Received: 26 April 2019 | Revised: 16 May 2019 | Accepted: 21 May 2019

DOI: 10.1002/ajh.25514

RESEARCH ARTICLE

Next-generation sequencing-defined minimal residual disease before stem cell transplantation predicts acute myeloid leukemia relapse

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Funding information
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Abstract

In acute myeloid leukemia (AML), the assessment of post-treatment minimal residual disease (MRD) may inform a more effective management approach. We investigated the prognostic utility of next-generation sequencing (NGS)-based MRD detection undertaken before hematopoietic stem cell transplantation (HSCT). Forty-two AML subjects underwent serial disease monitoring both by standard methods, and a targeted 42-gene NGS assay, able to detect leukemia-specific mutant alleles (with >0.5% VAF) (mean 5.1 samples per subject). The prognostic relevance of any persisting diagnostic mutation before transplant (≤27 days) was assessed during 22.1 months (median) of post-transplant follow-up. The sensitivity of the NGS assay (27 MRD-positive subjects) exceeded that of the non-molecular methods (morphology, FISH, and flow cytometry) (11 positive subjects). Only one of the 13 subjects who relapsed after HSCT was NGS MRD-negative (92% assay sensitivity). The cumulative incidence of post-transplant leukemic relapse was significantly higher in the pre-transplant NGS MRD-positive (vs MRD-negative) subjects (P = .014). After adjusting for TP53 mutation and transplant conditioning regimen, NGS MRD-positivity retained independent prognostic significance for leukemic relapse (subdistribution hazard ratio = 7.3; P = .05). The pre-transplant NGS MRD-positive subjects also had significantly shortened progression-free survival (P = .038), and marginally shortened overall survival (P = .068). In patients with AML undergoing HSCT, the pre-transplant persistence of NGS-defined MRD imparts a significant, sensitive, strong, and independent increased risk for subsequent leukemic relapse and death. Given that NGS can simultaneously detect multiple leukemia-associated mutations, it can be used in the majority of AML patients to monitor disease burdens and inform treatment decisions.

1 | INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive malignancy of clonal, immature myeloid cells with a five-year overall survival of less than 30%.[1] For patients with intermediate or high-risk disease, allogeneic hematopoietic stem cell transplantation (HSCT) offers the best opportunity for durable remission, but it also entails significant risk for transplant related morbidity and mortality.[2,3] Laboratory methods to accurately identify those patients most likely to relapse after HSCT, would provide an opportunity for risk-based therapeutic intervention.[4] Traditional

prognostic factors for post-HSCT outcomes include: AML disease control at the time of transplant, functional status, comorbidities, degree of HLA match, and underlying disease risk stratification.[5] Of these factors that are actionable, disease control at the time of transplant is of increasing interest, as there are now a variety of laboratory methods to monitor minimal residual disease (MRD) status, beyond standard morphologic criteria.

Morphologic assessment of the percentage of bone marrow myeloblasts has long been the traditional laboratory method for monitoring the response to AML therapy, and "complete remission" (CR), defined as <5% bone marrow myeloblasts, is a consensus therapeutic goal. More recent studies, however, have shown that a variety of other laboratory methods for more sensitively measuring submicroscopic MRD can be utilized as consistent, independent prognostic factors for AML relapse and survival.[6-11] These more sensitive MRD techniques include multicolor flow cytometry (MFC), RT-PCR for overexpressed genes, single-gene PCR for commonly mutated genes, fluorescent in situ hybridization (FISH), and next-generation sequencing (NGS).[6] The use of MRD monitoring prior to HSCT, has the potential to improve outcomes in HSCT patients, particularly for those patients with poor prognosis MRD studies for whom additional anti-leukemic intervention may be available. To extend the availability of prognostic MRD monitoring to a larger fraction of transplanted AML patients - including those with undetectable disease by traditional lab methods - we have performed longitudinal NGS on serial pre- and post-treatment samples. By using NGS, we could simultaneously assess a large panel of genes and thus identify a broader spectrum of potential MRD targets, including any of the heterogeneous leukemia-associated mutations that were identified at diagnosis. In this study, we evaluated NGS-defined molecular MRD at a standard time point for pre-transplant AML disease monitoring (within 30 days prior to HSCT), and established its clinical prognostic utility for predicting post-transplant leukemic relapse and death.

2 | METHODS

2.1 | Patients

In our institution, patients diagnosed with AML undergo routine NGS-based mutation profiling, both at their initial leukemia diagnosis, and at multiple subsequent post-treatment time points. For this single institution retrospective cohort study, we searched the clinical laboratory information systems and the CIBMTR (Center for International Blood and Marrow Transplant Research) transplant database and identified 42 subjects that met all of the following criteria: 1) diagnosed with AML following consensus WHO guidelines; 2) underwent allogeneic HSCT from February 2013 through June 2015 at our institution; 3) NGS had been performed both at diagnosis and at a pre-transplant time point, defined as less than 30 days prior to HSCT; 4) had at least one trackable pathogenic mutation detected at diagnosis; and 5) post-transplant clinical followup was available for a minimum of 350 days. The study was approved by the OHSU institutional review board.

2.2 | Treatment

An anthracycline + cytarabine based induction chemotherapy regimen was used in 41 patients. One patient received a high dose cytarabine based regimen. Fourteen of the 42 patients received "re-induction" chemotherapy due to disease persistence, either at the time of their nadir bone marrow biopsy or at the time of the recovery bone marrow biopsy. Twenty-nine of the patients underwent consolidation chemotherapy prior to transplant. The remaining thirteen patients went directly to transplant without consolidation. The transplant methodology used in our institution has been described.[12] Briefly, subjects underwent transplant with varying conditioning regimens based on HCT-CI, pre-transplant disease status, and type of HSCT donor.

2.3 | Next-generation sequencing

The NGS methodology used in this study has been previously described.[13] Briefly, we sequenced on a PGM sequencer (Life Technologies) using a customized Ampliseq panel of 42 genes relevant to myeloid leukemogenesis. Details of the genes and exons sequenced are provided in supplemental Table S1. The customized bioinformatics pipeline was a combination of the manufacturer's analysis pipeline, and a lab-developed variant annotation algorithm using public databases (COSMIC, dbSNP, ExAC, etc.). Our CLIA-approved protocol also includes supplemental single-gene non-NGS assays for the common insertion/deletion mutations that can be missed by NGS, including those in FLT3 (ITD) and CEBPA. The only mutations that were considered as trackable MRD targets for post-treatment monitoring were those that were both: 1) present in the pre-treatment sample within a predominating leukemia clone, and 2) classified as pathogenic or likely pathogenic (Tier I or Tier II) by consensus NGS somatic mutation interpretation guidelines.[14] For post-treatment samples, NGS reads at genomic coordinates specific for a subject's unique set of trackable mutations were manually inspected for mutant sequencing reads and total sequencing reads [to determine a residual variant allele fraction (% VAF)]. The NGS sequencing coverage across all time points and all genes averaged 1900. The lower limit of detection (LLOD) for post-treatment mutations was conservatively defined using the beta inverse function with a probability of 95%, as per consensus guidelines.[15,16] At the average sequencing coverage of 1900 reads, the 95% LLOD was 0.24%. The LLOD is, by definition, higher in samples with poorer coverage such that at a minimum 1000 coverage (constituting 83% of the samples), LLOD was 0.4%, and at a minimum 500 coverage (constituting 93% of the samples), LLOD was 0.7%.

2.4 | Non-NGS disease monitoring

Post-HSCT disease status was determined by morphologic, flow cytometric, & cytogenetic/FISH analysis of bone marrow specimens following standard guidelines.[17-19] Minimal residual disease by flow cytometry was performed by incubating 100 000 leukocytes with an 8-color fluorescent antibody panel including CD2, CD3, CD4, CD5, CD7, CD8, CD11b, CD13, CD14, CD15, CD16, CD19, CD20, CD33, CD34, CD45, CD56, CD64, CD117, CD123, and HLA-DR (Becton Dickinson

Biosciences). Minimal residual disease was assessed using an 8-color FACSCANTO II instrument (BD Biosciences) with WinList software (Verify). Residual disease was defined as any myeloid blast population with an abnormal pattern of antigen expression above a sensitivity limit of 0.5%. Minimal residual disease analysis by FISH was performed using standard protocols with a panel of probes (Abbott) targeting recurrent aberrations in AML. The lower limit of detection for scoring FISH-positive cells (among 200 scored cells) was established as the 95% upper confidence limit, of the percentage of positive signals observed in 200 normal non-cancer cells as per consensus guidelines.[15,16] For this study, "complete remission" (CR) was defined as the post-treatment absence of any leukemic blasts by morphology, flow cytometry, and cytogenetics/FISH. Four of the 11 subjects without a pre-transplant CR had detectable leukemia only by flow cytometry or FISH (not by morphology). Leukemic relapse was defined as the post-transplant presence of any documented leukemia cells (by any laboratory method).

2.5 | Statistical analysis

Descriptive statistics were used to summarize and compare the demographic, disease, and clinical characteristics of the subjects (and compare MRD groups). A Fisher's exact or Chi-squared test was used to compare categorical variables. A two-sample *t*-test or Mann-Whitney *U* test was used to compare continuous variables. To assess post-HSCT outcomes, we conducted competing risks analysis, which calculates the cumulative incidence of leukemic relapse in the presence of competing risks (non-leukemic death). Cumulative incidence curves of leukemic relapse in MRD-positive vs MRD-negative subjects were compared using Gray's test,[20] and the effect of MRD status was assessed, adjusting for potential confounding factors, using Fine and Gray's subdistribution hazard model.[21] The clinical or disease characteristics found to have significant association with leukemic relapse in univariable analysis ($P < .15$) were included in the multivariate model. Progression-free survival (PFS) and overall survival (OS) were compared between MRD-positive and MRD-negative subjects using the Kaplan-Meier method and the log-rank test. For the progression-free survival analyses, subjects alive without any evidence of leukemia were censored at the time of last follow-up (median 22.1 months after transplant; minimum 11.5 months), and failure events included leukemic relapse or non-relapse mortality. Statistical significance was defined as a $P < .05$. All statistical analyses were performed using SAS version 9.4.

3 | RESULTS

3.1 | Patient and disease characteristics

The demographic and disease characteristics of the 42 transplanted AML subjects are summarized in Table 1. Patient-specific details are shown in supplemental Table S2. ELN adverse risk patients were predictably over-represented in this transplanted cohort (compared to a large unbiased AML cohort),[22] and a significant minority of subjects had higher-risk secondary AML. Multi-gene NGS (42 target genes) before therapy showed the expected heterogeneity of mutated genes. In particular, there were 99 leukemia-associated, pathogenic, trackable mutations in 23 distinct genes, with an average of 2.4 mutations per subject (range 1-6)(supplemental Table S3). The most commonly mutated genes were FLT3 (8 internal tandem duplications and 3 tyrosine kinase domain mutations), NPM1 (n = 10), RUNX1 (n = 9), TET2 (n = 8), TP53 (n = 8), DNMT3A (n = 7), CEBPA (n = 6), NRAS (n = 6), WT1 (n = 6), IDH1 (n = 3), IDH2 (n = 3), PTPN11 (n = 3), SF3B1 (n = 3), and SRSF2 (n = 3). The median mutant allele frequency at diagnosis was 40% (IQR, 29-49).

3.2 | Response to therapy

To assess treatment efficacy, the subjects were monitored by NGS (as well as routine bone marrow morphology, cytogenetics/FISH, and flow cytometry) at serial time points after induction. Only 3% of the AML patients from our institution lack an NGS-detectable mutation (in 42 target genes) that precludes subsequent molecular MRD monitoring (unpublished data). The 42 subjects (all of whom, as per study eligibility criteria, had a trackable mutation) underwent 5.1 (mean) serial NGS assays throughout their disease follow-up. The number of trackable and persisting mutations in the sequenced samples that were collected at diagnosis, after induction chemotherapy, and before HSCT (≤ 27 days) are detailed in supplemental Table S4. After HSCT, possible disease recurrence was regularly evaluated by routine clinical and laboratory studies during a post-transplant follow-up period of 22.1 months (median). During this time an additional 2.9 (mean) post-transplant NGS samples per subject were evaluated for evidence of recurring disease.

In addition to having had an NGS assay less than a month before HSCT (a necessary eligibility criteria for this study), 28 subjects had NGS performed on an earlier post-induction sample (median 1.8 months after diagnosis)(supplemental Table S4). At this post-induction time point, 25 of the 28 (89%) subjects had achieved a complete remission (CR; with no detectable leukemic blasts), and the 3 non-CR subjects had low-level residual leukemia (with 1%, 4%, and 7% bone marrow blasts by MFC). In comparison, using the more sensitive sequencing assay, 54% of the subjects (15/28) had detectable NGS-defined minimal residual disease at this same post-induction time point (including the 3 subjects not in CR) (supplemental Table S4). In these 15 subjects with detectable disease post-induction, 27 mutations persisted at low levels (in 13 different genes)(mean VAF = 4.0%)(supplemental Table S4). The VAF of these post-induction persisting mutations was 16-fold reduced (median), compared to the pre-treatment diagnostic sample. The most common persisting leukemic clones post-induction had mutations in TP53 (6 mutations in 4 subjects), DNMT3A (5 mutations in 4 subjects), and TET2 (3 mutations in 2 subjects).

3.3 | Pre-transplant minimal residual disease

NGS was performed on all 42 subjects no more than 30 days prior to HSCT (median 0.6 months before HSCT and median 3.6 months after diagnosis) (supplemental Table S4). At this pre-transplant time point, 11 subjects (26%) had failed to achieve CR with detectable residual leukemia by morphology, flow cytometry, or cytogenetics/FISH ("Not CR" in Table 1). Expectedly, all 11 of these non-CR subjects also had

TABLE 1 Patient and disease characteristics

		MRD NEG N = 15	MRD POS N = 27	*P*	ENTIRE COHORT N = 42
Sex, No. (%)	Male	9 (60)	17 (63)	.85	26 (62)
Age at diagnosis, years [median (IQR)]		55 (42-61)	63 (61-67)	.0022	63 (52-65)
AML diagnosis, No. (%)	De novo	13 (87)	12 (44)	.0097	25 (60)
	Secondary	2 (13)	15 (56)		17 (40)
ELN genetic risk, No. (%)	Favorable[a]	5 (33)	2 (7)	.11	7 (17)
	Intermediate	4 (27)	8 (30)		12 (29)
	Adverse	6 (40)	17 (63)		23 (55)
Number of mutations at diagnosis, mean ± SE		1.8 ± 0.28	2.7 ± 0.27	.046	2.4 ± 0.21
VAF of diagnostic mutations (%), median (IQR)		38 (21-50)	42 (31-49)	.43	40 (29-49)
Cycles of induction chemotherapy, No. (%)	One	10 (67)	18 (67)	1.0	28 (67)
	Two	5 (33)	9 (33)		14 (33)
Pre-transplant disease status, No. (%)	CR1	13 (87)	15 (56)	.0031	28 (67)
	CR2 (after prior relapse)	2 (13)	1 (4)		3 (7)
	Not CR	0 (0)	11 (41)		11 (26)
Diagnosis to transplant, months [median (IQR)]		4.4 (3.6-6.7)	4.1 (3.1-5.3)	.28	4.2 (3.3-5.3)
HLA antigen match, No. (%)	Matched donor	13 (87)	25 (93)	.61	38 (90)
	Mismatched donor	2 (13)	2 (7.4)		4 (10)
HSCT regimen, No. (%)	Ablative	9 (60)	5 (18)	.01	14 (33)
	Reduced intensity (RIC)	6 (40)	15 (56)		21 (50)
	Non-myeloablative (NMA)	0 (0)	7 (26)		7 (17)
Comorbidities (HCT-CI), No. (%)	0-1	3 (20)	6 (22)	.29	9 (22)
	2-4	9 (60)	10 (37)		19 (45)
	5+	3 (20)	11 (41)		14 (33)
Performance status (KPS), No. (%)	>90	7 (47)	9 (33)	.39	16 (38)
	<90	8 (53)	18 (67)		26 (62)
Post-transplant follow-up time, months [median (IQR)]		20.9 (18.7-22.1)	25 (19.2-25.4)	.11	22.1 (19.1-25.2)
Outcome, No. (%)	Remission	11 (73)	10 (37)	.028	21 (50)
	Leukemic Relapse	1 (6.7)	12 (44)		13 (31)
	Non-Relapse Mortality	3 (20)	5 (19)		8 (19)

Abbreviations: CR, complete remission; HCT-CI, hematopoietic cell transplantation- comorbidity index; HLA, human leukocyte antigen; IQR, inter-quartile range; KPS, Karnofsky Performance Status; MRD, minimal residual disease (by NGS); VAF, variant allele frequency.

[a]The 7 subjects with ELN-defined favorable risk genetics were transplanted: after primary induction failure (n = 2); after attaining a post-relapse second CR (n = 2); or due to high-risk diagnostic features such as secondary AML (n = 1); therapy-related AML (n = 1); or hyperleukocytosis (n = 1).

persisting leukemia-associated mutations (22 persisting mutations) by NGS. Persisting mutations (n = 21) were also found in 16 additional subjects, with no other evidence of residual disease. This confirmed the increased analytical sensitivity of NGS - as compared to morphology, flow cytometry, and/or cytogenetics/FISH - for detecting low levels of minimal residual disease. In fact, NGS was able to detect residual molecular disease in more than half (52%; n = 16) of the 31 subjects who had achieved a CR.

The 27 subjects with detectable molecular MRD before transplant shared several disease characteristics traditionally associated with higher-risk AML. They included older age, higher prevalence of secondary AML, the failure to achieve a traditional CR, and a trend toward adverse risk ELN-defined genetics (Table 1). The number of pre-treatment mutations was also higher (P = .046) in these MRD-positive subjects (mean 2.7), as compared to the MRD-negative subjects (mean 1.8), perhaps due to the higher frequency of secondary

AML (Table 1). The VAF of these pre-treatment mutations, however, was not significantly different between the MRD-positive and MRD-negative groups (Table 1).

In these 27 subjects with persisting NGS-defined minimal residual disease before transplant, there were a total of 72 leukemia-associated pathogenic mutations that had been previously detected at diagnosis (in 20 different genes). Despite intensive chemotherapy, 43 of these 72 mutations (60%) persisted before transplant (in 16 different genes) (supplemental Table S4). The pre-transplant MRD burden of clonal leukemia averaged 12% VAF (median 1%) in the 27 subjects with persisting mutations (table 2), which represented a 41-fold (median) reduction relative to the same 72 mutations at diagnosis. A significant 30% minority of the persisting mutations (n = 13; in 10 subjects) were present at a VAF below 2.5%, which is the theoretical mutation burden corresponding to the 5% residual level of morphologically detectable leukemic blasts that typically defines "complete remission". In comparison, on the other end of this skewed distribution, 13 mutations (in 8 subjects) persisted at a VAF above 30% (including 4 subjects without any classically-detectable residual leukemia cells), suggesting that the treatment resistant residual myeloid clone may not always maintain the same undifferentiated morphology and/or immunophenotype as the original leukemic blast. Residual pre-transplant molecular disease burdens were expectedly significantly higher in the 11 MRD-positive subjects with classically-detectable leukemic blasts (mean 19% VAF) vs the 16 MRD-positive subjects in complete remission (mean 7.2% VAF)(P = .016).

The most common persisting myeloid clones before transplant had mutations in DNMT3A (7 mutations in 6 subjects), TET2 (6 mutations in 5 subjects), RUNX1 (5 mutations in 5 subjects), TP53 (5 mutations in 5 subjects), SF3B1 (3 mutations in 3 subjects), and SRSF2 (3 mutations in 3 subjects) (Figure 1). These are the same genes that are often mutated in age-related clonal hematopoiesis and the earlier preleukemic phases of multi-stage myeloid malignancy evolution - with biochemical functions in the cell's epigenetic or RNA splicing pathways.[11,23]

The 15 subjects with no detectable pre-transplant leukemia by NGS (and also by traditional methods) had a total of 27 leukemia-associated mutations (in 15 different genes) that had been identified before treatment. The most common gene mutations that completely cleared in every subject were in NPM1 (n = 10), NRAS (n = 6), and CEBPA (n = 6). In comparison, of the 43 mutations that persisted before transplant (in 27 subjects), 95% (n = 41) were in one of 14 "likely to persist" genes (RUNX1, TET2, TP53, DNMT3A, SF3B1, SRSF2, PTPN11, IDH1, IDH2, BCOR, U2AF1, ASXL1, EZH2, and JAK2). Conversely, of the 56 mutations that completely cleared after chemotherapy, 75% (n = 42) were in one of 9 "likely to clear" genes (FLT3, WT1, NPM1, NRAS, CEBPA, GATA2, NOTCH1, KIT, and KRAS) - that often function as transcription factors or in signal transduction pathways. The pre-transplant persistence vs clearance status of each of the 23 mutated genes is shown in Figure 1.

3.4 | Post-transplant outcome

All 42 subjects underwent HSCT within 27 days (median 19) of their pre-transplant NGS study, a median of 127 days after diagnosis. The only significant transplant-related variable that differed between the subjects with vs without pre-transplant NGS-defined MRD was the more frequent use of an ablative regimen in the (younger) subjects who achieved pre-transplant MRD-negativity (Table 1). Of note, the pre-transplant NGS result was not considered in the decision as to which pre-transplant conditioning regimen to use. During a median

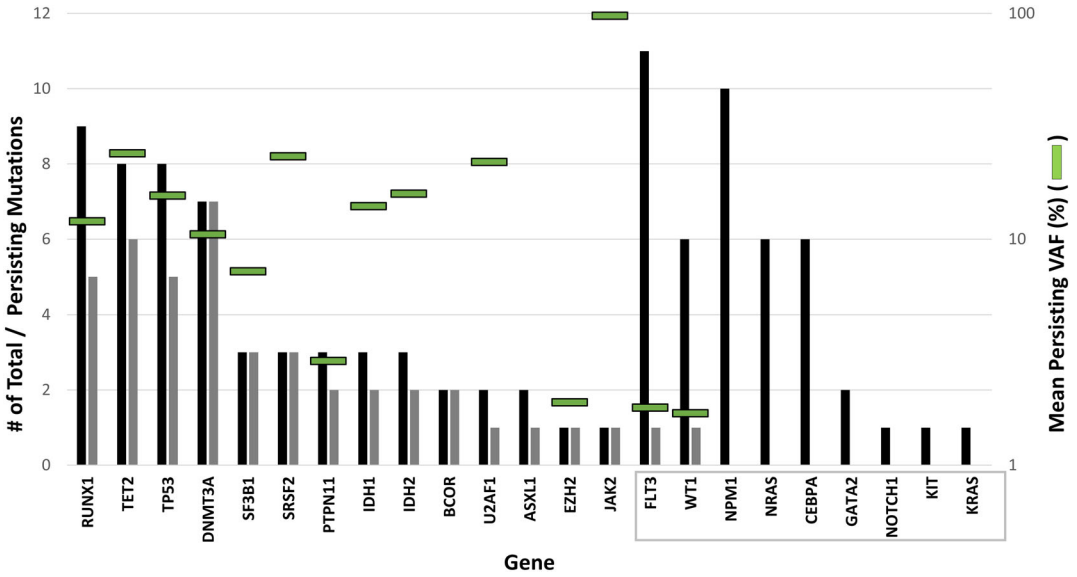

FIGURE 1 Persisting mutations before transplant. The number of detectable mutations within each gene are shown (left axis) before any therapy (black bars) and after therapy, no more than 30 days before transplant (gray bars). For those genes with persisting pre-transplant mutations, the mean variant allele frequency (percent VAF) is indicated (right axis; log scale; gray/green bricks). Genes with mutations that are likely to clear are shown within a box on the X-axis. VAF, variant allele fraction [Color figure can be viewed at wileyonlinelibrary.com]

post-transplant follow-up of 22.1 months (range 11.5-34.8), leukemic relapse occurred in 13 subjects, and 8 subjects died from non-relapse transplant-related causes (6 due to graft-vs-host disease and 2 due to infection) (Table 1). Of the 13 subjects who relapsed, 12 (92%) had NGS-detectable minimal residual disease prior to transplantation. In comparison, among the 21 remission subjects who survived without relapse or transplant-related mortality, a significantly smaller fraction (n = 10; 48%) had been MRD-positive prior to transplant (P = .028). The pre-transplant persistence of an NGS-defined mutation was thus a 92% sensitive (but not specific) prognostic biomarker for leukemic relapse, with only one subject without MRD ultimately relapsing. With non-relapse mortality treated as a competing risk, the cumulative incidence of leukemic relapse was significantly higher in the pre-transplant MRD-positive, as compared to the MRD-negative subjects (Figure 2A) (P = .014).

In a competing risk regression model, the unadjusted subdistribution hazard ratio (sHR) for leukemic relapse was 8.3 (95% CI, 1.2-59) (P = .036) in the subjects with (vs without) NGS-defined MRD (Table 2). The other demographic and prognostic risk variables were similarly evaluated. The only other significant predictors of leukemic relapse were the use of a non-myeloablative conditioning regimen (P = .02), and the presence of a diagnostic TP53 mutation (P = .02) (Table 2). Of the 6 subjects with a TP53 mutation, 4 relapsed and one died from an infection (the only one who cleared a TP53 mutation). These 3 variables imparting a significant univariate relapse risk were included in a multivariable competing risk model. It confirmed NGS-defined pre-transplant MRD as an independent prognostic predictor of leukemic relapse (sHR = 7.3; 95% CI, 0.96-56; P = .055), after adjusting for TP53 mutation and transplant conditioning regimen (Table 2). The progression free-survival was also significantly shorter for the pre-transplant MRD-positive (vs MRD-

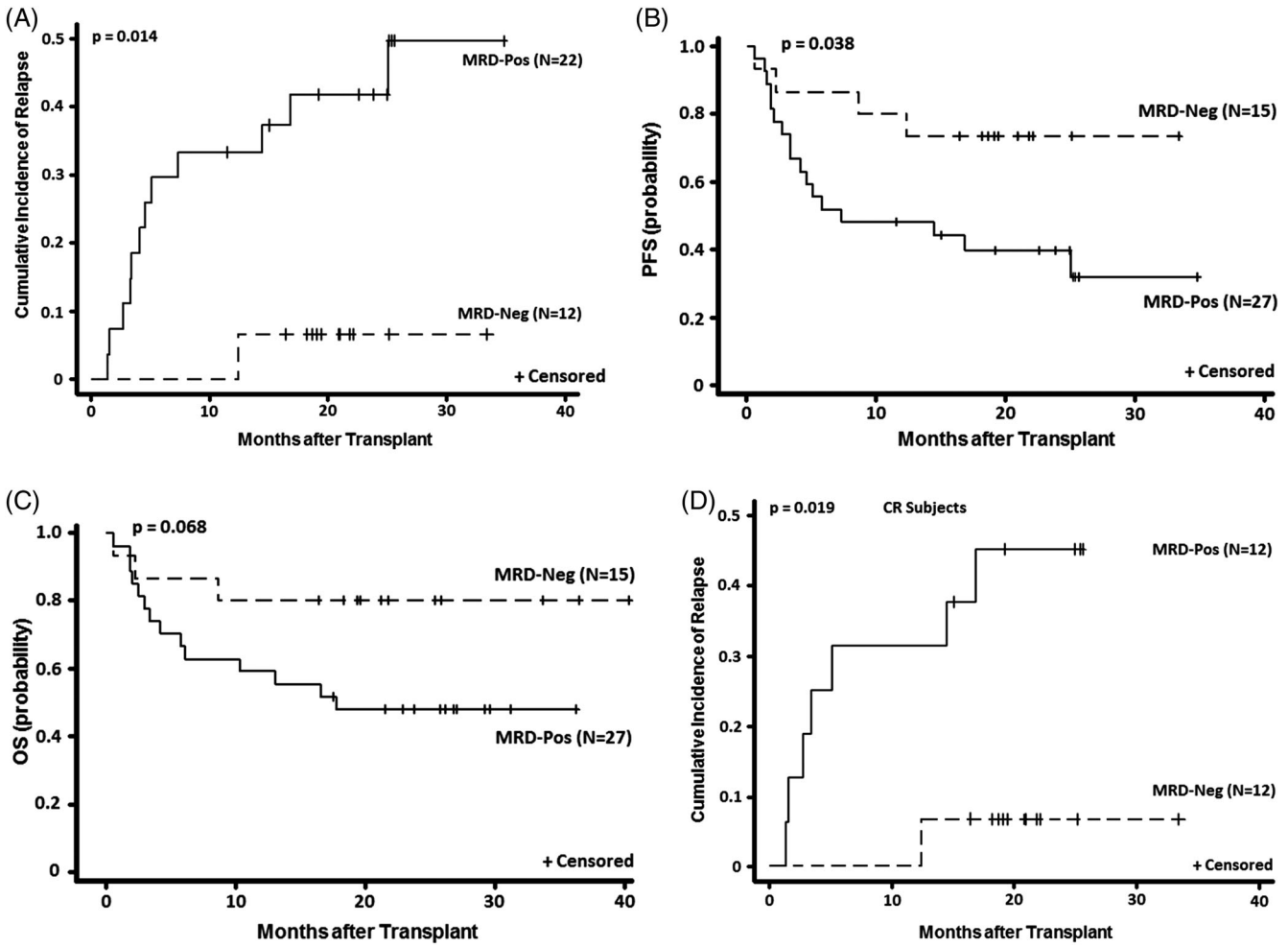

FIGURE 2 Pre-transplant NGS-defined MRD predicts post-transplant outcomes. Cumulative incidence of relapse (CIR) (panel A) among subjects with or without NGS-detectable MRD before HSCT. Cumulative incidence curves are not shown in panel A for the 8 subjects with non-relapse mortality, who were analyzed as a competing risk. Progression-free survival (PFS, panel B) and overall survival (OS, panel C) in MRD-positive vs MRD-negative subjects. Panel D shows the cumulative incidence of relapse in subjects with or without NGS-defined MRD, among the subset of subjects with a complete remission (CR), excluding the 11 MRD-positive subjects failing to achieve a CR before transplant (by non-molecular methods). Cumulative incidence curves are not shown in panel D for the 7 CR subjects with non-relapse mortality who were analyzed as a competing risk. For all panels, MRD-positive subjects are depicted with a solid line and MRD-negative subjects with a dashed line. MRD, minimal residual disease

TABLE 2 Univariate and multivariate analysis of prognostic factors for leukemic relapse

		Univariate analysis			Multivariate analysis		
		hazard	95% CI	*P*	hazard	95% CI	*P*
MRD (by NGS)	Positive (n = 27)	8.3	(1.2, 59)	.036	7.3	(0.96, 56)	.055
Sex	Male (n = 26)	0.58	(0.20, 1.7)	.32			
Age at diagnosis	1 year	1.0	(0.96, 1.1)	.87			
AML diagnosis	Secondary (n = 17)	1.7	(0.58, 4.8)	.34			
ELN genetic risk				.91			
	Favorable (n = 7)	REF	REF				
	Intermediate (n = 12)	0.79	(0.12, 5.2)	.81			
	Adverse (n = 23)	1.1	(0.22, 5.1)	.94			
Number of mutations at diagnosis	1 mutation	0.87	(0.60, 1.3)	.46			
VAF of diagnostic mutations		1.0	(0.96, 1.0)	.73			
Cycles of induction chemotherapy	One (n = 28)	0.53	(0.18, 1.5)	.24			
Pre-transplant disease status	[a]Not in CR (n = 11)	1.9	(0.65, 5.3)	.25			
Diagnosis to transplant	Months	1.0	(0.99, 1.0)	.57			
HSCT regimen				.022			.046
	Ablative (n = 14)	REF	REF		REF	REF	
	Reduced Intensity (RIC; n = 21)	1.18	(0.28, 5.0)	.82	0.34	(0.059, 1.9)	.22
	Non-myeloblative (NMA; n = 7)	4.3	(1.1, 16)	.031	1.5	(0.32, 6.6)	.63
Comorbidities (HCT-CI)				.76			
	0-1 (n = 9)	REF	REF				
	2-4 (n = 19)	0.8	(0.19, 3.4)	.77			
	5+ (n = 14)	1.3	(0.30, 5.3)	.75			
Performance status (KPS)	<90 (n = 26)	0.63	(0.22, 1.9)	.40			
TP53 mutation at diagnosis	Mutation present (n = 6)	4.0	(1.2, 13)	.021	6.4	(1.5, 26.5)	.011

Abbreviations: HSCT, hematopoietic stem cell transplant; NGS, next-generation sequencing; VAF, variant allele frequency.
[a]CR1 and CR2 combined (no relapse events after CR2).

negative) subjects (*P* = .038)(Figure 2B). In addition, there was a trend toward shorter overall survival in these pre-transplant MRD-positive (vs MRD-negative) subjects (*P* = .068)(Figure 2C).

Given this finding that the pre-transplant persistence of a leukemic clone, as measured by a sensitive NGS method, is a strong independent prognostic marker for post-transplant leukemic relapse, we also assessed the prognostic relevance of persisting leukemic blasts as measured by less sensitive morphologic, cytogenetic, and/or flow cytometric methods (ie, the failure to achieve a CR). Although there were likely too few non-CR subjects for a definitive conclusion, there was a possible trend toward a higher cumulative relapse incidence in the 11 subjects with vs the 31 subjects without detectable pre-transplant leukemic blasts by these non-molecular methods (*P* = .25)(Table 2). NGS-defined pre-transplant MRD, however, remained a significant strong and independent prognosticator of future leukemic relapse, even when these 11 non-CR subjects were excluded from the analysis (HR = 8.3, 95% CI 1.1-63, *P* = .019)(Figure 2D).

4 | DISCUSSION

In this retrospective study of AML subjects who underwent HSCT at a single center, we have demonstrated that, using NGS to serially monitor the pre-treatment leukemic clone, the persistence of molecular MRD in the month before transplant had significant independent prognostic value for predicting subsequent leukemic relapse and death. NGS-defined MRD was a significant risk factor for leukemic relapse by both univariate and multivariate analysis, independent of pre-treatment ELN genetic risk and other non-molecular pre-and post-treatment predictors of AML outcome. Unlike most other methods for determining MRD, because NGS simultaneously assesses mutations in most AML driver mutations, the vast majority of patients will have a trackable mutation at diagnosis that can serve as an applicable MRD marker. In our institution, 97% of AML patients have a trackable MRD mutation using NGS, compared to 89%-93% in two recent large studies, using a similarly-sized NGS panel.[10,24] In contrast, other methods for determining MRD are applicable to a smaller fraction of AML patients that have either a unique stable flow cytometric immunophenotypic profile, a structural chromosomal alteration (detectable by FISH), or a leukemia-specific driver mutation that can be sensitively detected by a single-gene quantitative (or digital) PCR assay. The expanded applicability of MRD monitoring to a larger fraction of AML patients, as now afforded by NGS, may have immediate and direct clinical care ramifications, given the consensus AML guidelines from ELN which include a new "complete remission

without MRD" response criteria category, that requires MRD testing by molecular or flow cytometric methods.[3]

Besides being applicable to a larger fraction of AML patients (ie, clinical sensitivity), NGS-based MRD methods can also be more analytically sensitive for the detection of low-level residual clones. In our cohort, pre-transplant MRD was thus detectable in more than double the number of subjects (n = 27) using NGS (limit of detection <0.5%), compared to the cumulative application of 3 traditional non-molecular MRD detection methods (morphology, cytogenetics/FISH, and MFC; n = 11). This increased analytical sensitivity for NGS was also seen at an earlier post-induction time point, at which we detected 5 times as many MRD-positive subjects with NGS relative to the same 3 non-molecular methods. This increased method-dependent MRD diagnostic yield also directly translated to the prediction of clinical outcomes. NGS-based MRD positivity was thus a strong and independent prognostic marker for post-transplant relapse. However, traditional non-molecular MRD detection methods not only failed to detect residual disease in the majority of subjects who subsequently relapsed (8 of 13), but also showed only a marginally significant correlation with post-transplant outcomes. Other AML studies have shown that standardized flow cytometric MRD detection methods do provide independent prognostic value.[10,25] Our study either used less sensitive MFC detection methods (perhaps due to the known inter-lab imprecision of MFC-based MRD)[26] and/or lacked sufficient statistical power (and was not designed) to adequately address this MRD method comparison question. Although single-gene PCR assays can be even more analytically sensitive than NGS, with MRD detection limits in the 0.1% range,[27] the applicable genetic alterations targeted by these single-gene PCR assays (PML-RARA, BCR-ABL1, NPM1, CBFB-MYH11, RUNX1-RUNX1T1) are present in only ~40% of AML patients,[27] leaving the majority of patients with no trackable single-gene MRD marker.

Several recent AML studies have shown that the persistence of leukemia-associated mutations after the initial course of standard induction chemotherapy imparts a significantly increased risk of subsequent leukemic relapse and death.[10,11,28-31] For those patients undergoing HSCT, we have extended these conclusions by also assessing NGS-defined MRD at the subsequent pre-transplant time point (within 30 days of HSCT), and showing that a persisting leukemia-associated mutation was a sensitive (but not specific), strong (hazard ratio ~8), and independent predictor of leukemic relapse. Other recent AML studies have also reported a significant post-transplant relapse risk in subjects with a persisting pre-transplant mutation.[7,32] However, compared to our study, with only one MRD-negative relapse subject (and a 92% assay sensitivity), the sensitivities of the NGS MRD assays were comparatively low in both of these other studies - at 67%[32] and 77%,[7] respectively. In addition, unlike these other studies that evaluated MRD at only a single pre-transplant time point (and for reference,[7] using only NGS and not MFC), we evaluated NGS-based MRD at several supplemental time points (mean 5.1 NGS samples per subject), thus allowing a more detailed analysis of evolutionary clonal kinetics.

Although the spectrum of mutations in our AML cohort was expectedly heterogeneous,[10,11,22,31] the only pre-treatment mutated gene that individually imparted a significant risk of post-transplant relapse was TP53, a well-known biomarker of particularly poor outcomes in AML.[33] Neither the number of pre-treatment mutations nor the mutant allele burden at diagnosis predicted subsequent post-transplant outcomes. In contrast, the pre-transplant persistence of any leukemia-associated mutation was a strong and independent risk factor for leukemic relapse. At this pre-transplant time point, there was a striking dichotomy of mutated genes. The "likely to clear" genes (FLT3, WT1, NPM1, NRAS, CEBPA, GATA2, NOTCH1, KIT, and KRAS) encode signaling and/or transcriptional regulatory proteins, while the "likely to persist" genes (TET2, DNMT3A, ASXL1, RUNX1, SF3B1, TP53, SRSF2, PTPN11, IDH1, IDH2, BCOR, U2AF1, EZH2, and JAK2) predominantly function as epigenetic modifiers or RNA splicing factors and are the same mutations that are typically found early during preleukemic clonal evolution and in age-related clonal hematopoiesis (CHIP).[10,11,31]

The prognostic relevance of these post-treatment persisting preleukemic mutations has been a controversial question, with some studies suggesting that persisting preleukemic mutations (particularly in the DNMT3A gene) impart no (or minimally) significant prognostic utility.[10,34-37] In contrast, our study, as well as several others, has shown that the post-treatment persistence of any leukemia-associated mutation, including preleukemic mutations, is associated with an inferior outcome.[8,28,31] Some of these studies had large enough cohorts to be able to specifically modify their definition of "MRD-positive" to either include or exclude persisting pre-leukemic (mostly DNMT3A) mutations. Even with these analogous analysis methods, however, some studies were able to show that the inclusion vs exclusion of preleukemic mutations significantly weakened the subsequent prognostic utility of the MRD biomarker (for long-term outcomes),[10,36] while other studies showed no such difference.[31] Both the post-treatment timing at which MRD is assessed and the intensity of the subsequent post-induction treatment regimen are likely to significantly contribute to the ultimate prognostic utility of pre-leukemic persisting mutations. Our study, for example, used an MRD time point prior to HSCT (median 3.6 months after diagnosis) that was later in the treatment cycle (after more cumulative chemotherapy), than all of the MRD studies that found preleukemic persisting mutations to be non-prognostic, when assessed at an earlier post-induction or first-remission time point.[10,34-37] The standard initial induction chemotherapy used to achieve a first remission often fails to effectively clear preleukemic mutations.[8,10,31,37] The subsequent intense conditioning regimen used prior to stem cell transplantation, has been shown to be preferentially most effective in clearing those clones that are the most resistant to the prior induction chemotherapy.[8] The confirmed MRD prognostic utility of any persisting mutation (without excluding preleukemic mutations) in patients with subsequent HSCT (our study and[8]), vs the apparent lack of prognostic utility for persisting preleukemic mutations in those who did not get transplanted[10,34-37] may then be partly attributable to the more intense treatment given before transplantation. Clones with these same preleukemic mutations (particularly in DNMT3A) have been shown to be resistant to standard AML induction chemotherapy.[38-40] They maintain a proliferative repopulation advantage,[41] and are the

evolutionary source for subsequent clones appearing at relapse,[40,42,43] These preleukemic mutations would thus seem to meet the definitional criteria for a clinically useful MRD biomarker.[27] Excluding these common persisting preleukemic mutations from the practical definition of "MRD" would then increase the specificity of an NGS-based MRD assay but at the cost of reducing the assay's sensitivity and real-world clinical applicability. A rigorously-powered study to quantitatively address the important question of which individual AML gene mutations, when persisting after treatment, impart the highest risk for poor outcomes will require a large number of enrolled subjects pooled from many collaborating institutions.

Our findings suggest that serial NGS-based MRD monitoring of AML patients can provide practical prognostic information at a key decision-making pre-transplant time point. Other post-treatment MRD time points have been shown to provide analogous risk stratification information.[8,10,11,28-31] The translation of this prognostic information into practical therapeutic actions is the next obvious target for intense investigation. Should some patients with detectable MRD thus be given additional pre-emptive therapy before proceeding to transplant conditioning? Which patients? What therapies? At what time points? And most importantly, would such an MRD-driven decision-making protocol lead to improved outcomes? Although some trials using flow cytometry or single-gene PCR-based MRD methods have inferred a potential benefit for MRD-directed therapeutic decision-making,[44-46] randomized prospective clinical trials of MRD-directed AML (non-APL) therapy, although ongoing in some centers,[47] have yet to yield clinically actionable consensus data.

The "actionable" clinical benefit of MRD monitoring is not restricted to direct changes in therapy. An MRD-positive test result often informs, for example, a more frequent subsequent disease monitoring schedule, such that, if clonal outgrowth is indeed progressing, it can be detected (and treated) earlier. Other key variables that will likely impact the ultimate "actionability" of MRD-based assays include the lab method used to determine MRD (NGS vs MFC vs single-gene PCR), the low-level analytical sensitivity of that method, and practical concerns such as test standardization, reproducibility, turnaround time, and cost. Although the NGS-based MRD testing employed in our study was substantially more sensitive and prognostically informative compared to other MRD detection methods, it remains underreimbursed by most American health care payors. That diminishes its routine clinical implementation beyond specialized research centers.

The limitations of this study include its small size and the single-site retrospective enrollment that was unavoidably biased toward high-risk (transplant-eligible) disease. The small minority of AML patients that were excluded from our study due to the absence of a detectable NGS mutation at diagnosis (~3%), could represent an additional minor source of selection bias. Pre-transplant monitoring of these same diagnostic mutations proved to be a sensitive MRD predictor of disease progression, with only one MRD-negative subject with a subsequent relapse. The specificity of the MRD assay, however, was a substantially less valuable predictive tool. There were thus 10 MRD-positive subjects having long-term remissions - perhaps due to either the absence of an aggressive leukemic phenotype in some persisting (perhaps preleukemic) clones, and/or the treatment-related suppression of these residual clones by the transplant conditioning regimen. These "false-positive" MRD determinations might, at present, limit this assay's practical clinical utility as a trigger for subsequent pre-emptive therapeutic intervention. Additional technical and/or analytical refinements in these NGS MRD assays will then be necessary to transition these assays from a strictly qualitative presence versus absence biomarker to a more sophisticated quantitative marker with a validated cutoff value for distinguishing an indolent persisting clone imparting minimal relapse risk from a high-risk persisting clone that will likely progress. In the interim, given the minimal number of false-negative MRD determinations, the current NGS MRD assay may best be utilized to identify low-risk MRD-negative patients, who may not require additional anti-leukemic intervention.

In summary, this study has demonstrated that pre-transplant MRD, as measured by a multi-gene NGS-based method, is a strong, sensitive, independent prognostic biomarker for predicting subsequent poor AML outcomes. Other recent studies confirm the clinical utility of MRD-based AML disease monitoring at several other pre-transplant[10,11,28-31] and post-transplant[8] time points. These results are informing a new era of AML management whereby routine post-treatment MRD monitoring will be used to directly inform the presence of deep remissions, stratify the risk of relapse, predict those patients that may not require additional toxic therapies, and identify early impending relapse. Despite this progress toward a "personalized diagnostics" program in the management of AML, many unanswered questions remain to be addressed, including: defining the optimal MRD laboratory method (how many targets? what sensitivity threshold?); defining the optimal post-treatment time point; expanding applicability to other AML treatment regimens (including targeted therapies); defining high- and low-risk persisting gene mutations; decreasing turnaround time; and standardizing and economizing the MRD assay. The ultimate confirmation of the clinical utilty of NGS-based MRD in AML will require a large prospective trial, ideally with an MRD-triggered intervention arm.

ACKNOWLEDGMENTS

We thank the entire Knight Diagnostic Laboratory team for contributing invaluable technical, administrative, and methodologic advice. Funding for this study was kindly provided by the OHSU Department of Pathology and Knight Cancer Institute.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Press RD, Eickelberg G, Froman A, et al. Next-generation sequencing-defined minimal residual disease before stem cell transplantation predicts acute myeloid leukemia relapse. *Am J Hematol*. 2019;1–11. https://doi.org/10.1002/ajh.25514