Multi-gene measurable residual disease assessed by digital polymerase chain reaction has clinical and biological utility in acute myeloid leukemia patients receiving venetoclax/azacitidine

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Abstract

Venetoclax with azacitidine (ven/aza) is a lower-intensity therapeutic regimen that has been shown to improve outcomes in elderly patients with acute myeloid leukemia (AML). Measurable residual disease (MRD) using flow cytometry is a valuable tool for the prediction of relapse in AML using conventional therapies and ven/aza; however, the prognostic value for broad-scale molecular MRD after ven/aza treatment is less clear. We aimed to determine the utility of retrospective assessment using multi-gene molecular MRD by droplet digital polymerase chain reaction (ddPCR). We found this approach correlates with outcomes in a cohort of patients receiving frontline ven/aza for AML. The predictive value of ddPCR MRD persisted when *NPM1* mutations were removed from analysis, as well as after adjustment for the impact of stem cell transplant on outcomes. Late achievement of MRD negativity, including after SCT, was still associated with superior outcomes compared to persistently detectable MRD. We further explored the impact of ven/aza on the burden of different classes of mutations, and identified the persistence of splicing factor mutations, commonly associated with MDS, as a consistent finding after ven/aza treatment. These data add to our understanding of the effects of ven/aza on AML disease biology and provide details on molecular depth of remission that can guide prospective trials in the future.

Introduction

Measurable residual disease (MRD) is a sensitive tool in the post-treatment setting to predict relapse in acute myeloid leukemia (AML).[1,2] The majority of clinical MRD studies in AML have been performed via multi-channel flow cytometry (MCF).[1,3] Despite its utility as a prognostic marker, 20-30% of MCF MRD-negative patients relapse.[1] Targeted molecular MRD has also proven to be a powerful tool for predicting outcomes in AML patients,[3-6] however, no molecular MRD platform is widely established for clinical use in AML. This is in part because of a lack of standardized methods and thresholds to monitor molecular MRD,[3] and uncertainty around which persistent mutations have immediate prognostic value.[7,8]

Approximately 20% of adults with AML have recurrent chromosomal translocations and 27% have *NPM1* mutations,[9,10] limiting the utility of standard quantitative polymerase chain recation (qPCR) whose use is largely restricted to these mutation subtypes. However, over 95% of patients have single nucleotide variants or small insertions/deletions[9,10] which can be captured by droplet digital PCR (ddPCR) or next-generation sequencing (NGS). NGS platforms are in general quite expensive and historically have had low sensitivity (2-3% limit of detection) prohibiting their use as MRD tools, although more sensitive platforms are being validated.[6,11] ddPCR has been identified as a highly sensitive and precise modality for mutation monitoring in AML and other malignancies, with limits of detection reported to be 10^{-4} to 10^{-5},[12] but its primary limitation has been assay specificity to individual mutations or hotspots.[13,14] Accordingly, to date the range of mutations evaluated by

ddPCR has been limited.

The introduction of venetoclax with hypomethylating agents (HMA) or low-dose cytarabine for the treatment of elderly patients with AML has led to enhanced remission rates and improved survival in a poor-risk patient population.[15-17] While promising, venetoclax combinations do not have universal efficacy,[18-20] and most patients will ultimately relapse in the absence of consolidative stem cell transplant (SCT).[21] Given the high likelihood of relapse in these patients,[18] and poor prognosis when it occurs,[18,22] it is important to identify early warning signs so that, where possible, these patients might be offered other treatment options to prevent relapse. While there are now four retrospective studies confirming the prognostic value of MCF MRD in patients receiving venetoclax-based therapies,[23-26] robust data on the relevance of molecular MRD for venetoclax-based therapies are lacking. To date, only *NPM1*-based molecular MRD has been evaluated in this context.[27,28] Our laboratory has previously provided preliminary ddPCR data demonstrating molecular depth of remission in cohorts of patients receiving venetoclax/azacitidine (ven/aza),[29-31] but has not published long-term outcome data on all ven/aza patients at our center based on ddPCR MRD status.

Here we demonstrate the feasibility of retrospectively detecting MRD with a broad panel of ddPCR assays for mutations identified by diagnostic NGS in adult AML patients. We confirm the association of ddPCR MRD status with outcomes in patients receiving ven/aza, including a subset of patients proceeding to SCT. Our findings also illuminate the relative responsiveness of different AML subclones to ven/aza selective pressure. To our knowledge, this is the first report of molecular MRD beyond *NPM1* mutations in the context of venetoclax-based therapy.

Methods

Patient selection

For this retrospective analysis, all patients ≥18 years of age with a diagnosis of AML treated at the University of Colorado were considered. Inclusion/exclusion criteria are summarized in Figure 1 and detailed in the *Online Supplementary Appendix*. A total of 64 patients were evaluated for MRD by ddPCR. All patients signed Colorado Multiple Institution Review Board (COMIRB)-approved consent for collection of tissue used in this analysis, and an additional IRB approval allowed the retrospective demographic and outcome data analysis.

DNA extraction

Genomic DNA was extracted from whole bone marrow aspirates in the Children's Hospital Colorado Molecular Diagnostics Core using the Qiagen QIAsymphony DSP DNA kits, as per institutional standards. Concentration and quality of DNA were evaluated via Qubit spectrophotometer. DNA was stored at -20°C.

Droplet digital polymerase chain reaction measurable residual disease monitoring

Based on diagnostic NGS results, a total of 50 AML-associated mutations were evaluated in this patient cohort (*Online Supplementary Table S1*). Mutations in *DNMT3A, TET2*, and *ASXL1* ("DTA mutations") were excluded from evaluation given the previous literature showing lack of correlation of these clonal hematopoiesis mutations with relapse outcomes.[8] In general, large insertions/deletions (such as *FLT3* ITD) were not amenable to ddPCR assay design and were excluded from evaluation. Mutation-specific primer/probe ddPCR assays were purchased commercially from BioRad or were custom designed. All assays were validated to a limit of detection of 0.02-0.15% variant allelic frequency (VAF). ddPCR was performed with 150 nanograms gDNA input on a BioRad QX200 Droplet Digital PCR instrument and data were analyzed via the BioRad QuantaSoft Analysis Pro v1.0.596 software. Additional details about assay design and validation are described in the *Online Supplementary Appendix*.

Retrospective chart review

Demographic information, diagnostic mutational data, dates of bone marrow evaluations and concurrent disease status, and outcome data were extracted from the electronic medical record.

Histologic assessment of dysplasia

Archived bone marrow slides from remission time points corresponding to ddPCR MRD evaluations were reviewed by two hematopathologists (ME and BJS) and the degree of dysplasia and involved lineages were scored according to clinical guidelines.[32]

Statistical analyses

Relapse-free survival (RFS) and overall survival (OS) were defined from the date of diagnosis to the respective endpoint. If no events occurred, individuals were censored at date of last follow-up. Median survival times were created using Kaplan-Meier product-limit estimates. Kaplan-Meier curves and Mantel-Cox log-rank tests were used to compare survival times. Summary statistics for variables of interest are presented alongside *P* values for the corresponding non-parametric tests based on variable class: Kruskal-Wallis for continuous variables, χ^2 test for categorical variables with expected call counts greater than or equal to 5, and Fisher exact tests for categorical variables where any expected cell counts do not meet this requirement. Two multivariate Cox proportional hazard models were explored with transplant, age at diagnosis, ddPCR MRD status, and type of mutation as covariates of interest: the first using OS outcome definition and the second using RFS outcome definition.

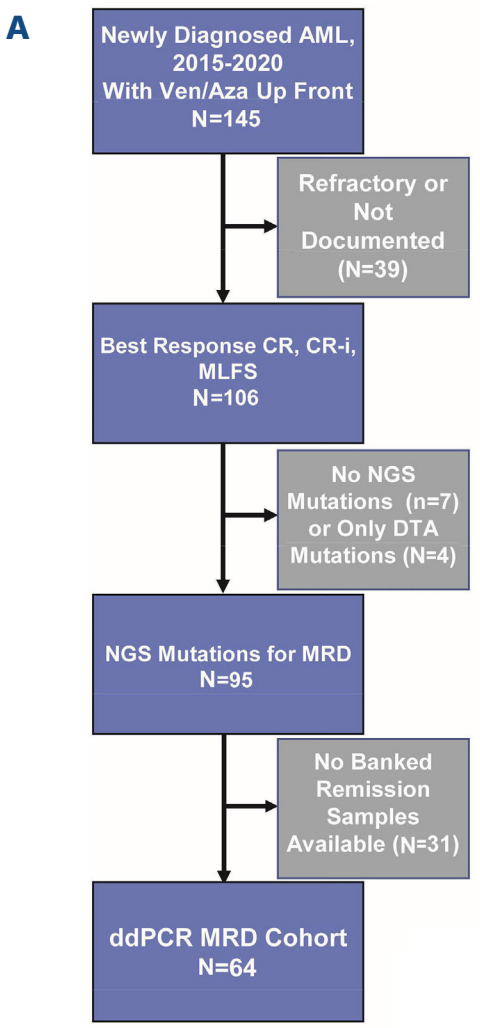
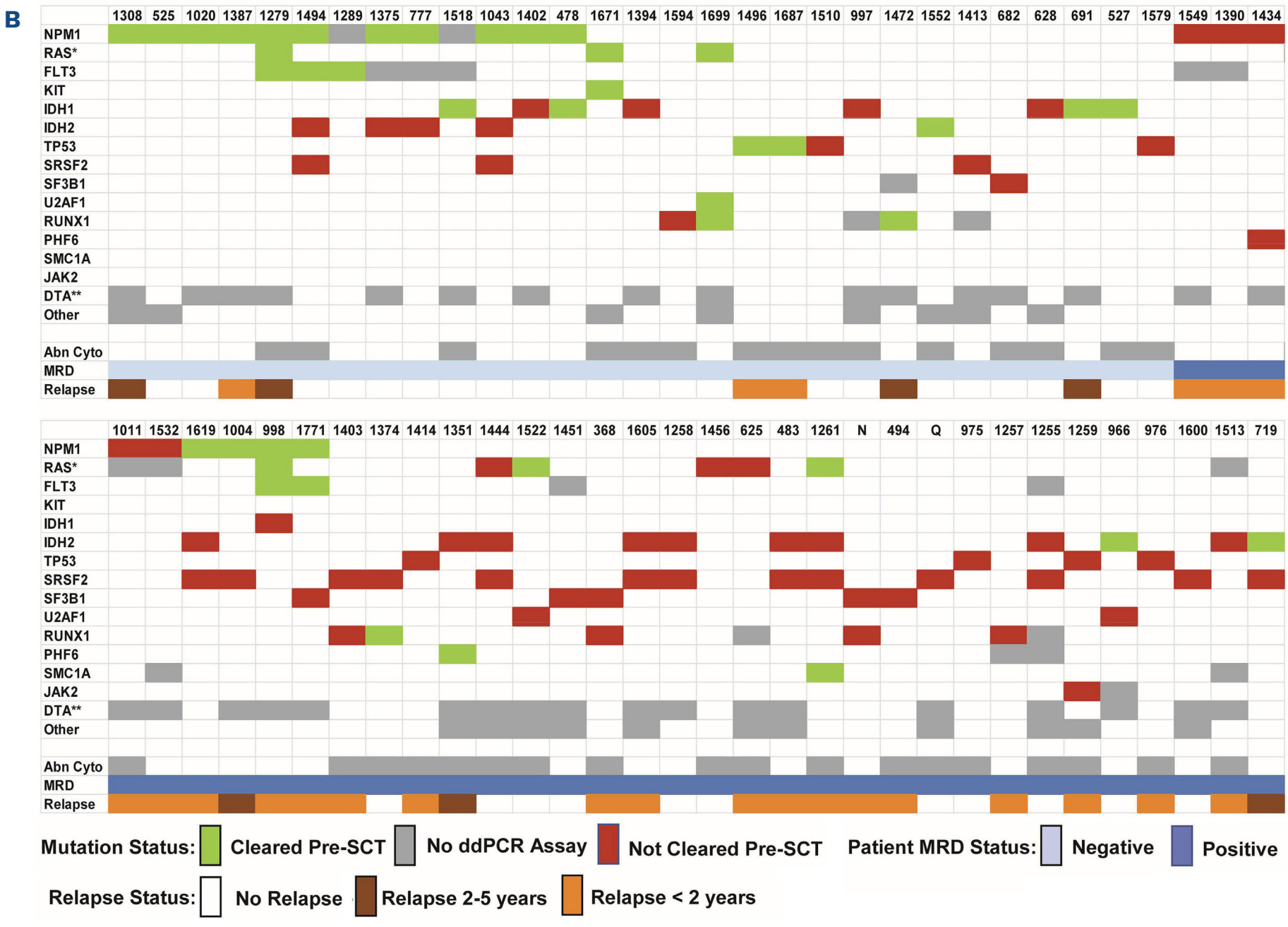


Figure 1. Cohort selection and mutational details. (A) Algorithm for patient cohort selection. Patients with acute myeloid leukemia (AML) newly diagnosed and induced with venetoclax/azacitidine (ven/aza) at the University of Colorado between 2015 and 2020 were evaluated for the present study. Patients who were primary refractory to therapy, those without diagnostic mutations amenable to droplet digital polymerase chain reaction (ddPCR), or those for whom no remission bone marrow samples were available were excluded from analysis. A final cohort of 64 patients was selected for ddPCR measurable residual disease (MRD) analysis. (B) Co-mutation table for individual patients. Numbers listed across the top of the table are research identification numbers for individual patients. Rows list specific acute myeloid leukemia (AML)-associated genes and squares are colored if a patient has a given mutation. Mutations in green cleared with ven/aza treatment (pre-transplant); mutations in red did not clear with ven/aza alone. Mutations in gray (including cytogenetic abnormalities ["Abn Cyto"]) were not evaluated by ddPCR. Overall MRD status (factoring in stem cell transplant [SCT]) is shown at the bottom, with MRD-negative patients on the left of the plot and MRD-positive patients on the right. Relapse status is also shown at the bottom of the plot. Note: all *FLT3* mutations monitored via ddPCR were tyrosine kinase domain mutations (TKD) and those excluded from monitoring were *FLT3* internal tandem duplications.



Results

Demographics and disease biology

Figure 1A describes inclusion/exclusion criteria for the patients in our ven/aza cohort. A date range of January 1, 2015 through December 31, 2020 was chosen for this study; prior to 2015 ven/aza was not used at our site, while patients diagnosed subsequent to 2020 had insufficient follow-up for outcome measurements. A total of 145 patients received ven/aza as frontline therapy. Of those, 39 individuals (27%) had primary refractory disease and therefore were not eligible for MRD evaluation. Another 31 (21%) had no remission bone marrow samples available retrospectively. Finally, 11 (8%) had no amenable mutations for ddPCR MRD monitoring. The final 64 patients (44%) were included in this analysis. These 64 individuals had a median of three (range, 1-7) mutations identified by targeted NGS at diagnosis (VAF 8-80%). The frequency of mutations in our patient cohort closely followed that described in larger cohorts of adult AML patients[9,10] (*Online Supplementary Figure S1*). Figure 1B shows a plot of mutation co-occurrence in individual patients. After eliminating DTA mutations (*DNMT3A, TET2,* and *ASXL1*), which are associated with clonal hematopoiesis,[8] patients had a median of two (range, 1-6) mutations, of which at least one (and up to 5) mutation was monitored by ddPCR. A median of five (range, 1-14) post-remission bone

Table 1. Patient/disease characteristics by droplet digital polymerase chain reaction measurable residual disease status.

Characteristic	MRD-negative N=29	MRD-positive N=35	*P*[1]
Age in years at diagnosis, median (IQR)	69 (65-72)	76 (72-80)	<0.001
Sex, N (%) Female Male	 20 (69) 9 (31)	 15 (43) 20 (57)	0.037
ELN Group, N (%) Adverse Favorable Intermediate Unable to assess	 15 (52) 12 (41) 2 (6.9) 0 (0)	 20 (57) 6 (17) 7 (20) 2 (5.7)	0.067
SWOG Classification, N (%) Favorable Intermediate Unfavorable Unable to assess/unknown	 1 (3.4) 14 (48) 11 (38) 3 (10)	 1 (2.9) 19 (54) 12 (34) 3 (8.6)	0.93
TP53 status, N (%) Wild-type Mutant	 25 (86) 4 (14)	 31 (89) 4 (11)	>0.99
Percent blasts in marrow at diagnosis, median (IQR)	62 (30-76)	48 (28-66)	0.19
FAB Subtype, N (%) M0 M0/M1 M1 M2 M4 M5 Unknown	 1 (3.4) 14 (48) 4 (14) 6 (21) 2 (6.9) 2 (6.9) 0 (0)	 1 (2.9) 14 (40) 4 (11) 6 (17) 7 (20) 2 (5.7) 1 (2.9)	0.72
Treatment-related AML, N (%) No Yes	 24 (83) 5 (17)	 30 (86) 5 (14)	>0.99
Secondary AML, N (%) No Yes	 20 (69) 9 (31)	 21 (60) 14 (40)	0.46
Received allo transplant, N (%) No Yes	 14 (48) 15 (52)	 33 (94) 2 (5.7)	<0.001

[1]Wilcoxon rank sum test, Pearson's χ^2 test, Fisher's exact test; MRD: measurable residual disease; IQR: interquartile range; ELN: European LeukemiaNet; SWOG: Southwest Oncology Group; FAB: French-American-British morphology subtype; AML: acute myeloid leukemia; allo: allogeneic.

marrow time points were evaluated per patient. Patients were classified as ddPCR MRD-negative if they had undetectable VAF of all monitored mutations at any single remission time point (termed time of best response or TBR), including after SCT (*Online Supplementary Table S2*). Otherwise, they were classified as ddPCR MRD-positive. Twenty-nine patients (45%) achieved MRD negativity by ddPCR; 35 patients (55%) remained MRD-positive. Demographics of the patient cohort by ddPCR MRD status are shown in Table 1. The only significant factors associated with ddPCR MRD status were age, sex, and transplant status. There were no differences in genetic risk classification, including incidence of *TP53* mutations, initial marrow disease burden, French-American-British (FAB) AML subtype, or proportion of patients with therapy-related AML or AML secondary to MDS between MRD-positive and MRD-negative cohorts.

Molecular measurable residual disease by droplet digital polymerase chain reaction correlates with outcomes after venetoclax/azacitidine therapy

Stratification of the frontline ven/aza cohort into MRD-negative and MRD-positive groups by ddPCR, as above, demonstrated an association with notable differences in outcomes (Figure 2A-C). Median follow-up for the entire cohort was 21 months (95% confidence interval [CI]: 17.3-43.2). Two-year RFS and OS were 73% *versus* 20% (P<0.0001) and 73% *versus* 26% (P=0.00015) in the ddPCR MRD-negative *versus* MRD-positive groups, respectively. Two-year cumulative incidence of relapse (CIR) was 80% in the ddPCR MRD-positive group *versus* 28% in the ddPCR MRD-negative group (P<0.0001). No patient in either cohort relapsed beyond 5 years. Of note, three of the 29 MRD-negative patients had recurrence of detectable mutation(s) by ddPCR - all three patients relapsed. *Online Supplementary Figure S2*

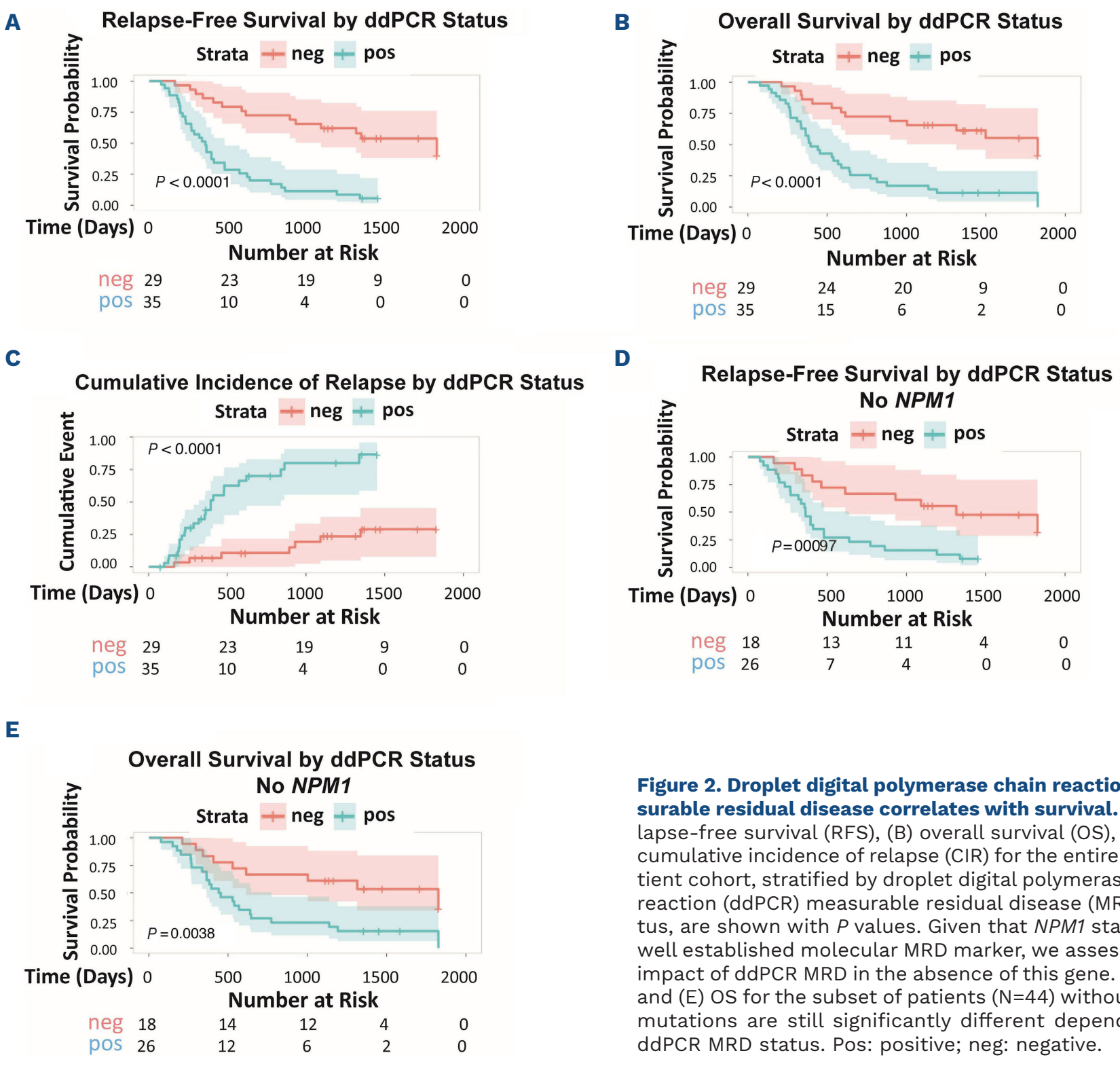


Figure 2. Droplet digital polymerase chain reaction measurable residual disease correlates with survival. (A) Relapse-free survival (RFS), (B) overall survival (OS), and (C) cumulative incidence of relapse (CIR) for the entire 64-patient cohort, stratified by droplet digital polymerase chain reaction (ddPCR) measurable residual disease (MRD) status, are shown with P values. Given that *NPM1* status is a well established molecular MRD marker, we assessed the impact of ddPCR MRD in the absence of this gene. (D) RFS and (E) OS for the subset of patients (N=44) without *NPM1* mutations are still significantly different depending on ddPCR MRD status. Pos: positive; neg: negative.

shows re-stratification of these individuals into the ddPCR MRD-positive group, which did not change the predictive value of the modality.

NPM1 is the only established gene mutation for clinically actionable MRD evaluation in adult AML,[3,5] whereas there is controversy about the prognostic value of other genes.[7] Twenty of our 64 patients had an *NPM1* mutation that was monitored by ddPCR. When we removed those patients from the analysis (and thereby the effect of *NPM1*-based MRD on prognosis), we still observed significant differences in RFS and OS in ddPCR negative and positive cohorts (Figure 2D, E). These data confirm for the first time that multi-gene molecular MRD is a valuable tool for risk stratification in patients with AML receiving ven/aza.

Time to relapse depends on the nature of persisting mutations

One of the challenges of molecular MRD is that ramifications for disease recurrence seem to differ based on the mutation in question, likely related to associations with disease ontogeny.[7,33] We evaluated the impact on survival and time to relapse for persistence of different classes of mutations: (i) *NPM1* mutation, (ii) "late" mutations (*FLT3* tyrosine kinase domain/TKD, *NRAS*, *PTPN11*), (iii) *IDH1* or *IDH2* mutations, (iiii) splicing factor mutations (*SRSF2, SF3B1, U2AF1*), or (iv) multiple. In the latter category we particularly noted persistence of co-occurring *IDH1/2* and splicing factor mutations in nine patients. For this specific analysis, we considered mutations "persistent" if they were detectable throughout all pre-SCT assessments, even if they cleared post-SCT. Patients with persistence of "late" mutations including *NPM1* had a significantly worse prognosis than other groups with persistent mutations (Figure 3A). Patients with persistent *IDH1/2* mutations, persistent splicing factor mutations, or multiple persistent mutations fared better, with no significant difference in RFS between these groups. We hypothesized that, in addition to any inherent differences in disease biology associated with these different mutations, time to relapse played a major role since this likely impacted whether a patient could be successfully salvaged by SCT or other therapies. We divided patients who relapsed (N=31) into those with no persistent mutations by ddPCR ("negative"), the mutation categories listed above, persistent *TP53* mutation, or a basket category of "other." We combined *NPM1* and other "late" mutations for statistical analysis given small sample sizes. Consistent with findings from previous mutational analyses, patients with persistence of *NPM1* or *RAS* pathway mutations had the shortest time to relapse (Figure 3B), which was significantly shorter than both those with no persistent mutations (P=0.03) and those with persistent splicing factor mutations (P=0.03). There was no significant difference in time to relapse when any of the other mutation groups were compared against those without persistent mutations (splicing factor *vs*. negative comparison shown). Of note,

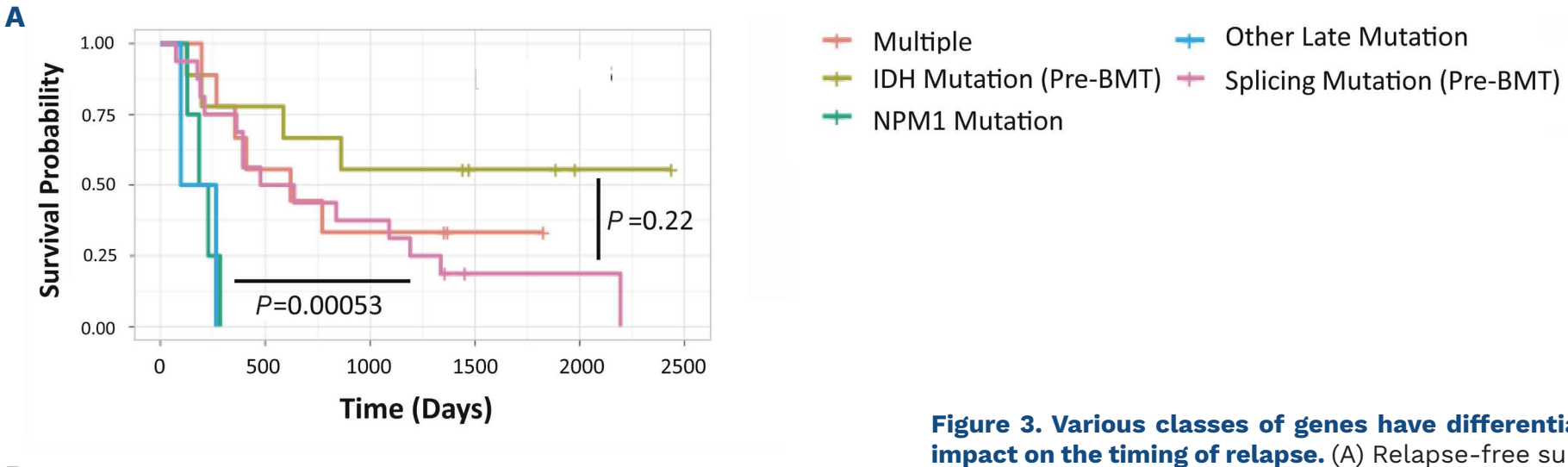


Figure 3. Various classes of genes have differential impact on the timing of relapse. (A) Relapse-free survival (RFS) was calculated for patients with persistent mutations pre-stem cell transplant (SCT) or in the absence of SCT, by category. *NPM1* and "late" mutations (signaling pathway mutations) when persistent portended the worst survival, whereas persistent *IDH1*, *IDH2*, or splicing factor mutations (or a combination thereof) were indistinguishable and had relatively improved RFS compared to *NPM1* and "late" mutations. (B) For patients who relapsed, time to relapse was evaluated based on whether the patient had no persisting mutations ("negative") *versus* persistent mutations of various classes. Once again, *NPM1* and *RAS* pathway mutations when persistent were associated with rapid onset of relapse compared to MRD-negative patients (P=0.03) and compared to patients with persistent splicing factor mutations (P=0.03). Ven/aza: venetoclax/azacitidine.

we did not see any differences in rate of clearance of IDH1 or IDH2 mutations based on whether they were clonal (VAF ≥40% at diagnosis) *versus* subclonal (VAF <40% at diagnosis), as shown in *Online Supplementary Table S3*.

Persistence of splicing factor mutations suggests an "myodisplatic syndrome reset" phenomenon

Splicing factor mutations such as *SRSF2*, *SF3B1*, and *U2AF1* have been shown to be associated with MDS and MDS-related ("secondary") AML.[33,34] These mutations are included in the new World Health Organization and International Consensus Classification algorithms as defining AML with MDS-related gene mutations.[35,36] Previous studies have demonstrated persistence of these mutations in the context of cytotoxic induction chemotherapy and epigenetic modifiers.[33,37] While there was variability in the clearance of other classes of mutations with ven/aza, splicing factor mutations uniformly persisted after ven/aza therapy with one exception (a patient with *U2AF1* mutation); three other patients cleared their splicing factor mutations only after SCT (Figure 4A). As can be seen in the figure, and summarized in detail in *Online Supplementary Table S4*, approximately half of patients with persistent splicing factor mutations relapsed unless they proceeded to consolidative SCT with disappearance of their mutation. The patients who did not receive SCT yet did not relapse had splicing factor mutation VAF between 0.5% and 50% at best response. While ven/aza is effective at clearing leukemia cells, including

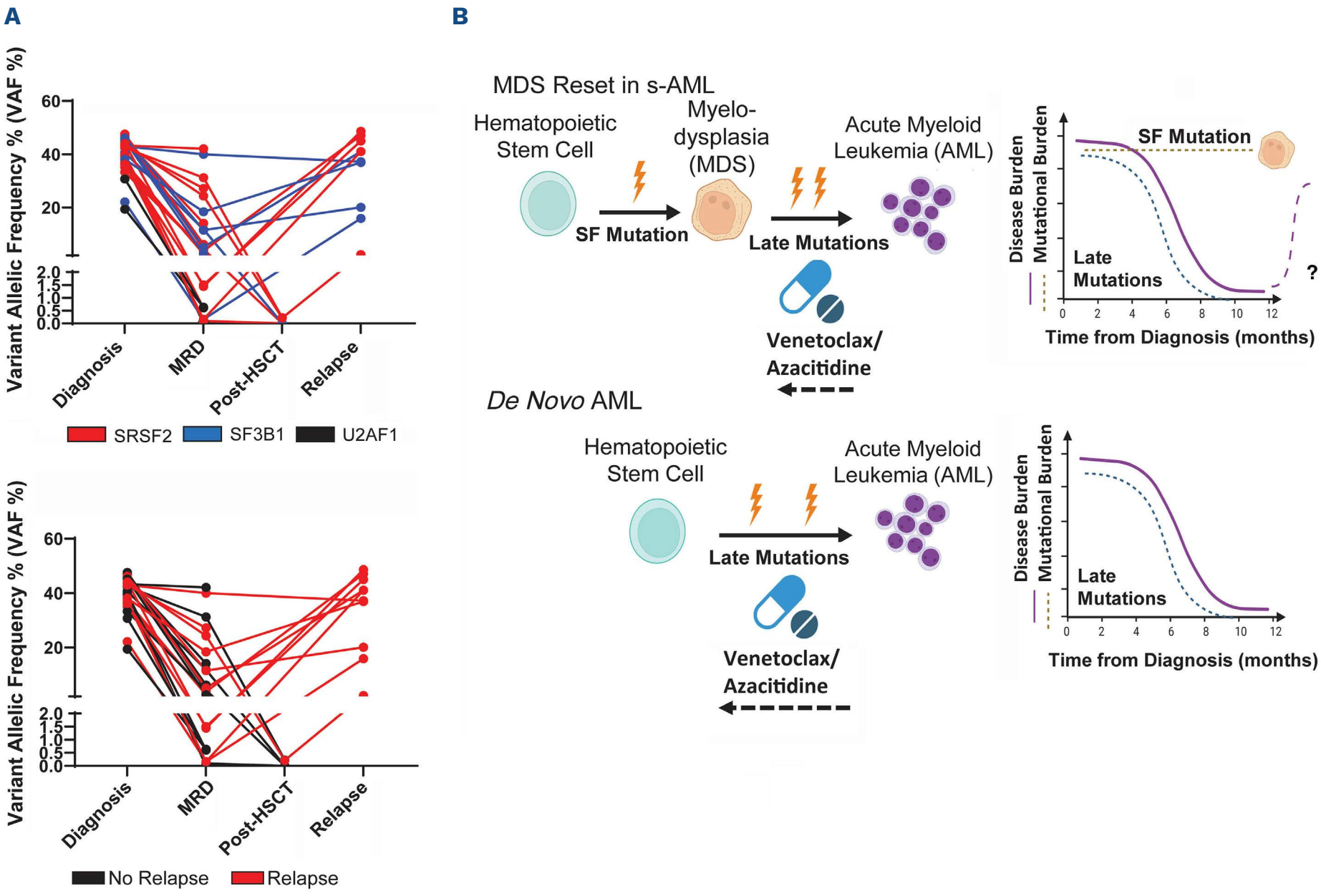


Figure 4. Splicing factor mutations persist in the absence of stem cell transplant, suggestive of persistence of an myelodysplastic syndrome clone. (A) The top graph shows individual patient time series by mutation, with variant allelic frequency (VAF) on the y-axis and time point during therapy on the x-axis. Measurable residual disease (MRD) signifies the lowest VAF achieved pre-transplant (if applicable). The bottom graph shows the same data but colored for outcome, with patients who relapsed in red and those who did not relapse in black. Save those who lost their splicing factor mutation after transplant, about half of these patients relapsed. (B) Schematic diagram of the "MDS reset" phenomenon (created with BioRender.com). Patients with myelodysplastic syndrome (MDS) preceding their acute myeloid leukemia (AML), as evidenced by dysplasia or splicing factor mutations (or both), tend to respond to venetoclax/azacitidine (ven/aza) with disappearance of their later mutations but persistence of MDS mutations. This reversion back to the pre-leukemic clone may ultimately lead to recurrence of AML at longer follow-up. Conversely, patients with *de novo* AML who have only later mutations and no dysplasia tend to lose all AML-associated mutations upon treatment with ven/aza and were more likely to be categorized as "MRD-negative" by droplet digital polymerase chain reaction. HSCT: hematopoietic stem cell transplant.

leukemia stem cells (LSC),[29] we hypothesized that the regimen may not effectively eradicate pre-leukemic stem cells and therefore essentially "resets" a patient's bone marrow to an MDS-like phenotype post-AML remission. We accessed bone marrow biopsy slides from these patients at all available MRD time points and sought to correlate dysplasia in the marrow (evaluated independently by 2 hematopathologists, MDE and BJS) with VAF of persistent splicing factor mutations. In total, there was available histology on 37 patients from our MRD cohort, 22 of whom had no splicing factor mutation and 15 of whom had a splicing factor mutation. Some were characterized clinically as having secondary AML (s-AML). Seventeen patients (6 with clinically defined s-AML) had no splicing factor mutations at diagnosis, nor did they have dysplasia meeting criteria for MDS on their remission marrows (*Online Supplementary Table S5*). Seven patients (1 with clinically defined s-AML), had a splicing factor present but no dysplasia on any follow-up marrows, despite persistence of these mutations in six of the patients. Five patients (2 with clinically defined s-AML) without splicing factor mutations had dysplasia - two with *TP53* mutations, one with *DNMT3A* mutation, and two with *IDH1* mutations - and in all cases the degree of dysplasia correlated with VAF of their primary mutation. Finally, eight patients had splicing factor mutations and dysplasia (4 with clinically defined s-AML). Two patients had poor-quality samples limiting correlation of histology with ddPCR; the other six patients had rising VAF of their splicing factor mutation that immediately preceded or coincided with re-emergence or increased prominence of dysplastic features. Thus, the incidence of dysplasia was enriched in but not exclusive to patients with splicing factor mutations in our cohort. In all cases of dysplasia with sufficient quality of remission samples, persistence or re-emergence of dysplasia correlated strongly with the VAF of splicing factor or other founder mutations (*TP53, IDH1*), suggestive of an MDS reset phenomenon (Figure 4B). There were no differences observed between groups with respect to initial bone marrow blast percentage, percent identified clinically as s-AML, proportion of patients with abnormal cytogenetics, or proportion of patients who relapsed (*Online Supplementary Table S6*).

Droplet digital polymerase chain reaction measurable residual disease does not correlate with muti-channel flow cytometry measurable residual disease in our patient cohort

Since MCF MRD is the current clinical standard for AML, we evaluated the agreement between MCF MRD and ddPCR MRD, considering only MCF MRD performed at a reference laboratory with a validated assay. Forty-one patients had such a test performed. Due to small sample size, likely time period bias (i.e., patients diagnosed more recently having higher likelihood of MCF MRD obtained), and sampling error (MCF MRD at fewer time points than ddPCR MRD), MCF MRD status did not correlate with outcomes (Figure 5), contrary

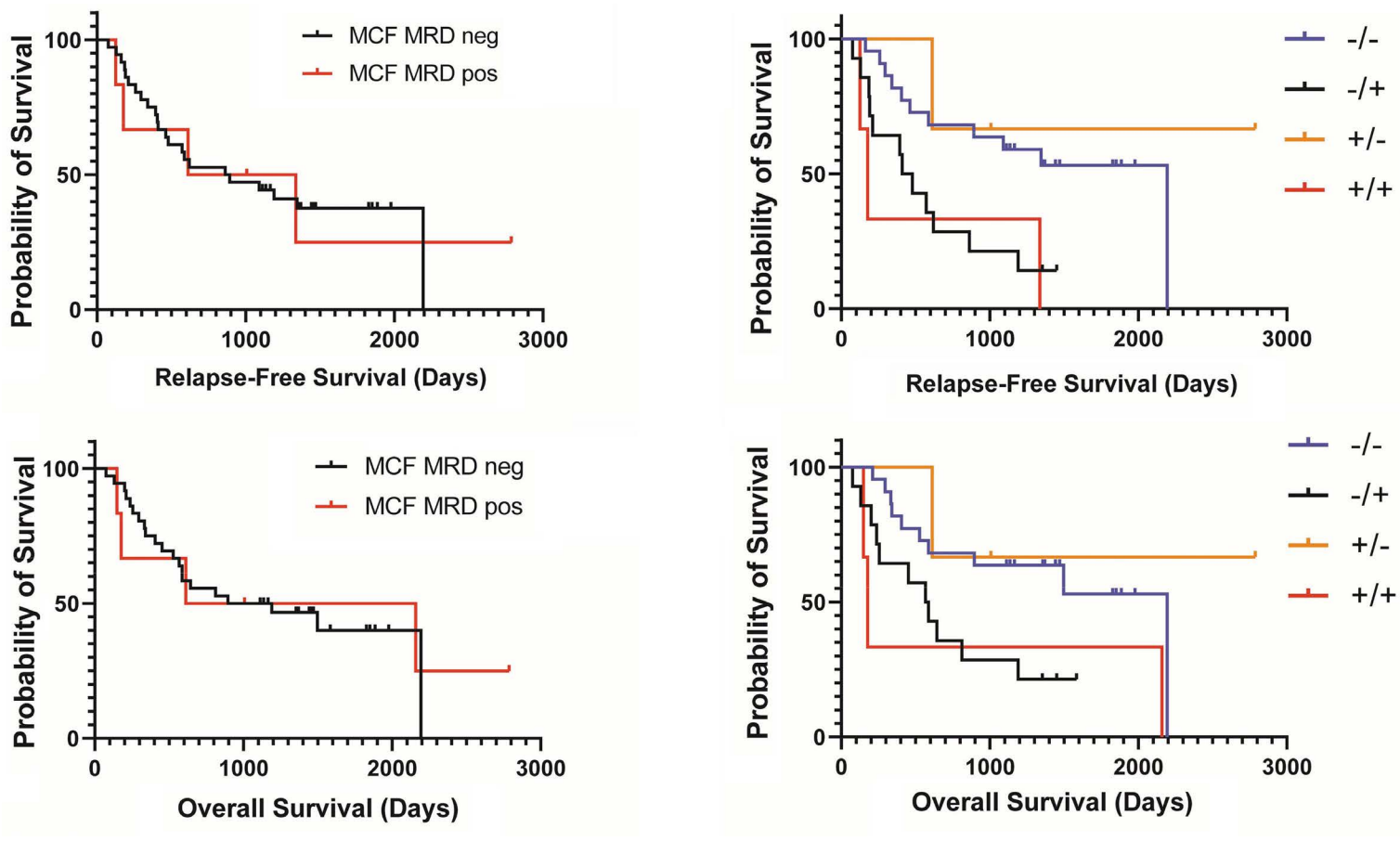


Figure 5. Multi-channel flow cytometry measurable residual disease was not prognostic of outcome in our patient cohort. (Left) Relapse-free survival (RFS) and overall survival (OS) by MCF measurable residual disease (MRD) status alone (N=41 patients). (Right) RFS and OS stratified by both multi-channel flow cytometry (MCF) MRD and droplet digital polymerase chain reaction (ddPCR) MRD in our patient cohort, where both were available (N=41 patients). Survival correlates better with ddPCR MRD. Blue lines = MCF MRD-negative (neg), ddPCR MRD-negative; black lines = MCF MRD-negative, ddPCR MRD-positive (pos); orange lines = MCF MRD-positive, ddPCR MRD-negative; red lines = MCF MRD-positive, ddPCR MRD-positive.

to what has been consistently shown in the literature[1,2] and to what we show for ddPCR MRD. While analysis of 41 patients is not sufficient to rigorously evaluate MCF *versus* ddPCR, we note that only three of 17 patients scored as MRD-positive by ddPCR were also positive by MCF; whereas 22 of 24 individuals scored as MRD-negative by ddPCR were also negative by MCF. The two MRD modalities were in agreement only 59.5% of the time due to most of the ddPCR MRD-positive patients being classified as MRD-negative by MCF. Nevertheless, within this 41-patient subset ddPCR MRD still stratified patients with differing RFS and OS (Figure 5). These findings suggest that ddPCR has considerably greater predictive power for relapse than MCF.

Correlation of digital droplet polymerase chain reaction measurable residual disease with survival persists in the absence of stem cell transplantation

We have previously published that patients receiving ven/aza and then proceeding to SCT have better outcomes than patients who receive ven/aza in the absence of consolidative

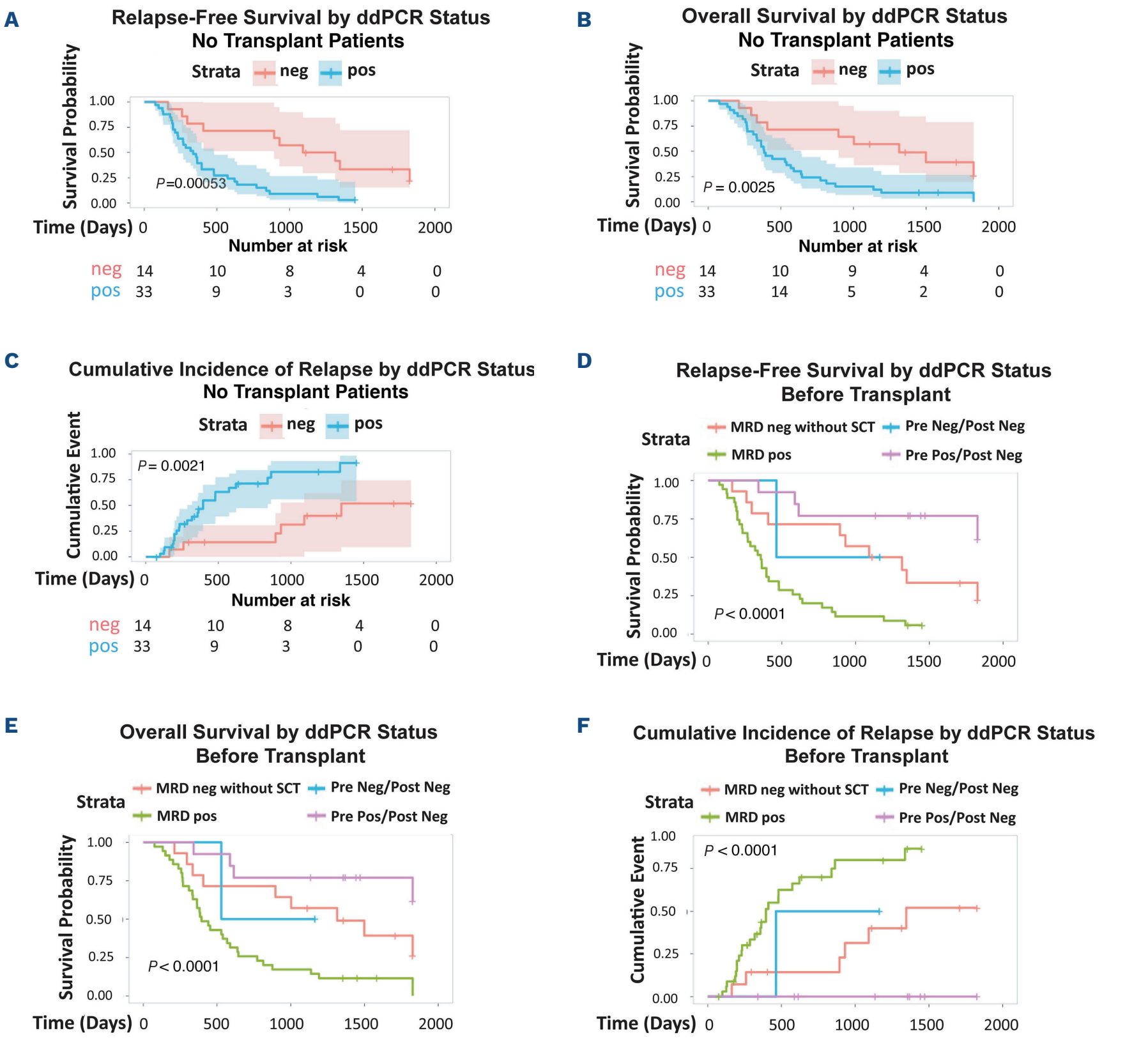


Figure 6. Droplet digital polymerase chain reaction measurable residual disease is a valuable prognostic tool after venetoclax/azacitidine with or without stem cell transplant. (A) Relapse-free survival (RFS), (B) overall survival (OS), and (C) cumulative incidence of relapse (CIR) of all venetoclax/azacitidine (ven/aza) patients who did not receive stem cell transplantation (SCT), stratified by droplet digital polymerase chain reaction (ddPCR) measurable residual disease (MRD) status. When the total cohort (N=64) was reclassified according to ddPCR MRD status pre-SCT, the presence of SCT did further improve outcomes relative to no SCT in the MRD-negative (neg) group, but MRD-positive (pos) patients still did the worst. (D) RFS, (E) OS, and (F) CIR.

SCT.[21] In the present cohort it was notable that the vast majority of individuals in the ddPCR MRD-negative group received SCT, many of them becoming MRD-negative only after SCT. Therefore we evaluated whether the predictive value of ddPCR MRD after ven/aza was only valid because of the role of SCT. We censored patients who received SCT and evaluated only patients receiving ven/aza alone for AML therapy (N=47). For this subset, ddPCR MRD status was still associated with differential RFS, CIR, and OS (Figure 6A-C), although this significance looked to be related to delays in relapse and death rather than total cure as the ddPCR MRD-negative patients without SCT still ultimately relapsed at high rates.

Next, we considered the full cohort (N=64) and divided the ddPCR MRD negative group into those negative prior to SCT *versus* those only negative after SCT (Figure 6D-F). There was a differential outcome for these groups. Patients who were ddPCR MRD-positive, including the two patients whose MRD persisted despite SCT, did worst. Patients who were ddPCR MRD-negative prior to transplant (N=2) or who did not receive SCT (N=14) had intermediate outcomes. Patients who became MRD-negative after SCT (N=13) had the best outcome, with CIR of 0%. These data support the hypothesis that, while SCT undoubtedly has benefit in this patient population, ddPCR MRD status is still an important variable to consider in clinical decision-making after ven/aza treatment.

Multivariate analysis confirms molecular measurable residual disease status as the sole predictor of outcome

We performed multivariate analysis using a Cox PH regression model to evaluate factors contributing to RFS and OS in this cohort. We considered as covariates age, SCT, ddPCR MRD status, and persistent mutation class. Results for both RFS and OS outcomes were similar: only ddPCR MRD-positive status in general and persistent *NPM1* mutation in particular were predictive of inferior outcomes with hazard ratios between 4 and 5, though wide confidence intervals indicate these measures are inflated (Table 2). These results confirm previous literature[27] demonstrating the value of monitoring *NPM1* mutational burden in patients with *NPM1*-mutant AML receiving ven/aza.

Discussion

This is the first study to demonstrate the utility of multigene ddPCR-based MRD for predicting outcomes in patients with AML receiving ven/aza. We show that ddPCR MRD status correlated with RFS, OS, and CIR, both including and excluding patients with *NPM1* mutations. We show that time to relapse is shortest in patients with *NPM1* or "late" signaling pathway mutations, but many patients with persistence of mutations such as *IDH1, IDH2*, and splicing factors also relapse in the absence of SCT, albeit with longer remission duration. We confirm the existence of an "MDS reset" phenomenon with ven/aza therapy, whereby persistence of MDS-associated mutations precedes or coincides with re-emergence of dysplasia. It remains to be seen whether "MDS reset" patients will have higher incidences of relapse with longer follow-up. However, this raises the question of whether venetoclax and HMA, currently in clinical trials for MDS, are capable of curing patients. Finally, we confirm the value of SCT as consolidative therapy for ven/aza, and demonstrate that MRD status can add value for SCT timing considerations. In contrast to MRD after cytotoxic chemotherapy, which is most prognostic post-induction,[3] we find that ddPCR MRD negativity at TBR, whether occurring early or late in therapy and whether pre-SCT or post-SCT, correlates with lowest relapse rates. These findings are in agreement with those published from the VIALE-A cohort,

Table 2. Multi-variate analysis of factors impacting relapse-free survival and overall survival.

Relapse-free survival		
Characteristic	**HR (95% CI)**	***P***
Transplant		
No	-	
Yes	0.72 (0.22-2.40)	0.59
Age at diagnosis	1.05 (1.00-1.10)	0.070
ddPCR MRD		
Negative	-	
Positive	4.86 (1.96-12.1)	<0.001
Persistent mutation (pre-SCT)		
None	-	
Multiple	0.50 (0.15-1.63)	0.25
IDH1 or I*DH2* mutation	0.63 (0.17-2.26)	0.47
NPM1 mutation	4.12 (1.12-15.2)	0.034
Other late mutation	2.58 (0.49-13.5)	0.26
Splicing factor mutation	0.44 (0.18-1.05)	0.064
Overall survival		
Characteristic	**HR (95% CI)**	***P***
Transplant		
No	-	
Yes	1.13 (0.34-3.73)	0.84
Age at diagnosis	1.04 (0.99-1.10)	0.10
ddPCR MRD		
Negative	-	
Positive	4.52 (1.84-11.1)	0.001
Persistent mutation (pre-SCT)		
None	-	
Multiple	0.47 (0.15-1.51)	0.20
IDH1 or I*DH2* mutation	0.32 (0.08-1.28)	0.11
NPM1 mutation	4.81 (1.27-18.2)	0.021
Other late mutation	3.02 (0.54-17.0)	0.21
Splicing factor mutation	0.43 (0.18-1.01)	0.053

HR: hazard ratio; CI: confidence interval; ddPCR: droplet digital polymerase chain reaction; MRD: measurable residual disease; SCT: stem cell transplant.

where MCF MRD negativity after multiple cycles of ven/aza showed no decrement in survival compared to MRD negativity after one cycle.[23] Conversely, patients receiving SCT did best when they proceeded to SCT with MRD, suggesting that low-level persistence of mutations post-ven/aza can be an impetus for early SCT in eligible patients.

ddPCR has been described as highly sensitive and relatively cost-effective yet limited in clinical utility due to its restriction to one mutation per assay. Indeed, previous reports utilizing ddPCR in the context of AML have been restricted to a handful of frequently occurring mutations.[14,38] We utilized 50 unique ddPCR assays for the current ven/aza cohort, which were a mixture of 27 commercially available assays and 23 custom designed constructs. Since the inception of our project, availability of commercial assays has expanded; today only eight of our custom assays would not be available for purchase from an established vendor. Based on our retrospective experience, rapid design and ordering of custom assays can typically occur in 1-2 days and delivery of both custom and commercial assays in 2 weeks. Given the 2–3-week turnaround for diagnostic NGS, a provider could have a relevant ddPCR assay around the time of completion of cycle 1 of ven/aza, allowing for early MRD assessment. We acknowledge differences in cost and person-hours between laboratory-grade assay validation *versus* CLIA certification of an assay for prospective clinical use. However, newer iterations of ddPCR such as the development of dropoff assays for hotspot mutations[39,40] could further simplify the workflow and make development of ddPCR for clinical MRD more appealing. It is worth noting that only 8% of the attrition in our ven/aza patient cohort was due to inability to design an assay for patient-relevant mutations, suggesting that >90% of patients would be eligible for ddPCR MRD monitoring. In addition, while targeted gene panels for NGS will likely become the standard of care for molecular MRD in much of the United States and Europe over the next decade, under-resourced countries might preferentially benefit from the relatively inexpensive (~US $5/sample) and less labor- and time-intensive ddPCR workflow based on more focused diagnostic mutational assessment.

A limitation of the present study was our relatively small cohort size relative to other historic MRD analyses in the literature, limiting our capacity to perform subgroup analyses. However, we note that the number of patients in our cohort was comparable to existing MRD evaluations of ven/aza patients,[24,26,28] with the exception of the VIALE-A cohort.[23] Ours is also the most comprehensive molecular MRD analysis of ven/aza patients to date.[27,28] Although our data suggest that the receipt of SCT did not fully explain the beneficial effects of ddPCR MRD-negative status in our cohort, given our small sample sizes we cannot fully rule out that disease biology or patient factors such as age do not impact our results. Therefore, to enable more in-depth subset analysis we are continuing these studies in ven/aza patients.

Finally, although ddPCR MRD strongly correlated with outcomes in our cohort, we were not able to monitor every mutation in every patient, particularly *FLT3* ITD and other large insertions/deletions. The addition of a *FLT3* ITD assay to our panel, which was not logistically possible at the time of our analysis and was a limitation of our MRD coverage, would further add to the power of this modality and is an active area of development. This could have impacted our results and led to misclassification of patients as MRD-negative who actually had residual disease. An ultra-sensitive NGS platform such as those recently described[6,8,41,42] is another alternative for molecular MRD monitoring, although currently these platforms are more labor-intensive and more costly than ddPCR. Consortium efforts to standardize molecular MRD for future studies are underway and will be essential to the establishment of this modality for clinical use.

In summary, multi-gene molecular MRD utilizing ddPCR is feasible and correlates with outcomes after ven/aza therapy. While persistence of AML-associated mutations such as *NPM1* portend more imminent relapse, consideration of other mutations such as *IDH1, IDH2*, and potentially splicing factor mutations may also provide a comprehensive assessment of disease status and contribute to clinical decision-making.

Disclosures

DAP has received research funding and served as a consultant to Abbvie. The other authors have no conflicts of interest to disclose.

Contributions

ACW conceptualized the project, designed the assays, performed ddPCR experiments, collected clinical data from the electronic medical record, and analyzed data. MM and AM performed ddPCR experiments and data analysis. BMS, JAG, DAP, and CTJ contributed to the design of the project and provided feedback on the work. JAG and DAP consented patients for biobanking and clinical data acquisition. NM, JY, and ALT performed diagnostic and relapse NGS and processed bone marrow samples for DNA isolation. MDE and BJS reviewed bone marrow slides for dysplasia to correlate with persistence of splicing factor mutations. All authors contributed to the writing of the manuscript.

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Data-sharing statement

Original data, primer/probe sequences, and protocols are available upon request by contacting the corresponding author.

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