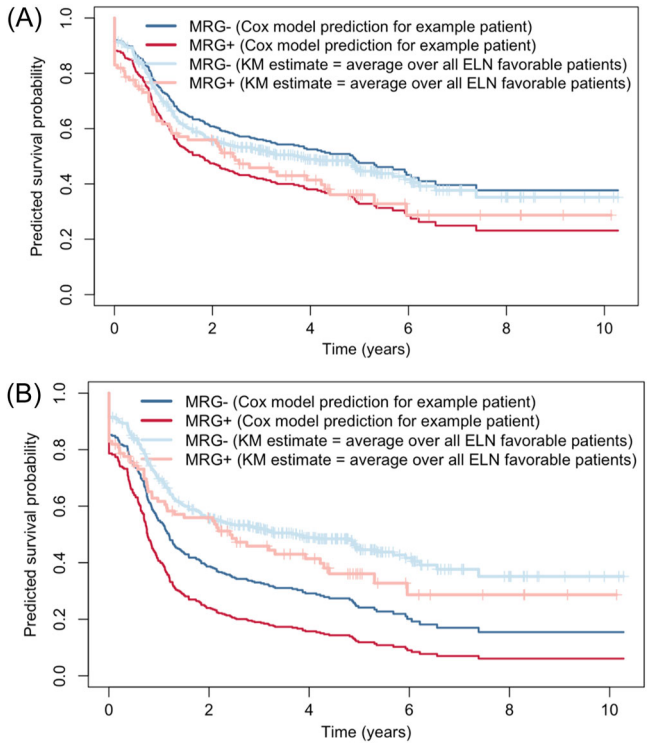


FIGURE 4 **Outcome of two exemplary patients with 2022 ELN favorable-risk *NPM1*^{mut} AML by the presence of myelodysplasia-related gene (MRG) mutations predicted from the Cox model.** Event-free survival (EFS) by the presence of MRG mutations for two exemplary patients with 2022 ELN favorable-risk *NPM1*^{mut} AML, aged 58, female, *de novo* disease, without **(A)** and with **(B)** *DNMT3A*^{R882} co-mutation. The shaded curves illustrate Kaplan–Meier estimates over all 2022 ELN favorable-risk patients (with or without MRG mutations) and are identical in panels A and B. Bolded curves show EFS predicted from the multivariable model for the two exemplary patients; the patient exhibiting a *DNMT3A*^{R882} co-mutation **(B)** overall has a significantly inferior outcome compared to the same patient without a *DNMT3A*^{R882} co-mutation, due to the highly significant detrimental effect of *DNMT3A*^{R882} co-mutation. KM, Kaplan-Meier.

One open question raised by 2022 ELN is whether MRG co-mutations abrogate the positive prognostic impact of *NPM1*^{mut} AML, in particular those cases classified as favorable-risk.[6] In our study, 470 of 568 (83%) *NPM1*^{mut} AML were categorized as 2022 ELN favorable. In this risk group, MRG co-mutations had no impact on outcome in the univariable analysis (Figure 3); however, in the multivariable analysis, they were associated with inferior EFS (Table 2). One likely explanation for the discrepancy between the results from univariable and multivariable analyses is the different co-mutational pattern of *NPM1*^{mut}/MRG^{neg} versus *NPM1*^{mut}/MRG^{pos} AML, whereby the significantly higher frequency of *FLT3*-ITD and *DNMT3A*^{R882} mutations in the *NPM1*^{mut}/MRG^{neg} cases, both of which have a highly significant negative effect on EFS, led to virtually overlapping EFS curves between *NPM1*^{mut}/MRG^{pos} and *NPM1*^{mut}/MRG^{neg} AML in Kaplan-Meier analysis (Figure 3). The negative prognostic effect of MRG mutations based on multivariable analysis is visualized in Figure 4 showing predicted EFS curves resulting from the Cox model for two exemplary patients, one with and one without *DNMT3A*^{R882} co-mutation. Similarly, in the entire cohort of *NPM1*^{mut} AML MRG mutations also had no significant effect in univariable analysis; however, there was a trend for inferior EFS in multivariable analysis. MRG

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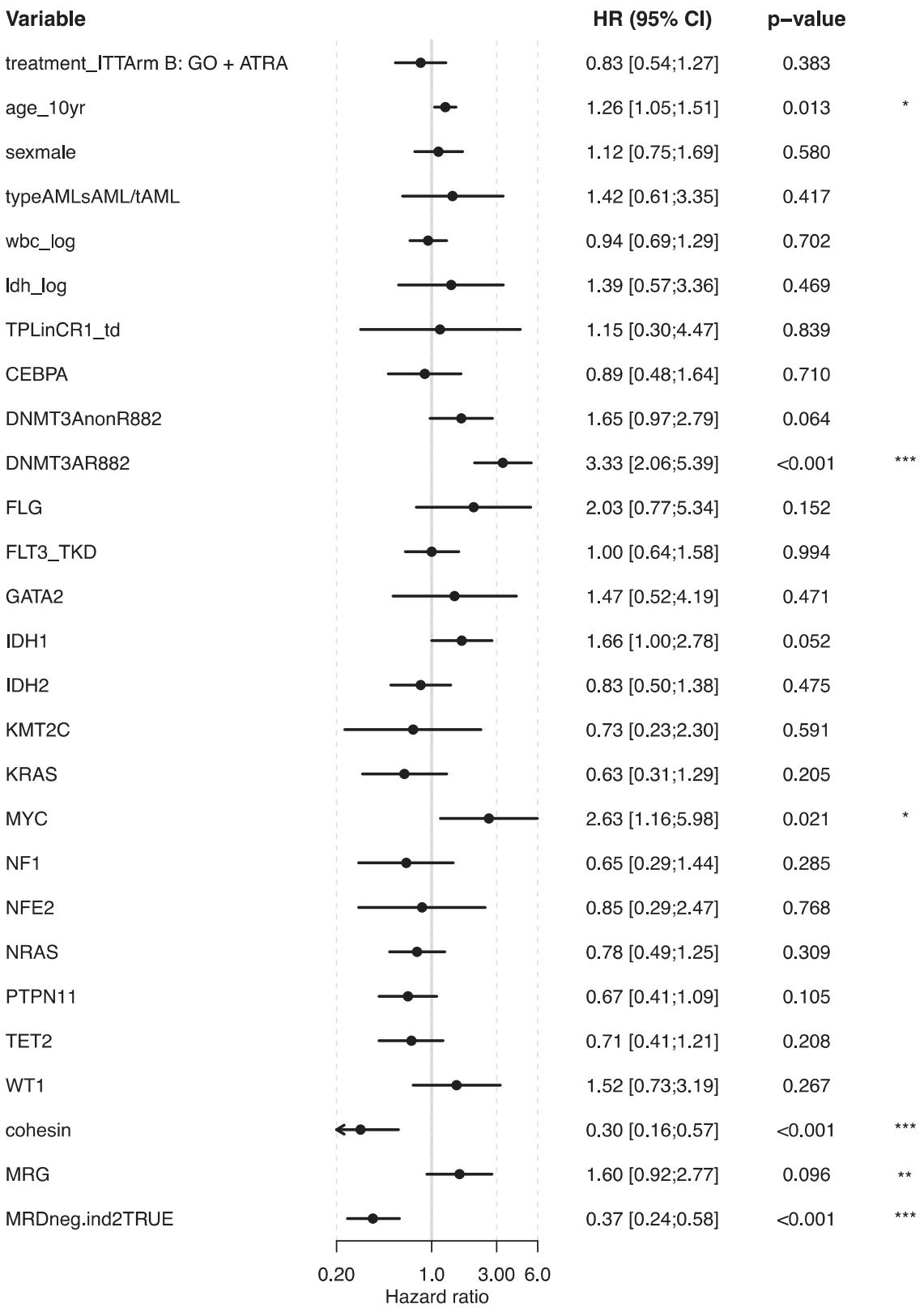


FIGURE 5 **Multivariable analysis of event-free survival (EFS) in patients with 2022 ELN favorable-risk *NPM1*^{mut} AML with inclusion of qPCR *NPM1* measurable residual disease (MRD) status in peripheral blood at landmark time point end of induction cycle 2**. ATRA, all-*trans* retinoic acid; CI, confidence interval; CR, complete remission; GO, gemtuzumab ozogamicin; HR, hazard ratio; LDH, lactate dehydrogenase; MRD, measurable residual disease; s-AML, secondary AML; t-AML, therapy-related AML; TKD, tyrosine kinase domain; TPL, allogeneic transplantation; WBC, white blood cells. All genes mutated in ≥3% of cases were included in the model. *Cohesin complex genes: *RAD21*, *SMC1A*, *SMC3*, *STAG2*. **Myelodysplasia-related genes (MRG): *ASXL1*, *BCOR*, *EZH2*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, and *ZRSR2*. ***Variables with highest significance ($p < 0.001$).

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mutations did not affect OS, neither in the favorable-risk group nor in the total cohort.

To validate our findings, we analyzed a cohort of 435 intensively treated patients with *NPM1*^{mut} AML from our previous targeted sequencing study in 1540 intensively treated patients.[3] The prevalence of MRG mutations (11.3%) in *NPM1*^{mut} AML was somewhat lower than in the AMLSG 09-09 discovery cohort (18.1%), likely due to the lower median age (50 years) in the independent cohort, but almost identical to the frequency (12%) found in the NCRI study, which included mostly younger patients with a median age of 53 years.[15] Outcome analyses in the independent cohort were comparable to those of the AMLSG 09-09 cohort. In univariable analysis, the presence of MRG mutations did not impact EFS in the entire cohort (only in the 2022 ELN favorable group), whereas in multivariable analysis, MRG mutations had a significant negative prognostic impact on EFS in the entire cohort as well as the 2022 ELN favorable-risk subset; no MRD data were available for the independent cohort.

In the ELN favorable-risk group, poor prognostic factors for EFS in addition to MRG mutations were age, *DNMT3A*^{R882} hot spot, and *IDH1* mutations, whereas cohesin complex gene co-mutations and treatment with GO were associated with superior EFS. The beneficial effect of GO for EFS in the favorable-risk group was supported by multivariable analysis for CIR (Supporting Information S1: Table S6). This beneficial effect of GO in *NPM1*^{mut} AML is consistent with the data from the ALFA and the NCRI studies.[15,28]

The 2022 ELN recommendations explicitly emphasize that not only genetic abnormalities at the time of diagnosis but also results from MRD analysis should be taken into consideration for a comprehensive genetic risk assessment.[6] For *NPM1* MRD, qPCR results from peripheral blood post cycle 2 have been shown to be most informative for prognosis and are currently considered an important clinical decision point.[18,25–27] In our study, *NPM1*^{mut} qPCR MRD negativity had a highly significant favorable effect on EFS ($p < 0.001$) (Figure 5). When restricting the MRD status post induction cycle 2 to a subset of CR/CRi patients with available data in multivariable analysis for EFS, MRG mutations no longer showed an unfavorable effect; *DNMT3A*^{R882} and *MYC* mutations remained unfavorable, while cohesin gene mutations were favorable.

Recent studies analyzed the impact of MRG mutations in *NPM1*^{mut} AML, which yielded controversial results, with either a negative or no impact on outcome.[11–15] The NCRI group reported on 1098 *NPM1*^{mut} AML of which 644 cases had NGS and MRD (post course 2) data available.[15] The study included 39% *NPM1*^{mut}/*FLT3*-ITD co-mutated cases, which somewhat limits the comparability to our study where the frequency was only 17.3%. In the NCRI study, among baseline genetic factors, *FLT3*-ITD, *DNMT3A*^{mut}, *WT1*^{mut}, and non-ABD type *NPM1*^{mut} were independently associated with poorer OS; in this study, the authors did not find a significant impact of MRG co-mutations.[15] Similarly, in our study, *FLT3*-ITD and *DNMT3A*^{mut} (but only the R882 hot spot mutation) were independent poor prognostic factors for OS, *WT1*^{mut} only reached significance for EFS, and *NPM1*^{mut} type had no prognostic impact (data not shown). When including MRD status in blood post cycle 2 in the multivariable model of the NCRI study, *FLT3*-ITD, *DNMT3A*^{mut}, and non-ABD type *NPM1*^{mut} remained significant, while MRD negativity was associated with a significantly improved OS with a hazard ratio of 0.31, similar to the effect found for MRD negativity for EFS in our study (HR 0.34). Furthermore, the investigators confirmed their previous finding that patients with *NPM1*^{mut} AML who achieve MRD negativity in blood post cycle 2 do not benefit from allogeneic HCT.[29] Similarly, in our study, there was no benefit for allogeneic HCT neither in the entire cohort nor in the 2022 ELN favorable-risk group. Both NCRI and our study provide further support for the current ELN recommendations not to assign patients with *NPM1*^{mut} AML who are in molecular remission post cycle 2 a priori to allogeneic HCT.

The negative impact of concurrent *FLT3*-ITD in *NPM1*^{mut} AML is well established. However, with the introduction of FLT3 inhibitors the natural history of AML with *FLT3* mutations has changed.[30] None of the patients in our and the NCRI cohort received midostaurin.[15] Furthermore, the recent development of NGS-based *FLT3*-ITD MRD monitoring may complement qPCR *NPM1*^{mut} monitoring in assessing the risk of relapse in *NPM1*^{mut}/*FLT3*-ITD co-mutated cases.[31–33] *WT1* mutations have been shown to be associated with *FLT3*-ITD and to confer poor outcomes.[34] The poor prognostic effect of *WT1* found in the NCRI study is consistent with previous data from the RATIFY trial showing that in AML with *NPM1*^{mut} and *FLT3*^{mut}, *WT1* mutations had a significant negative effect.[34] The effect of *WT1*^{mut} in our study was less pronounced compared to the NCRI study, which may be explained by the considerably lower frequency of cases with concurrent *FLT3*-ITD. *DNMT3A* mutations (in particular R882 hot spot mutations), which are significantly associated with *NPM1*^{mut} AML, have previously been shown to have a negative prognostic effect on outcome.[35] The data from our clinical AMLSG 09-09 trial suggested that the negative prognostic impact of *DNMT3A* mutations may be largely overcome by the addition of GO to intensive chemotherapy.[17] Unlike in the NCRI study, in our study, *IDH1* co-mutation exerted a significant inferior effect on both EFS and OS, even more pronounced in the favorable-risk subset, whereas *IDH2*^{R140} mutations had a beneficial effect for OS in the favorable-risk and entire cohort. Overall, these effects are consistent with data from a meta-analysis of *IDH*^{mut} AML.[36] In a phase 1 trial, the combination of the IDH inhibitors ivosidenib and enasidenib with intensive chemotherapy has shown promising results in *IDH1*- and *IDH2*-mutated AML, respectively.[37] These combinations are currently being investigated in a randomized phase 3 trial (NCT03839771). Cohesin gene mutations are known to be associated with *NPM1*^{mut} AML; however, so far no data with regard to prognostic impact specifically in *NPM1*^{mut} AML were reported,[3,38,39] except the recent NCRI study,[15] in which the cohesin genes were not identified as a relevant prognostic factor.

In conclusion, in 2022 ELN favorable-risk *NPM1*^{mut} AML, MRG co-mutations at baseline predicted for inferior EFS; however, this effect was no longer present when including qPCR *NPM1*^{mut} MRD status in blood post cycle 2 in the survival model. Based on our data, we propose not to consider MRG co-mutations for risk stratification in *NPM1*^{mut} AML if molecular MRD data post cycle 2 are available. Beyond the current use of GO and FLT3 inhibitors, the treatment landscape for *NPM1*^{mut} AML is going to change with the development of IDH, BCL2, and menin inhibitors,[40] thereby likely improving the outcome of *NPM1*^{mut} AML with its various co-mutations.

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AUTHOR CONTRIBUTIONS

Sibylle Cocciardi, Hartmut Döhner, and Konstanze Döhner designed the study. Sibylle Cocciardi, Nina Weiß, Silke Kapp-Schwoerer, Isabelle Schneider, Annika Meid, and Sabrina Skambraks performed experiments and validated data. Maral Saadati, Daniela Späth, and Axel Benner performed statistical analyses. Walter Fiedler, Michael W. M. Kühn, Ulrich Germing, Karin T. Mayer, Michael Lübbert, Elli Papaemmanuil, Felicitas Thol, Michael Heuser, Arnold Ganser, Hartmut Döhner, and Konstanze

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Döhner collected, assembled, analyzed, and interpreted data. Sibylle Cocciardi, Hartmut Döhner, and Konstanze Döhner 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 STATEMENT

Verena I. Gaidzik: Advisory Board: Jazz Pharmaceuticals, Abbvie, Boehringer-Ingelheim, Speakers Bureau: Pfizer, Janssen, Abbvie, Travel Support: Abbvie. Walter Fiedler: Personal fees and non-financial support from AbbVie; grants, personal fees, and non-financial support from Amgen and Pfizer; and personal fees from Jazz Pharmaceuticals, Celgene, MorphoSys, Ariad/Incyte, Stemline Therapeutics, Clinigen, Daiichi Sankyo, Otsuka and Servier outside the submitted work; in addition, research support from Apis; patent issued with Amgen; support for medical writing for Amgen, Pfizer, and AbbVie. Michael W. M. Kühn: receives honoraria and is a consultant for Kura Oncology, Jazz Pharmaceuticals, Bristol-Myers Squibb/Celgene, and Abbvie, and is on the speakers' bureau of Gilead. Karin T. Mayer: advisory role with honoraria for Bristol Myers Squibb; Travel Support from Novartis, Celgene, Roche, Amgen, Pfizer, Jazz Pharmaceuticals, Astellas, Teva. Michael Lübbert: advisory role with honoraria from AbbVie, Astex Pharmaceuticals, Imago BioSciences, Janssen, Otsuka, Syros; Research support (to institution) from Janssen; clinical research support with study drug from Cheplapharm. Elli Papaemmanuil: founder, equity holder, and holds fiduciary roles in Isabl Inc. Felicitas Thol: advisory role with honoraria for AbbVie, BMS, Novartis, Menarini, Rigel, Astellas. Michael Heuser: declares honoraria from Abbvie, Eurocept, Jazz Pharmaceuticals, Janssen, Novartis, Takeda; paid consultancy for Abbvie, Agios, BMS, Daiichi Sankyo, Glycostem, Jazz Pharmaceuticals, Kura Oncology, Novartis, Pfizer, PinotBio, Roche, Tolremo; and research funding to his institution from Abbvie, Agios, Astellas, Bayer Pharma AG, BergenBio, Daiichi Sankyo, Glycostem, Jazz Pharmaceuticals, Loxo Oncology, Novartis, Pfizer, PinotBio, Roche. Lars Bullinger: advisory roles for Abbvie, Amgen, Astellas, Bristol-Myers Squibb, Celgene, Daiichi Sankyo, Gilead, Hexal, Janssen, Jazz Pharmaceuticals, Menarini, Novartis, Pfizer, Sanofi, Servier; as well as research funding from Bayer, Jazz Pharmaceuticals. Hartmut Döhner: advisory role with honoraria for AbbVie, AstraZeneca, Gilead, Janssen, Jazz, Pfizer, Servier, Stemline, Syndax; clinical research funding (to institution) from AbbVie, Astellas, Bristol Myers Squibb, Celgene, Jazz Pharmaceuticals, Kronos Bio, Servier. Konstanze Döhner: consultancy with honoraria: AbbVie, Janssen, Jazz, Novartis, Bristol Myers Squibb, Celgene; Clinical Research Funding to Institution: Novartis, AbbVie, Astellas, Bristol Myers Squibb, Celgene, Jazz Pharmaceuticals, Kronos Bio, Servier. The remaining authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Access to de-identified data and supporting information can be requested by any qualified researchers who engage in rigorous, scientific research. Data will be provided following review and approval of a research proposal, Statistical Analysis Plan, and execution of a Data Sharing Agreement. The data will be accessible for 12 months, with possible extensions considered. For requests, please contact konstanze.doehner@uniklinik-ulm.de.

ETHICS STATEMENT

The study was approved by the institutional review board at each participating center. Written consent was given by all patients according to the Declaration of Helsinki.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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