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

Amanda C. Winters,[1] Mohd Minhajuddin,[2] Brett M. Stevens,[2] Ajay Major,[2] Grace Bosma,[3] Diana Abbott,[3] Nicholas Miltgen,[4] Ji Yuan,[4] Amy L. Treece,[5] Bradford J. Siegele,[6] Mark D. Ewalt,[7] Jonathan A. Gutman,[2] Craig T. Jordan[2] and Daniel A. Pollyea[2]

[1]Center for Cancer and Blood Disorders, Department of Pediatrics, University of Colorado, Aurora, CO; [2]Division of Hematology, Department of Medicine, University of Colorado, Aurora, CO; [3]Department of Biostatistics and Informatics, University of Colorado, Aurora, CO; [4]Molecular Diagnostics, Children's Hospital Colorado, Aurora, CO; [5]Pediatric Pathology, Children's of Alabama, Birmingham, AL; [6]Department of Pathology, University of Colorado, Aurora, CO and [7]Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY, USA

Correspondence: A.C. Winters
amanda.winters@childrenscolorado.org

Received: June 19, 2023.
Accepted: December 7, 2023.
Early view: December 14, 2023.

https://doi.org/10.3324/haematol.2023.283790

Supplementary Materials

Supplementary Methods.

Patient selection. The University of Colorado began using the venetoclax/azacitidine (ven/aza) regimen in early 2015, as part of a multi-institutional phase II trial (NCT02203773). Between that date and the end of 2020, a total of 145 patients were diagnosed with AML at the University of Colorado and received ven/aza induction (**Figure 1**). These dates were chosen to allow sufficient follow-up for outcomes. Exclusion criteria for the current study included refractory disease or undocumented disease status after ven/aza (n=39); no diagnostic mutations identified via clinical targeted NGS (RainDance Thunderbolts Myeloid Panel) (n=7); and no availability of at least one post-remission bone marrow sample for DNA extraction (n=31). In addition, patients with only clonal hematopoiesis-associated mutations (*DNMT3A, TET2*, *ASXL1*) were excluded from molecular MRD evaluation (n=4)(1).

Droplet digital PCR assay design and validation. Custom assays were designed using the ThermoFisher Custom TaqMan Assay Design Tool or Primer Express v3.0.1 software. Assays were validated with an annealing temperature gradient to identify optimal PCR conditions, and limits of detection (LoD) were quantified through evaluation of background signal from at least 8 replicates of wild-type DNA (cord blood mononuclear cells) and serial dilutions of mutant positive control DNA into wild-type DNA background. For all experiments, samples were analyzed in duplicate in combination with cord blood genomic DNA as a wild-type control and mutation-specific DNA standards (Horizon Discovery) or patient diagnostic genomic DNA as mutation-positive controls.

Supplementary Table 1. List of droplet digital PCR (ddPCR) assays utilized in the present study. Diagnostic mutations were prioritized for measurable residual disease (MRD) quantitation as follows: (1) single nucleotide variants (SNVs) recurrently seen in the adult AML literature, (2) small insertions/deletions (indels) recurrently seen in the adult AML literature, or (3) for patients without mutations meeting either of these 2 criteria, patient-specific SNV or indel assays were developed. Assay design for complex indels such as *FLT3* ITD was not attempted. For SNVs, the mutation is labeled according to the resulting amino acid change for easy recognition. For indels the nucleotide change is reported. Limits of detection (LoD) for each assay are shown as validated with 150 nanograms input genomic DNA. BioRad (B) commercial assays are wet-lab validated to 0.1% LoD.

ddPCR Assay/ Mutation	LoD (VAF %)	BioRad or Custom	ddPCR Assay/ Mutation	LoD (VAF %)	BioRad or Custom
NPM1 c.859_860 ins TCTG	0.02	C	U2AF1 S34F	0.1	C
NPM1 c.863_864 ins CTTG	0.02	C	U2AF1 R156H	0.1	B
IDH2 R140Q	0.1	B	PTPN11 E76Q	0.02	C
IDH2 R172K	0.1	B	PTPN11 I56V	0.15	C

IDH2 R172W	0.1	B	PTPN11 G60V	0.02	C
IDH2 R140L	0.1	B	RUNX1 R201X	0.1	C
IDH1 R132C	0.1	B	RUNX1 R201Q	0.1	B
IDH1 R132H	0.1	B	RUNX1 W106S	0.1	B
IDH1 R132G	0.1	B	RUNX1 R166Q	0.1	B
SRSF2 P95H	0.03	C	RUNX1 S141L	0.1	B
SRSF2 P95L	0.09	C	RUNX1 c.423_424 dup	0.02	C
SRSF2 P95R	0.05	C	RUNX1 c.811delA	0.05	C
SRSF2 c.284_307 del	0.15	C	RUNX1 R191X	0.04	C
JAK2 V617F	0.1	B	RUNX1 c.273_274insGGGGGGCGC	0.02	C
NRAS G12D	0.06	C	TP53 H193R	0.1	B
NRAS Q61K	0.02	C	TP53 Y205F	0.1	B
NRAS G12V	0.1	B	TP53 I251N	0.05	C
NRAS G13C	0.1	B	TP53 E258G	0.1	B
SF3B1 K700E	0.07	C	TP53 R273H	0.1	B
SF3B1 K666M	0.1	B	TP53 V272M	0.1	B
SF3B1 K666N	0.05	C	TP53 c.376-1G>A splice site	0.1	C
SF3B1 G740E	0.1	B	PHF6 c.1009delinsCT	0.02	C
FLT3 D835Y	0.1	B	PHF6 I314T	0.1	C
FLT3 D839G	0.1	B	SMC1A E687X	0.1	B
KIT N822Y	0.1	B	SMC1A R807H	0.1	B

VAF = variant allelic frequency; B = BioRad commercial assay; C = custom-designed assay

Supplementary Results.

ddPCR MRD evaluation at specific time points was not feasible in our retrospective cohort. We assessed the availability of bone marrow samples from all 64 patients in our cohort at specific time points in therapy: post-cycle 1 of ven/aza, post-cycle 4 of ven/aza, and (for patients receiving SCT) immediately pre-SCT. **Supplementary Table 2** shows availability of samples and MRD status where able to be assessed. Almost all patients had bone marrow sample available post-cycle 1 of ven/aza; however, no patients were MRD negative by ddPCR at this time point. The post-cycle 4 time point had a significant drop-off in number of patients

with available bone marrow material for MRD assessment, leaving only 6 patients in the MRD negative group. Finally, a minority of patients in our cohort proceeded to SCT, which also limited cohort size at the pre-SCT time point. Therefore, we chose “time of best response” (TBR) as our MRD assessment time point for this analysis.

Supplementary Table 2. Number of patients with available bone marrow at individual time points post-therapy.

Number of Patients by Category	Post-Cycle 1	Post-Cycle 4	Pre-Transplant
MRD pos	57	28	13
MRD neg	0	6	2
Data missing	7	22	2
Not applicable*	0	8	47

*Not applicable = time point not achieved or did not receive transplant

Supplementary Table 3. Clonality status of *IDH1* and *IDH2* mutations did not impact rates of mutation clearance by droplet digital PCR.

IDH1	Mutation Cleared	Mutation Persistent	p-value (Fisher’s exact)
Clonal	1	4	
Subclonal	3	2	0.12
IDH2			
Clonal	2	10	
Subclonal	1	1	0.39

Supplementary Table 4. Persistence of splicing factor mutations ultimately leads to relapse in approximately half of patients receiving venetoclax/azacitidine.

	MRD negative	MRD positive (VAF <10%)	MRD positive (VAF ≥10%)
Relapse	0	7	3
No Relapse	4*	9	3

p-value from Fisher’s exact test = 0.13 (not significant) *3 of 4 became MRD negative after SCT

MRD = measurable residual disease; VAF = variant allelic frequency; SCT = stem cell transplant

Supplementary Table 5. Correlation of Dysplasia with Persisting Mutations.

Patient	s-AML?	SF Mutation (VAF)	Other Mutation (VAF)	Dysplasia ?	Dysplasia vs VAF	Initial Blast %	Karyotype	Outcome
1351	no	n/a	IDH2 (42%)	no		79.5	-7	relapse >2y
1434	no	n/a	PHF6 (45%)	scant*		63	normal	relapse <2y
998	no	n/a	IDH1 (41%)	scant*		58	normal	relapse <2y

1289	no	n/a	FLT3 (11%), NPM1 (42%)	scant*		76.5	normal	CR (no SCT)
1387	no	n/a	NPM1 (41%)	scant*		78.5	normal	relapse <2y
527	yes	n/a	IDH1 (28%)	no		47	-7, +8	CR (no SCT)
1532	yes	n/a	NPM1 (41%), SMC1A (39%)	no		20	normal	relapse <2y
1259	yes	n/a	TP53 (11%), JAK2 (26%)	no		53	complex	relapse <2y
1456	no	n/a	PTPN11 (22%)	no		35	inv(16), +8, +22	CR (no SCT)
975	no	n/a	TP53 (19%)	no		49.5	complex	CR (no SCT)
691	no	n/a	IDH1 (14%)	no		60	normal	relapse >2y
1671	yes	n/a	NRAS (13%), KIT (8%)	no		15.5	t(8;12;21)	CR (no SCT)
1375	yes	n/a	IDH2 (45%), NPM1 (43%)	no		82.5	normal	CR (SCT)
1518	yes	n/a	IDH2 (44%)	scant*		64	-13	CR (no SCT)
1308	no	n/a	NPM1 (39%)	no		96.5	normal	relapse >2y
1020	no	n/a	NPM1 (38%)	no		85	normal	CR (no SCT)
1279	no	n/a	NPM1 (40%), FLT3 (16%)	scant*		71	add(6)	relapse >2y
1414	no	n/a	TP53 (44%)	yes	correlate (both stable)	28.5	complex	relapse <2y
1011	yes	n/a	DNMT3A , NPM1 (42%)	yes	correlate (both stable)	79	-Y	relapse <2y
976	yes	n/a	TP53 (74%)	yes	correlate (fall/rise)	27	complex	relapse <2y
1402	no	n/a	IDH1 (35%), NPM1 (44%)	yes	dysplasia disappeare d prior to fall in IDH1	80	normal	CR (SCT)
478	no	n/a	IDH1 (21%)	yes	correlate (both decrease)	58	normal	CR (no SCT)
1261	no	SRSF2 (38%)	IDH2 (51%), SMC1A (64%)	no		70.5	+8, +15	relapse <2y
1374	no	SRSF2 (26%)	RUNX1 (14%)	no		40	tetraploidy	CR (no SCT)

1043	no	SRSF2 (47%)	IDH2 (47%), NPM1 (34%)	no		66.5	normal	CR (SCT)
1699	no	U2AF1 (37%)	RUNX1 (40%), NRAS (36%)	no		20	normal	CR (no SCT)
1522	no	U2AF1 (28%)	NRAS (15%)	scant*		58	add(17)	CR (no SCT)
966	yes	U2AF1 (47%)	IDH2 (47%)	no		87	normal	CR (no SCT)
1605	no	SRSF2 (44%)	IDH2 (47%)	scant*		51	normal	relapse <2y
494	no	SF3B1 (22%)		yes	correlate (fall/rise)	25	t(1;3)	relapse <2y
Q	no	SRSF2 (40%)		yes	correlate (both decrease)	42.5	-7	CR (no SCT)
682	yes	SF3B1 (38%)		RS	rising VAF led dysplasia	22	-7	CR (SCT)
368	yes	SF3B1 (35%)		RS	rising VAF led dysplasia	37	-Y	relapse <2y
N	no	SF3B1 (46%)		yes	missing data	20.5	normal	relapse <2y
719	yes	SRSF2 (unk)	IDH2 (45%)	yes	rising VAF led dysplasia	21	normal	relapse >2y
1004	no	SRSF2 (32%)	NPM1 (37%)	yes	missing data, also acquired -7	60	normal	relapse >2y
1258	yes	SRSF2 (45%)	IDH2 (45%)	yes	rising VAF led dysplasia	28	normal	CR (no SCT)

s-AML: secondary AML; SF: splicing factor; VAF: variant allelic frequency; CR: complete remission; SCT: stem cell transplant; 2y: 2 years; scant: not meeting MDS criteria; unk: unknown

Supplementary Table 6. Comparisons between mutational/dysplasia groups show no significant biological differences.

Group	Patient Range from Supp Table 5	Initial Blast %, median (IQR)	s-AML, n (%)	Abnormal Cytogenetics, n (%)	Relapses, n (%)
SF-/Dysplasia-	1351::1279	63 (49.5-78.5)	6 (35%)	8 (47%)	9 (53%)
SF-/Dysplasia+	1414::478	58 (28.5-79)	2 (40%)	3 (60%)	3 (60%)
SF+/Dysplasia-	1261::1605	58 (45.5-68.5)	1 (14%)	3 (43%)	2 (29%)
SF+/Dysplasia+	494::1258	26.5 (21.8-38.4)	4 (50%)	4 (50%)	5 (63%)
p-value*		0.06	0.30	0.52	0.50

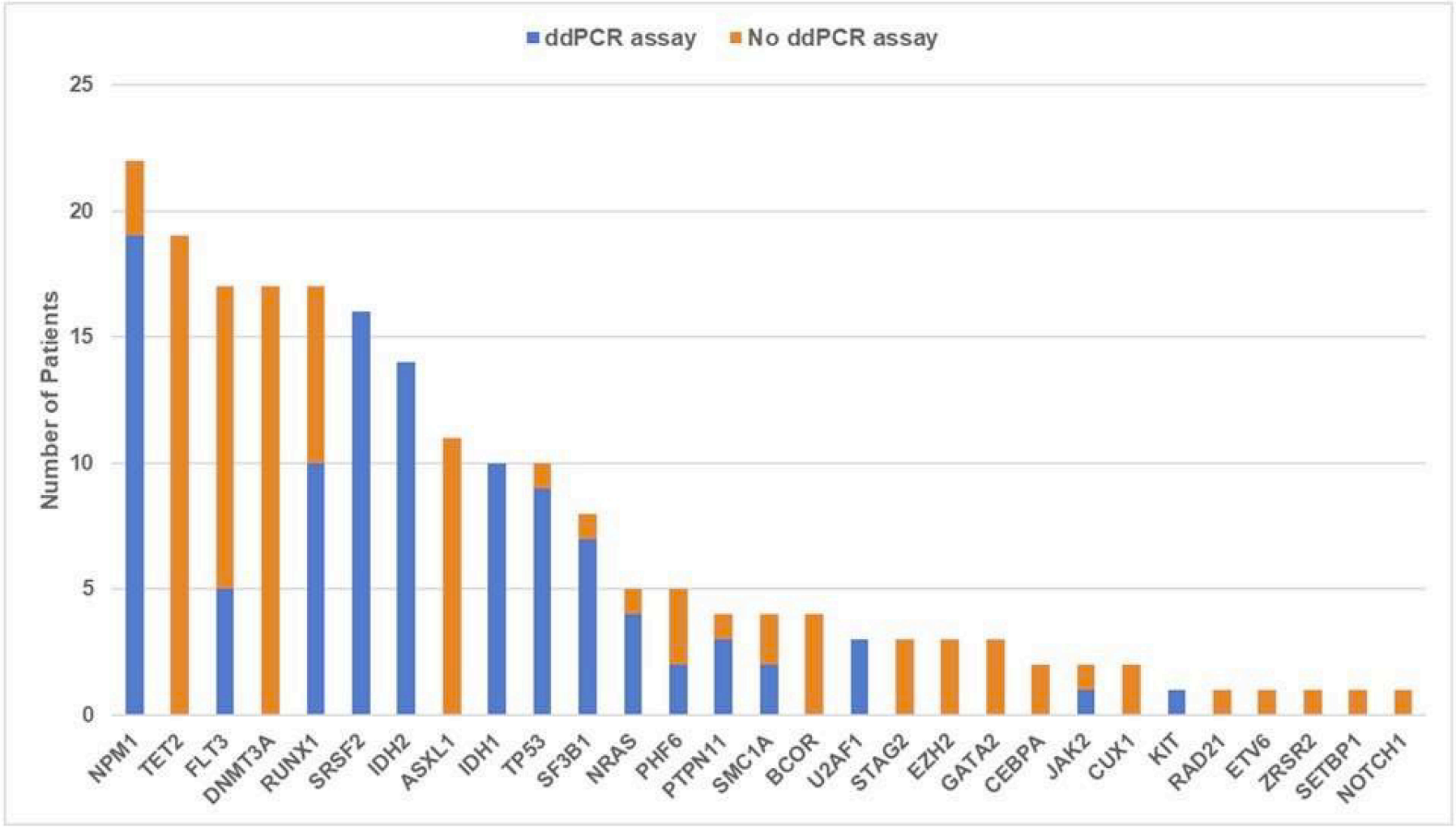
SF: splicing factor; IQR: interquartile range; s-AML: secondary AML; *p-values calculated via Kruskal-Wallis (blast %) and Fisher exact test combining Dysplasia+ groups and Dysplasia- groups (other fields)

Supplementary Figure 1. Frequency of mutations in this cohort closely mirrors large-cohort AML mutation frequency. Sum of all mutations by gene was based on diagnostic next-generation sequencing (NGS), with ratios shown of those that were monitored by droplet digital PCR (ddPCR) and those that were not. For example, *FLT3* tyrosine kinase domain (TKD) mutations were included in ddPCR measurable residual disease (MRD) monitoring, but *FLT3* internal tandem duplications (ITD) were not due to challenges with assay design.

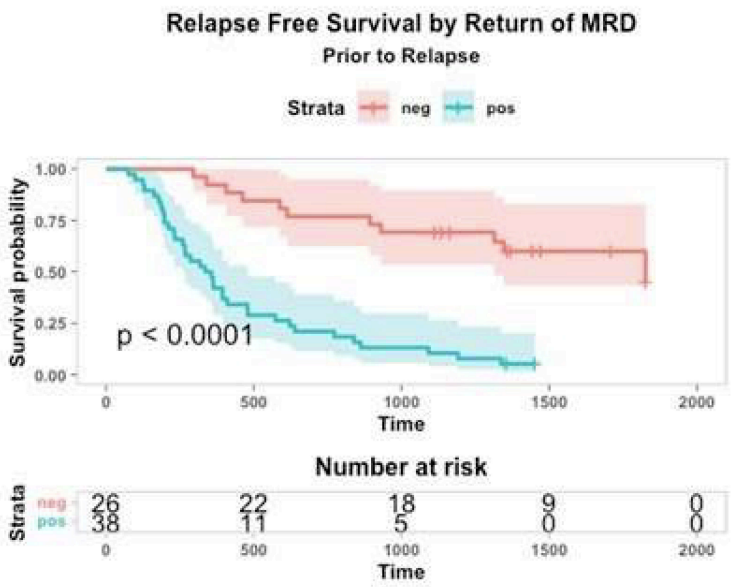
Supplementary Figure 2. Re-stratification of measurable residual disease (MRD) negative patients with MRD recurrence as "MRD positive" did not change significance of droplet digital PCR (ddPCR) MRD status for outcomes. Three individuals who were MRD negative at time of best response (TBR) subsequently had recurrence of detectable mutation(s) by ddPCR. Re-stratification of these patients as MRD positive is shown and significance for (a) relapse-free survival and (b) overall survival is re-demonstrated.

References

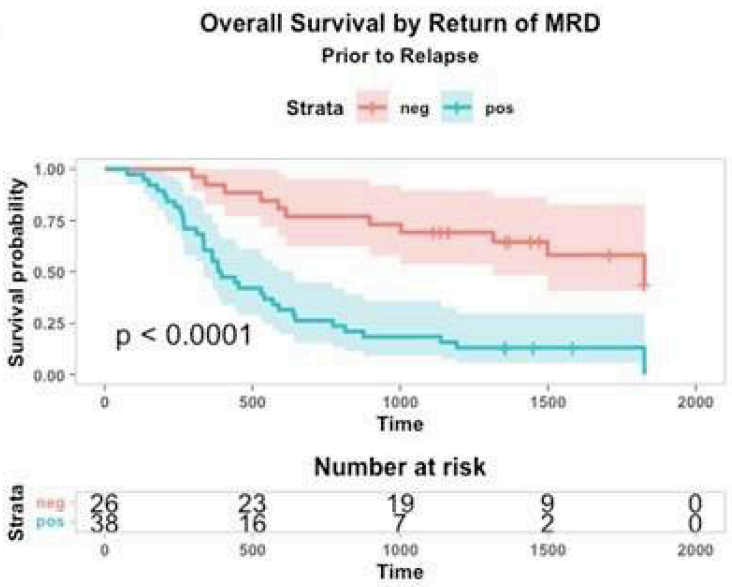
1. Jongen-Lavrencic M, Grob T, Hanekamp D, Kavelaars FG, Al Hinai A, Zeilemaker A, et al. Molecular Minimal Residual Disease in Acute Myeloid Leukemia. N Engl J Med. 2018;378(13):1189-99.


ddPCR assay
No ddPCR assay
Number of Patients
25
20
15
10
5
0
NPM1
TET2
FLT3
DNMT3A
RUNX1
SRSF2
IDH2
ASXL1
IDH1
TP53
SF3B1
NRAS
PHF6
PTPN11
SMC1A
BCOR
U2AF1
STAG2
EZH2
GATA2
CEBPA
JAK2
CUX1
KIT
RAD21
ETV6
ZRSR2
SETBP1
NOTCH1

a.


Relapse Free Survival by Return of MRD
Prior to Relapse
Strata neg pos
Survival probability
1.00
0.75
0.50
0.25
0.00
p < 0.0001
0
500
1000
1500
2000
Time
Number at risk
Strata
neg
pos
26 22 18 9 0
38 11 5 0 0
0
500
1000
1500
2000
Time

b.


Overall Survival by Return of MRD
Prior to Relapse
Strata neg pos
Survival probability
1.00
0.75
0.50
0.25
0.00
p < 0.0001
0
500
1000
1500
2000
Time
Number at risk
Strata
neg
pos
26 23 19 9 0
38 16 7 2 0
0
500
1000
1500
2000
Time