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ORIGINAL REPORT

Serum 2-Hydroxyglutarate Production in *IDH1*- and *IDH2*-Mutated De Novo Acute Myeloid Leukemia: A Study by the Acute Leukemia French Association Group

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ABSTRACT

Purpose
Mutated isocitrate dehydrogenases (IDHs) 1 and 2 produce high levels of 2-hydroxyglutarate (2-HG). We investigated whether, in acute myeloid leukemia (AML), serum 2-HG would predict the presence of *IDH1/2* mutations at diagnosis and provide a marker of minimal residual disease (MRD).

Patients and Methods
Serum samples from 82 patients at diagnosis of de novo AML (*IDH1/2* mutated, n = 53) and 68 patients without AML were analyzed for total 2-HG and its ratio of D to L stereoisomers by mass spectrometry. We measured 2-HG levels and molecular markers of MRD (*WT1* and *NPM1*) in serial samples of 36 patients with *IDH1/2* mutations after induction therapy.

Results
In patients with AML with *IDH1/2* mutations, 2-HG serum levels were significantly higher than in patients with *IDH1/2* wild type ($P < .001$). Area under the receiver operating characteristic curve was 99%. The optimum diagnostic cutoff between *IDH1/2* mutated and normal was 2 μmol/L (sensitivity, 100%; specificity, 79%). Quantification of the D/L stereoisomers increased specificity (100%; 95% CI, 83% to 100%) compared with total 2-HG ($P = .031$). In patients with *IDH2* R172 mutations, 2-HG levels were higher relative to those with other *IDH1/2* mutations ($P < .05$). During follow-up, serum 2-HG levels showed strong positive correlation with *WT1* and *NPM1* ($P < .001$). After induction therapy, total 2-HG serum levels < 2 μmol/L were associated with better overall ($P = .008$) and disease-free survival ($P = .005$).

Conclusion
Serum 2-HG is a predictor of the presence of *IDH1/2* mutations and outcome in these patients. Discrimination between D/L stereoisomers improved specificity.

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INTRODUCTION

Mutations in the isocitrate dehydrogenase (IDH) *IDH1* and *IDH2* genes revealed a novel mechanism of cancer formation and uncovered new therapeutic opportunities. These enzymes normally catalyze a Krebs cycle–like reaction, namely the conversion of isocitrate to alpha-ketoglutarate (aKG) coupled with NADPH (nicotinamide adenine dinucleotide phosphate) production, which is essential for oxidative stress response and several metabolic pathways. Mutated IDH enzymes produce the D stereoisomer of 2-hydroxyglutarate (2-HG) from aKG.[1] As a result, tumor cells accumulate massive amounts of 2-HG, which can interfere with aKG-dependent dioxygenases, including histone and DNA demethylases, such as TET2.[2-4] In vitro data and a mouse model concurrently indicated that in hematopoietic cells, *IDH1/2* mutations lead to major alterations of epigenetic marks throughout the genome.[5,6] *IDH1/2* mutations are found in 15% to 20% of patients with AML ($\leq$ 30% in patients with normal karyotype).[7-9] *IDH1/2* mutations are thought to be an early oncogenic event in AML.[10] The clinical impact of these mutations may depend on the specific mutation (eg, *IDH2* R140, R132, or R172) and on the presence of associated mutations, such as *FLT3* internal tandem duplication (*FLT3-ITD*) or *NPM1*.[8,11-13] Detection of *IDH1/2* mutations and quantitation of 2-HG in blood may therefore

provide markers proximal to the mechanism of disease, which could help to optimize treatment and follow-up of this subset of AML. Several studies have shown increased levels of total serum 2-HG in small cohorts of patients with AML with *IDH1/2* mutations,[14-19] but none have investigated long-term follow-up or measured the specific product of the mutated enzymes (ie, D stereoisomer *v* unrelated (L) stereoisomer of 2-HG).

We measured levels of total 2-HG (ie, sum of D and L stereoisomers) and the ratio between the two stereoisomers (D to L) in serum or plasma from a cohort of 82 patients with de novo AML and 68 patients without AML. We tested the diagnostic value of these two markers to identify *IDH1/2* mutations as well as their prognostic significance and whehter they correlated with known minimal residual disease (MRD) markers.

PATIENTS AND METHODS

Study Population and Data Collection

Serum or plasma samples from 150 patients with (n = 82) and without AML (n = 68) were analyzed for 2-HG levels (Fig 1). Among those with AML, 53 had *IDH1/2* mutations, and 29 consecutive patients were *IDH1/2* wild type. Samples from patients with AML were collected at diagnosis between 2005 and 2012 through a collaborative network of French centers (Creteil, Dijon, Lille, Paris Saint Louis, and Villejuif) for the Acute Leukemia French Association (ALFA) group. All patients were investigated for *IDH1*, *IDH2*, *AML1/RUNX1*, *CEBPA*, *FLT3*, and *NPM1* mutations as described.[11] They all had intermediate-risk cytogenetics (except one because of karyotype failure), but the cohort was enriched in *IDH1/2*-mutated patients. Fifty-two patients were included in the ALFA-0701[20] and -0702 trials (ClinicalTrials.gov Identifier: NCT00932412). Thirty patients received intensive treatment according to ALFA protocols, combining anthracyclines and cytarabine.[21,22] All patients signed written informed consent.

For follow-up studies, we analyzed 171 serum or plasma samples from 36 patients with AML with *IDH1/2* mutations (at diagnosis, in complete remission [CR], or with refractory disease) after consolidation courses, during follow-up, and at relapse. Serums were analyzed along with molecular markers of MRD (*NPM1* mutation, *WT1* expression) as reported.[23,24] MRD levels were assessed using cDNA-based real-time quantitative polymerase chain reaction (PCR) and reported as the ratio of *NPM1* mutation or *WT1* transcript to 100 *ABL* transcript. *WT1* overexpression at diagnosis was defined as a ratio of *WT1* to *ABL* transcript > 25% in bone marrow (BM) or 5% in peripheral blood (PB) samples. Samples were collected at diagnosis, after induction, after consolidation course, during follow-up, and at relapse.

2-HG measurements in plasma samples of the 68 patients without AML were analyzed per routine diagnostic procedure at the Necker Hospital (Paris, France) for the workup of 50 consecutive patients with putative metabolic disorders or during the follow-up of known inherited metabolic diseases (n = 18). Basic characteristics of patients without AML are listed in Appendix Table A1 (online only).

Quantification of IDH1/2 Mutation Burden

Genomic DNA was extracted from BM or PB samples using conventional procedures. *IDH1*- (codon R132) and *IDH2*-targeted (codons R140 and R172) regions were amplified by PCR with primers (available on request) containing Ion Torrent (Life Technologies, Carlsbad, CA) adapters and unique barcodes to generate libraries. Pooled amplicon libraries were clonally amplified on Ion Spheres using the Ion Xpress Template 200 Kit (Life Technologies) and then bidirectionally sequenced on Ion Torrent Sequencer. The relative *IDH1/2* mutational burden was determined as number of normal to mutant reads.

2-HG Analysis

Plasma or serum samples (dry lithium heparin) from the reference population (n = 68) and patients with AML (n = 82) were analyzed in the Reference Center for Metabolic Disorders at Necker Hospital. To each 100-μL sample we added 0.5 nmoles of 1,2,3,4-13C4–labeled 2-HG, prepared by reduction of labeled aKG (Eurisotop, Saint-Aubin, France) as the internal standard. Analysis was performed in selected-reaction monitoring mode by gas chromatography–tandem mass spectrometry on a GC 450/300-MS triple quadrupole (Varian, Brüker Daltonics, Fremont, CA). Sample processing was performed as reported.[25] Calibration curves were linear between < 20 pmoles and > 400 nmoles. The limit of quantification was 50 pmoles, with a coefficient of variation < 10%. The laboratory participates in the International External Quality Assurance Programme for Quantitative Organic Acids (http://cms.erndimqa.nl/).

Statistical Analysis

Analyses were performed using the SAS software (version 9.2; SAS Institute, Cary, NC) and R language–based software (version 2.14; (http://www.r-project.org). Area under the receiver operating characteristic curve (ROC

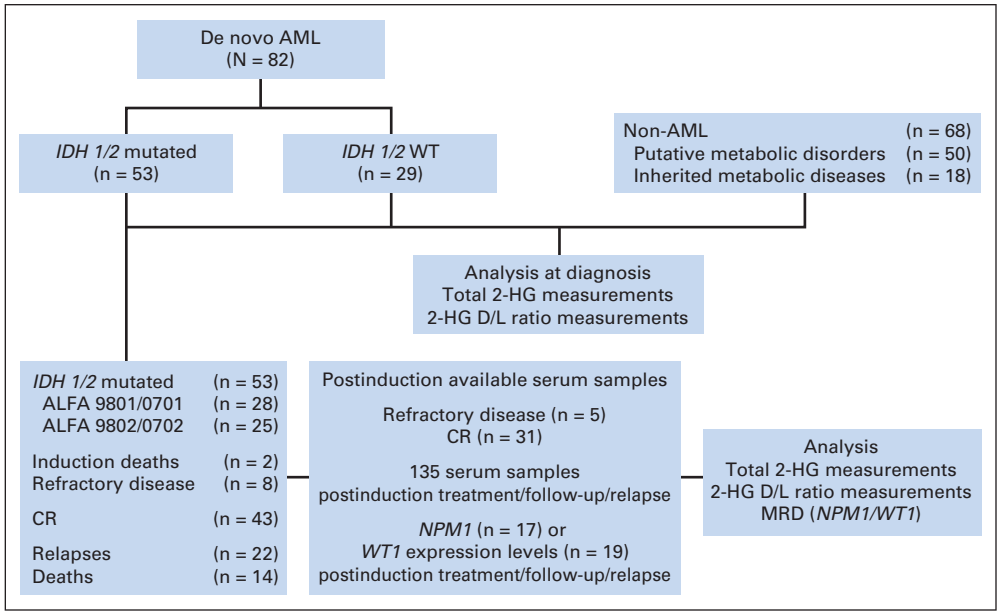

Fig 1. CONSORT diagram. 2-HG, D and L stereoisomers of 2-hydroxyglutarate; ALFA, Acute Leukemia French Association; AML, acute myeloid leukemia; CR, complete remission; MRD, minimal residual disease; WT, wild type.

AUC) was estimated using the *pROC* package (R software; 95% CIs estimated using de Long method). Correlations between 2-HG and hematologic parameters or molecular markers of follow-up were estimated with Spearman rank-order correlation coefficient. Progression-free survival (PFS) was estimated for patients in CR from the date of diagnosis to date of progression. Patients who died without progression were considered censored at the date of death. The Kaplan-Meier method was used to perform univariate survival analyses; the log-rank test was used to test statistical significance. Cox regression analysis was performed to identify independent prognostic parameters. All *P* values were two tailed, and the level of significance was $P < .05$.

RESULTS

IDH1/2 *Gene Mutation Profile in the Study Population*

Initial characteristics of 82 patients with de novo AML are summarized in Table 1, with details listed in Appendix Table A2 (online only). Fifty-three of these patients (64%) were had *IDH1/2* mutations and were compared with 29 consecutive patients with de novo AML without *IDH1/2* mutations. *IDH1/2* mutations were associated with

Table 1. Characteristics and Outcomes of Patients With AML According to *IDH* Mutation Status

Characteristic	Total		*IDH1* R132		*IDH2* R140		*IDH2* R172		*IDH* WT		*P*
	No.	%	No.	%	No.	%	No.	%	No.	%	
No. of patients	82	100.0	20	24.3	24	29.3	9	11.0	29	35.3	
Age, years											NS
Median	59		56		55		59		63		
Range	22-85		32-74		23-78		26-65		22-85		
Sex											
Male	42		10		10		4		18		
Female	40		10		14		5		11		
WBC, g/L											NS
Median	10.3		4.6		13.4		17.2		11		
Range	0.89-250		0.89-178		1-244		1.2-28.3		1-250		
Circulating blasts, %											NS
Median	40		43.5		30.5		70		39		
Range	0-97		1-91		1-94		6-83		1-97		
BM blasts, %											.04
Median	66		80.5		62		78		60		
Range	11-97		13-95		11-97		55-91		15-93		
FAB											
M0	6	7	1		1		2		2		
M1	27	33	12		4		6		5		
M2	14	17	3		7		1		3		
M4	10	12	0		4		0		6		
M5	19	23	3		8		0		8		
M6	2	2	1		0		0		1		
NC	4	5	0		0		0		4		
IDH mutation											
IDH1 R132C			10	50							
IDH1 R132G			3	15							
IDH1 R132H			7	35							
IDH2 R140L					2	8					
IDH2 R140Q					21	87					
IDH2 R140W					1	4					
IDH2 R172K							9	100			
Gene mutation											
IDH1 rs11554137*	12	15	4	20	2	8	1	11	5	17	
IDH1 V71I*	1	1							1	3	
FLT3 (ITD and *TKD)*	21	26	6	30	10	42	1	11	4	14	NS
NPM1	41	50	12	60	18	75			11	38	< .001
CEBPA	1	1			1	4					
MLL duplication	2	2			2	8					
RUNX1	1	1			1	4					
3-year OS											NS
IDH1		54									
IDH2 R140Q		74									
IDH2 R172K		56									
IDH WT		70									

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; FAB, French-American-British; NS, not significant; WT, wild type.
*Germ line polymorphisms *IDH1* G105G (rs11554137) and *IDH1* V71I were previously reported.

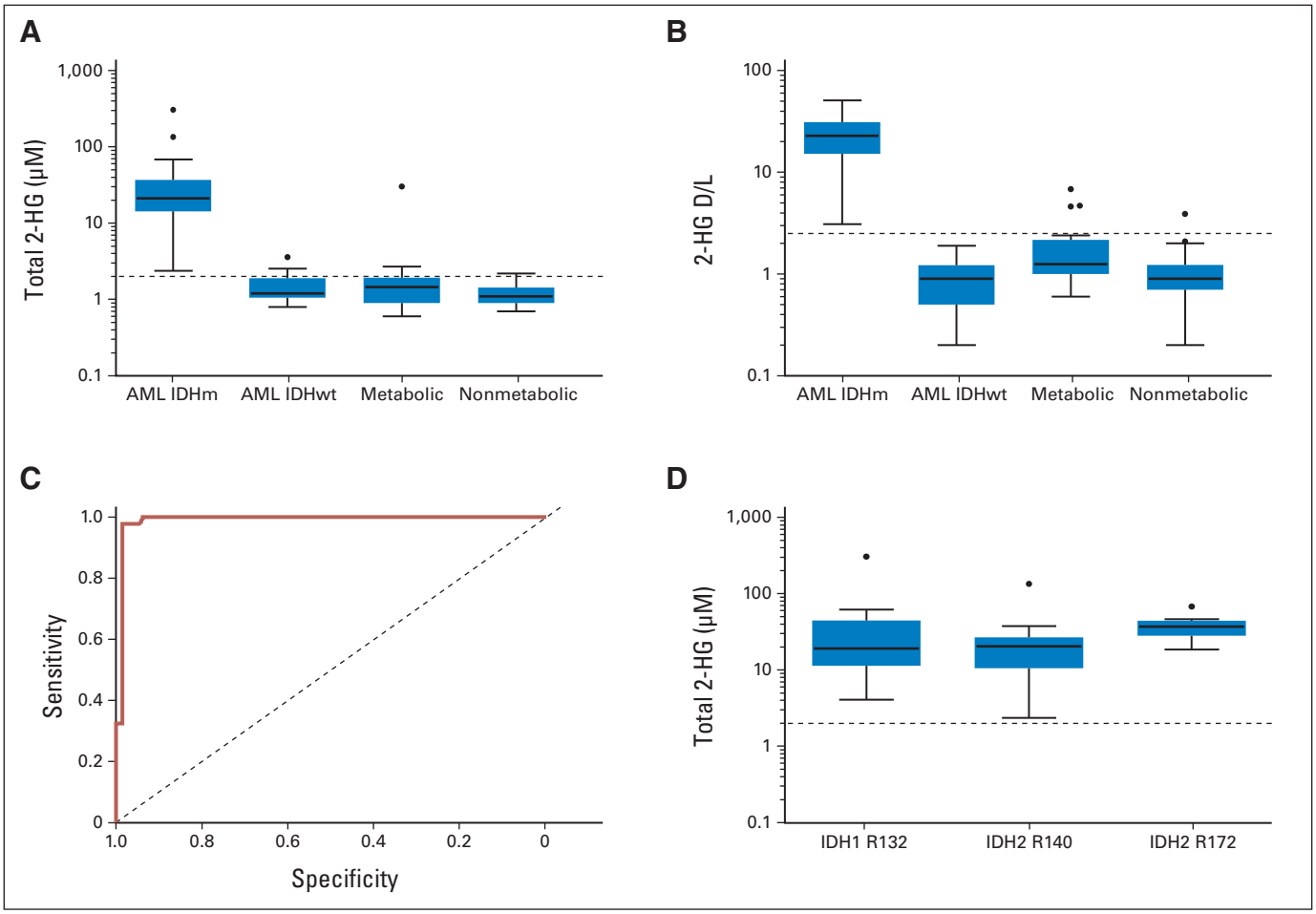

Fig 2. Diagnostic performance of total 2-hydroxyglutarate (2-HG; ie, D plus L stereoisomers) and D to L ratio. Boxplots of (A) total 2-HG and (B) D to L ratio in blood samples at diagnosis from patients with acute myeloid leukemia (AML) with *IDH* mutations (IDHm; n = 53) or without (*IDH* wild type [IDHwt]; n = 29), as compared with samples from metabolic (n = 18) and nonmetabolic reference populations (n = 50). Dashed lines indicate proposed screening cutoffs. (C) Area under receiver operator characteristic curve for total 2-HG levels for patients with and without AML at diagnosis (n = 150); *y*-axis, true-positive rate (sensitivity); *x*-axis, false-positive rate (1-specificity). (D) Boxplot comparing total 2-HG in serum from patients with different *IDH* mutations: *IDH1* R132 (n = 20), *IDH2* R140 (n = 24), and *IDH2* R172 (n = 9).

NPM1 mutations ($P < .001$) but not with *FLT3* mutations (neither ITD nor tyrosine kinase domain [TKD]) and had higher percentage of bone marrow blasts ($P = .04$) compared with *IDH1/2* wild type (Table 1).

2-HG Plasma Level and D to L Stereoisomer Ratio in Patients Without AML

We measured plasma 2-HG levels in a reference population that included 50 consecutive adult patients without metabolic disorders (nonmetabolic reference population) and 18 pediatric or adult patients with known inherited metabolic disease but without mutations in *IDH1/2* or D-2 or L-2 hydroxyglutarate dehydrogenase genes (metabolic reference population; Appendix Table A1, online only). The latter were meant to provide relevant information for metabolic disturbances that may affect 2-HG levels by acting upstream or downstream of the IDH1/2 enzymes. Because *IDH1/2* mutations produce the D stereoisomer and not the L stereoisomer, we calculated the ratio between D and L to determine which stereoisomer was responsible for increased total 2-HG. Indeed, increased levels of the L stereoisomer may confound the analysis of total 2-HG, producing false positives. Median total 2-HG (total 2-HG) levels were 1.1 μmol/L (range, 0.7 to 2.2 μmol/L) for the 50 nonmetabolic patients and 1.5 μmol/L (range, 0.6 to 30.4 μmol/L) for the 18 metabolic patients (Fig 2A). Metabolic patients had a higher median D to L ratio (1.3; range, 0.6 to 6.8) than nonmetabolic patients (0.9; range, 0.2 to 3.9; Wilcoxon rank sum test $P = .003$; Fig 2B) but had similar total 2-HG levels (median, 1.4; range, 0.6 to 30.4 *v* median, 1.1; range, 0.7 to 2.2 μmol/L, respectively; Wilcoxon rank sum test $P = .18$). One patient with a metabolic disorder had a 2-HG level as high as 30 μmol/L but a normal D to L ratio. Overall, these data suggest that some metabolic disturbances may significantly alter 2-HG levels, thereby confounding analysis of patients with AML.

Prediction of IDH1/2 *Mutational Status of Patients With AML at Diagnosis*

Patients with AML without *IDH1/2* mutations (n = 29) had median total 2-HG levels (1.2; range, 0.8 to 3.6 μmol/L) similar to those of nonmetabolic patients (1.1; range, 0.7 to 2.2 μmol/L) and metabolic patients (1.5; range, 0.6 to 30.4 μmol/L; Wilcoxon rank

sum test $P = .08$, Fig 2A). In contrast, patients with AML with *IDH1/2* mutations had a markedly higher median level (21.2; range, 2.4 to 305.9 μmol/L; $P < .001$). Median D to L ratio was 0.9 (range, 0.2 to 1.9) for *IDH1/2* wild-type patients with AML and 22.9 (range, 3.1 to 51.2) for patients with AML with *IDH1/2* mutations (Fig 2B). Total 2-HG level and D to L ratio that ensured maximum specificity at 100% sensitivity to detect *IDH1/2* mutations were approximately 2 μmol/L and 2.5, respectively. At these optimal screening cutoffs, among patients with AML, D to L ratio showed better specificity (100%) than total 2-HG (79%; McNemar test $P = .031$). Thus, only the D to L ratio led to complete separation between patients with AML with or without *IDH1/2* mutations. Nevertheless, this picture may be overoptimistic, because the inclusion of patients without AML reduced specificity of the D to L ratio to 96% (misclassifications included ratios between 3.9 and 6.8 from reference population; Fig 2B).

High values for either total 2-HG or D to L ratio were strongly predictive of the presence of *IDH1/2* mutations. Both had ROC AUCs > 99%, with 95% CI of 98% to 100% (Fig 2C; Appendix Fig A1A, online only). Interestingly, in two patients with AML, *IDH1/2* mutations that had not been detected with the classical Sanger method were revealed by high levels of 2-HG. Deep sequencing identified *IDH1* R132C and *IDH2* R140Q mutations (in patients No. 6 and 37, respectively; Appendix Table A2, online only) at allelic burdens of 10% and 20%, respectively.

Parameters Associated With High 2-HG Level at Diagnosis

We found a strong positive correlation between allelic burden and D to L ratio in patients with *IDH1/2* mutations ($P < .001$; $r^2 = 0.489$; Appendix Figure A2A, online only). D to L ratio also showed positive correlation with WBC counts ($P < .001$; $r^2 = 0.301$; Appendix Fig A2B, online only). Neither *FLT3-ITD* or *-TKD* mutations nor *NPM1* mutations were associated with D to L ratio or total 2-HG ($P = .89$ and $P = .1$, respectively).

Presence of the *IDH2* R172K mutation was associated with increased total 2-HG and D to L ratio compared with *IDH2* R140 and *IDH1* R132 mutations (Kruskal Wallis test $P = .02$ and $P = .04$, respectively; Fig 2D; Appendix Fig A1B, online only). This remained significant after adjustment for WBC counts, percentage of circulating blasts, and percentage of BM blasts (data not shown). Single nucleotide polymorphism rs11554137[26] observed in five patients carrying the *IDH1* wild-type coding sequence (patients No. 70, 71, 74, and 78; Appendix Table A2, online only) or polymorphic variant *IDH1* V71I[27] (patient No. 54; Appendix Table A2, online only) showed normal levels of 2-HG.

2-HG Level Reflects Clinical Status and Correlates With MRD Markers

The serial collection of total 171 serum samples from 36 patients with *IDH1* or *IDH2* mutations included time points at diagnosis, after induction treatment, during follow-up, and at relapse. Median 2-HG level and D to L ratio in samples at CR were significantly lower (1.3 μmol/L and 1.4, respectively) than those from patients who did not achieve CR after one induction course (6.2 μmol/L and 8.3) as well as from refractory patients (15.8 μmol/L and 23.1) at relapse (5.1 μmol/L and 8.5) and diagnosis (22.4 μmol/L and 21.1; Kruskal Wallis test $P < .001$ and $P < .001$, respectively; Fig 3; Appendix Fig A3A, online only).

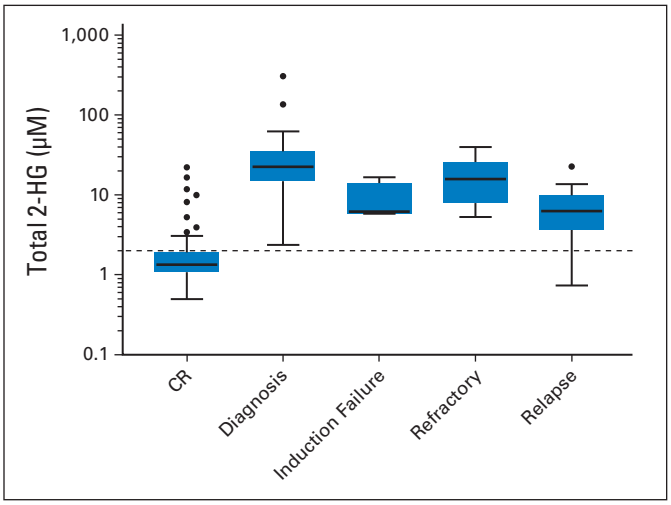

Fig 3. Correlation of total 2-hydroxyglutarate (2-HG; D plus L stereoisomers) level with clinical status. Induction failure indicates no achievement of complete remission (CR) after one induction course.

Finally, the relation between 2-HG level and MRD was assessed by comparing 2-HG levels with mutated *NPM1* burden (n = 17 patients) or *WT1* expression levels (n = 19 patients; Fig 4; Appendix Fig A3B, online only) at diagnosis, at CR, and during follow-up (persistent CR and relapsing patients). A positive correlation was observed between 2-HG level (either total 2-HG or D to L ratio) and *WT1* expression in blood (n = 48 paired samples), *WT1* expression in BM (n = 41), *NPM1* in blood (n = 37), and *NPM1* in bone marrow (n = 39; all correlations significant at $P < .001$; $r^2 > 0.3$; Fig 4; Appendix Fig A3B, online only). Thus, both total 2-HG and D to L ratio are good candidate markers of MRD.

Prognostic Impact of Postinduction 2-HG Levels in Patients With IDH1/2 Mutations

Median follow-up of the 53 patients with *IDH1/2* mutations was 2.9 years (95% CI, 2 months to 6.9 years). Two died during induction treatment, and eight were refractory (five of 20 with *IDH1* R132 and three of nine with *IDH2* R172 mutations). Forty-three of these patients (13 of 20 with *IDH1* R132, 24 of 24 with *IDH2* R140, and six of nine with *IDH2* R172 mutations) achieved CR (81%). After CR achievement, 22 relapsed, and 14 died.

In these 53 patients with *IDH1/2* mutations, univariate prognostic analyses of *IDH1* R132, *IDH2* R140Q, *IDH2* R172K, *FLT3-ITD*, *FLT3-TKD*, and *NPM1* mutations and initial levels of total 2-HG (> or < median level; median, 21.2; range, 2.4 to 305.9 μmol/L) revealed that at diagnosis, only the presence of *NPM1* mutation had a significant impact on outcome, because it predicted better overall survival (OS; $P = .005$) although not PFS (log-rank test $P = .1$; Appendix Fig A4, online only). The impact of *NPM1* on OS remained significant after adjustment for age (age as continuous covariable: hazard ratio [HR], 0.26; 95% CI, 0.08 to 0.79; $P = .02$; Appendix Table A3, online only).

Postinduction serum samples were available for 31 of the 43 patients who achieved CR. Median total 2-HG and D to L ratio

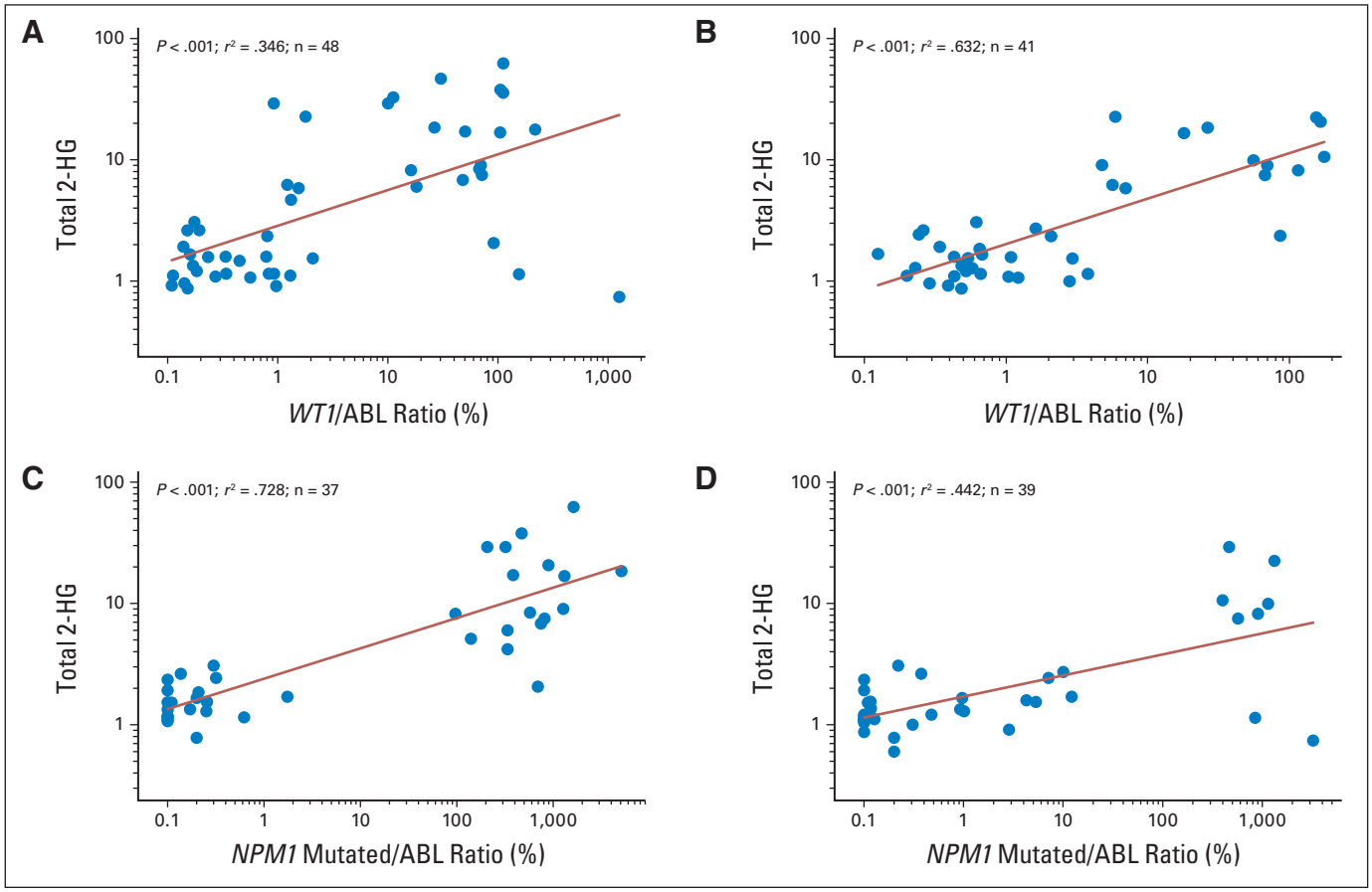

Fig 4. Correlation of total 2-hydroxyglutarate (2-HG; D plus L stereoisomers) level with minimal residual disease markers. Real-time quantitative polymerase chain reaction was used to monitor *WT1* expression level (relative to *ABL1*) in (A) blood and (B) bone marrow (BM) samples and estimate *NPM1* mutation copies (relative to *ABL1* copies) in (C) blood and (D) BM samples. Regression lines, associated *P* values, and r^2 values are indicated.

were 1.7 μmol/L (range, 0.7 to 25.9 μmol/L) and 1.88 (range, 0.7 to 23.2), respectively. In these 31 patients with *IDH1/2* mutations in CR, 15 relapsed, and four died (all after relapse). Univariate prognostic analyses of *IDH1* R132, *IDH2* R140Q, *FLT3-ITD*, and *NPM1* mutations and total 2-HG at CR revealed that only 2-HG level at CR had a significant impact on outcome (Table 2). This impact on PFS remained significant after adjustment for age (age as continuous covariable: HR, 4.37; 95% CI, 1.14 to 16.8; $P = .032$; Table 2). Patients with total 2-HG levels at CR < diagnostic cutoff (ie, < 2 μmol/L) had significantly better PFS ($P = .005$) and OS ($P = .008$; Fig 5). The diagnostic cutoff for the D to L ratio (2.5) at CR was not significantly prognostic of PFS ($P = .17$) or OS ($P = .13$).

Table 2. Prognostic Impact of 2-HG Level at CR in Patients With AML With *IDH1/2* Mutations

	PFS			OS*		
Variable	HR	95% CI	*P*	HR	95% CI	*P*
Total 2-HG (> 2 *v* ≤ 2 μmol/L)	**5.00**	**1.50 to 16.6**	**.009**	†		**.008**‡
Total 2-HG (> 2 *v* ≤ 2 μmol/L)§	**4.37**	**1.14 to 16.8**	**.032**			
D to L ratio (> 2.5 *v* ≤ 2.5)	2.53	0.84 to 7.58	.10	2.12	0.30 to 15.0	.45
NPM1	0.47	0.15 to 1.44	.19	0.25	0.03 to 1.77	.16
FLT3-ITD	1.09	0.36 to 3.32	.89	2.34	0.33 to 16.7	.40
IDH1 R132	2.31	0.29 to 18.5	.43	4.73	0.48 to 47.1	.18
IDH2 R140Q	0.52	0.16 to 1.67	.27	0.17	0.02 to 1.75	.14

NOTE. Univariate Cox regression analysis of PFS and OS. Bold font indicates significance.
Abbreviations: 2-HG, 2-hydroxyglutarate; AML, acute myeloid leukemia; CR, complete remission; HR, hazard ratio; OS, overall survival; PFS, progression-free survival.
*Not adjusted for age.
†HR impossible to estimate; no event in one of two groups.
‡Log-rank test.
§Age adjusted.

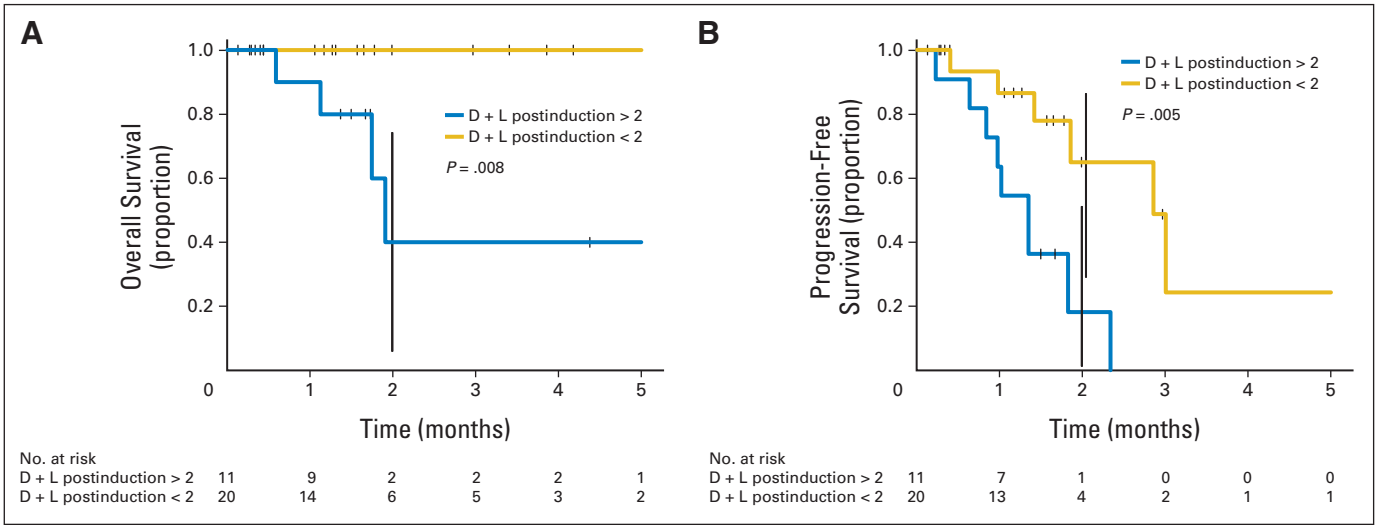

Fig 5. Kaplan-Meier survival curves of (A) overall and (B) progression-free survival for patients with serum levels of total 2-hydroxyglutarate > or < 2 μmol/L (blue *v* gold lines, respectively) at complete remission.

DISCUSSION

This study suggests that 2-HG released by blast cells in the serum is a biomarker of *IDH1/2* mutations, presumably reflecting the neomorphic enzymatic activity of the mutants. Total 2-HG level correlated with allelic burden and tumor mass. Consistently, 2-HG levels were significantly associated with clinical status (ie, CR *v* absence of CR) and correlated with available markers of MRD (ie, *NPM1* mutation or *WT1* overexpression). This suggests that serum 2-HG can serve as a marker of MRD. Patients who achieved CR with total 2-HG levels > diagnostic cutoff (ie, 2 μmol/L) had decreased PFS and OS.

Because 2-HG levels were elevated in the presence of low mutation burden, the biochemical assay for serum 2-HG may be an efficient, fast, and cost-effective complement to molecular analysis. In addition, serum 2-HG may be of particular interest in follow-up when standard molecular markers are not available (ie, presence of *IDH1/2* mutations but absence of *NPM1* mutations and/or normal *WT1* expression levels). This remains to be confirmed in future prospective studies. 2-HG levels may be informative beyond de novo AML (eg, *IDH1/2* mutations frequently occur in myelodysplastic syndromes and myeloproliferative diseases when they undergo transformation[28,29]).

As a national reference center for metabolic disorders, we extended the analysis to a reference population of patients with suspected or known metabolic conditions that could affect 2-HG production independent of *IDH1/2* mutations. This provided a framework to better appreciate the strength of the association between 2-HG and different clinical situations. We determined the cutoff of serum total 2-HG level (ie, 2 μmol/L) that detected all patients with AML with *IDH1/2* mutations while excluding the greatest fraction of patients and controls with normal IDH (thus achieving 89% specificity at 100% sensitivity). In addition, we reasoned that because only the D form is produced by *IDH1/2*-mutant enzymes,[1,27,30] specificity could further increase if we were to measure the level of the IDH-specific D form or ratio between D and the alternative L form rather than total 2-HG. Indeed, when we considered the entire sample of patients with AML and controls without AML, the D to L ratio (with cutoff at 2.5) provided better specificity than total 2-HG to predict *IDH1/2* mutations (96% *v* 89% at 100% sensitivity). D to L ratio actually led to complete separation between the two groups of patients with AML (*IDH1/2* mutations *v* *IDH1/2* wild type). The diagnostic performance of serum D stereoisomer alone was identical to that of the D to L ratio (data not shown). However, the D to L ratio has the additional advantage of being technically more reliable than measuring 2-HG concentrations (whether total or D stereoisomer), because its quantification does not require the addition of an internal standard. Therefore, we focused on the D to L ratio rather than the D stereoisomer as a complementary method to the more standard assay for total 2-HG.

Presence of *IDH1/2* mutations is relevant for prognosis and treatment of a subset of patients with AML.[13] Recent results further indicate that a small-molecule selectively inhibits 2-HG production by *IDH1* R132 mutation.[31] Therefore, we argue that total 2-HG can serve as a surrogate marker of treatment efficacy. In addition, our finding that total 2-HG level at CR was significantly predictive of outcome could be of particular interest for patients with a so-called favorable genotype (ie, *NPM1* mutations associated with *IDH1* or *IDH2* in absence of *FLT3-ITD*).[13] In these patients, elevated levels of total 2-HG after induction therapy could modify therapeutic options, notably making a stronger case for allogeneic stem-cell transplantation.

Finally, our data raise the possibility that additional metabolic markers could be useful in follow-up studies. Indeed, outcome was significantly predicted by total 2-HG, but not or only weakly by D to L ratio. We infer that other putative sources of metabolic dysfunction that involve the L form of 2-HG might affect outcome.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under

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Appendix

Table A1. Baseline Characteristics of Patients Without AML

Patient No.	Sex	Age (years)	FAB	WBC (g/L)	Circulating Blasts (%)	BM Blasts (%)	Caryotype	*IDH1* rs11554137	*IDH1*	*IDH2*	*FLT3*	*NPM1*	Other Mutation
1	F	63	M2	4.4	38	43	CN	Het	R132C		*FLT3-ITD*		
2	F	69	M1	1	11	69	CN		R132C				
3	M	60	M2	6.2	84	51	CN		R132C				
4	M	39	M6	0.89	20	13	CN		R132C				
5	M	74	M1	2.4	49	79	Trisomy 8		R132C				
6	M	34	M0	2.9	2	50	Trisomy 8	Het	R132C				
7	M	64	M1	2.4	20	85	CN	Het	R132C				
8	F	67	M1	1.4	1	85	CN		R132C			*NPM1*	
9	F	60	M1	10.01	96	93	CN	Het	R132C			*NPM1*	
10	F	53	M1	2	15	86	CN		R132C		*FLT3-ITD*	*NPM1*	
11	M	68	M1	1.6	26	72	CN		R132G		*FLT3-TKD*	*NPM1*	
12	F	67	M1	4.8	70	75	CN		R132G			*NPM1*	
13	M	48	M1	1.9	22	90	CN		R132G			*NPM1*	
14	F	71	M1	162	89	93	CN		R132H		*FLT3-ITD*		
15	F	47	M1	76.9	90	90	CN		R132H		*FLT3-ITD*	*NPM1*	
16	M	40	M5	178.6	90	95	CN		R132H		*FLT3-ITD*	*NPM1*	
17	F	49	M2	7.86	57	60	CN		R132H			*NPM1*	
18	M	53	M1	55.8	91	88	CN		R132H			*NPM1*	
19	M	32	M5	10.78	2	20	CN		R132H			*NPM1*	
20	F	46	M5	88.1	86	82	CN		R132H			*NPM1*	
21	M	53	M2	6.8	4	47	CN			R140L	*FLT3-TKD*	*NPM1*	
22	F	61	M4	11.8	30	60	CN			R140L		*NPM1*	
23	F	78	M5	15	70	67	CN			R140Q	*FLT3-ITD*		
24	M	68	M2	1	4	57	CN			R140Q			
25	M	75	M0	1.9	1	44	CN			R140Q			*RUNX1*
26	M	65	M5	17.6	3	22	CN			R140Q			*MLL* duplication
27	F	39	M1	118.9	94	86	CN			R140Q			*CEBPA*
28	F	54	M2	11.6	42	48	CN			R140Q			*MLL* duplication
29	F	23	M5	141	97	79	CN			R140Q	*FLT3-ITD*	*NPM1*	
30	M	53	M5	30.8	31	82	CN			R140Q	*FLT3-TKD*	*NPM1*	
31	M	59	M1	4	7	86	CN			R140Q	*FLT3-TKD*	*NPM1*	
32	F	40	M4	3.7	0	35	CN			R140Q		*NPM1*	
33	F	56	M5	30.1	9	52	CN			R140Q		*NPM1*	
34	F	70	M4	8.8	11	11	CN			R140Q		*NPM1*	
35	F	61	M2	2.8	2	45	CN			R140Q		*NPM1*	
36	M	55	M4	34.3	1	65	CN			R140Q		*NPM1*	
37	M	55	M2	2.3	5	52	CN			R140Q		*NPM1*	
38	F	63	M2	96	70	80	CN			R140Q	*FLT3-ITD*	*NPM1*	
39	F	57	M1	244	91	97	CN			R140Q	*FLT3-ITD*	*NPM1*	
40	M	47	M5	4.3	80	75	CN			R140Q	*FLT3-ITD*	*NPM1*	
41	F	41	M1	16.3	75	89	CN	Het		R140Q	*FLT3-ITD*	*NPM1*	
42	F	52	M5	1.3	68	64	CN	Het		R140Q		*NPM1*	
43	F	42	M5	25	60	20	CN			R140Q		*NPM1*	
44	M	42	M2	46.5	73	64	CN			R140W	*FLT3-TKD*	*NPM1*	
45	F	65	M1	18.6	82	89	CN			R172K	*FLT3-TKD*		
46	F	59	M1	28.3	50	61	CN			R172K			
47	F	59	M1	1.6	6	78	CN			R172K			
48	F	44	M1	34.2	83	91	CN			R172K			

(continued on following page)

Table A1. Baseline Characteristics of Patients Without AML (continued)

Patient No.	Sex	Age (years)	FAB	WBC (g/L)	Circulating Blasts (%)	BM Blasts (%)	Caryotype	*IDH1* rs11554137	*IDH1*	*IDH2*	*FLT3*	*NPM1*	Other Mutation
49	M	53	M1	17.2	70	75	Trisomy 10			R172K			
50	M	49	M1	1.2	2	60	CN			R172K			
51	M	26	M0	4.9	80	87	CN			R172K			
52	M	65	M0	27.3	84	83	CN			R172K			
53	F	62	M2	1.8	26	55	CN	Het		R172K			
54	M	72	NC	44	1	19	CN	Hom	V71I				
55	M	63	NC	11	20	86	CN				*FLT3-TKD*		
56	F	70	M1	1.2	1	74	Trisomy 8						
57	F	42	M4	44.7	59	39	CN						
58	F	46	M2	6.7	4	40	CN						
59	F	66	M5	76	68	60	CN						
60	F	55	M0	2.262	58	87	CN						
61	M	22	M5	134.9	95	84	CN						
62	M	37	M4	250	81	60	CN						
63	M	60	M1	2.9	4	16	CN						
64	M	61	M6	1.2	10	44	CN						
65	M	58	M5	3.9	15	62	Tetrasomy 8						
66	M	63	M1	1.6	3	37	CN						
67	M	73	M2	1.4	4	15	Y loss						
68	M	73	NC	3.7	9	20	Trisomy 8						
69	M	69	M0	44.4	94	92	CN						
70	F	57	M4	10.6	39	39	CN	Het					
71	M	77	NC	1.7	0	26	CN	Het					
72	F	70	M5	71	32	20	ND					*NPM1*	
73	M	78	M1	19.4	74	88	Monosomy 8					*NPM1*	
74	M	85	M4	5.6	26	82	CN	Het				*NPM1*	
75	M	48	M2	1.1	4	38	CN					*NPM1*	
76	M	52	M4	80.9	63	74	CN				*FLT3-ITD*	*NPM1*	
77	M	43	M5	1.9	57	61	CN				*FLT3-ITD*	*NPM1*	
78	F	63	M5	181.9	87	85	CN	Het			*FLT3-ITD*	*NPM1*	
79	F	46	M5	63.6	75	54	CN					*NPM1*	
80	F	69	M5	14.2	86	84	CN					*NPM1*	
81	F	51	M4	66	79	76	CN					*NPM1*	
82	M	71	M1	138	97	93	CN					*NPM1*	

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; CN, cytogenetically normal; FAB, French-American-British; het, heterozygous; hom, homozygous; NC, not classified; ND, not determined.

Table A2. Detailed Clinical Characteristics of Patients Without AML

Characteristic	Metabolic Population (No.)	Nonmetabolic Population (No.)
Total No. of patients	18	50
Age, years		
Median	17	41
Range	0.5-56	19-73
Sex		
Male	8	28
Female	10	22
Organic acidurias*	8	
Aminoacidopathies†	5	
Energy metabolism deficiencies‡	5	
Unexplained neurologic disorders§		34
Various disorders‖		16

Abbreviation: AML, acute myeloid leukemia.
*Including succinic semialdehyde dehydrogenase deficiency, glutaric aciduria type 1, propionic and isovaleric aciduria, and alkaptonuria.
†Including ornithine transcarbamoylase deficiency, arginase deficiency, lysinuric protein intolerance, cystathione beta-synthetase deficiency, and phenylketonuria.
‡Including pyruvate dehydrogenase deficiency and respiratory chain complex IV and I deficiency.
§Including cognitive impairment, pyramidal and or extrapyramidal symptoms, epilepsy, and hypotonia.
‖Including lethargy, vomiting, malnutrition, hypoglycemia, metabolic acidosis, ketoacidosis, hyperammonemia, microcephaly, and disorders of neural development.

Table A3. Prognostic Impact of 2-HG at Diagnosis in Patients With AML With *IDH1/2* Mutations

Variable	PFS			OS		
	HR	95% CI	*P*	HR	95% CI	*P*
D plus L > median	0.78	0.34 to 1.80	.56	1.04	0.32 to 3.34	.95
D to L ratio > median	0.62	0.27 to 1.44	.26	1.01	0.31 to 3.24	.99
NPM1	0.56	0.25 to 1.23	.15	0.26	0.08 to 0.79	**.02**
FLT3-ITD	1.09	0.45 to 2.62	.85	1.94	0.51 to 7.36	.33
FLT3-TKD	0.45	0.10 to 1.93	.28	0.93	0.20 to 4.24	.92
IDH1 R132	0.53	0.25 to 1.15	.11	0.51	0.19 to 1.37	.18
IDH2 R140Q	0.81	0.38 to 1.72	.58	1.85	0.59 to 5.75	.29
IDH2 R172K	1.44	0.50 to 4.19	.50	0.63	0.20 to 1.96	.20

NOTE. Univariate Cox regression analysis for PFS and OS. Continuous adjustment for age. Bold font indicates significance.
Abbreviations: 2-HG, 2-hydroxyglutarate; AML, acute myeloid leukemia; HR, hazard ratio; OS, overall survival; PFS, progression-free survival.

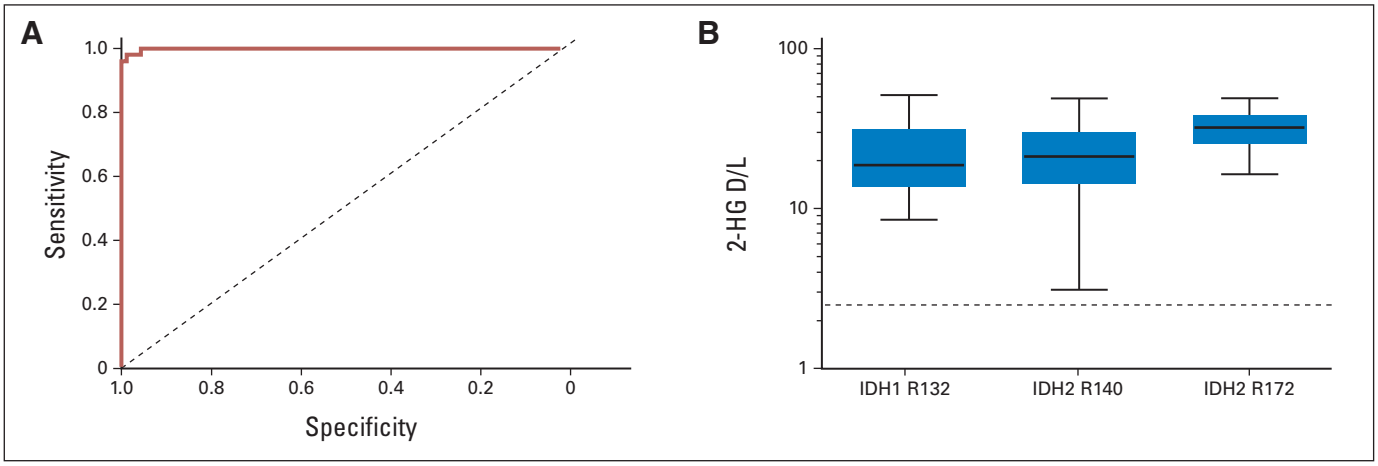

Fig A1. (A) Areas under receiver operator characteristic curve for 2-hydroxyglutarate (2-HG) and D to L ratio for patients with and without acute myeloid leukemia (AML) at diagnosis (n = 150). (B) Comparison of D to L ratio in serum from patients with AML at diagnosis with different mutation sites: *IDH1* R132 (n = 20), *IDH2* R140 (n = 24), and *IDH2* R172 (n = 9).

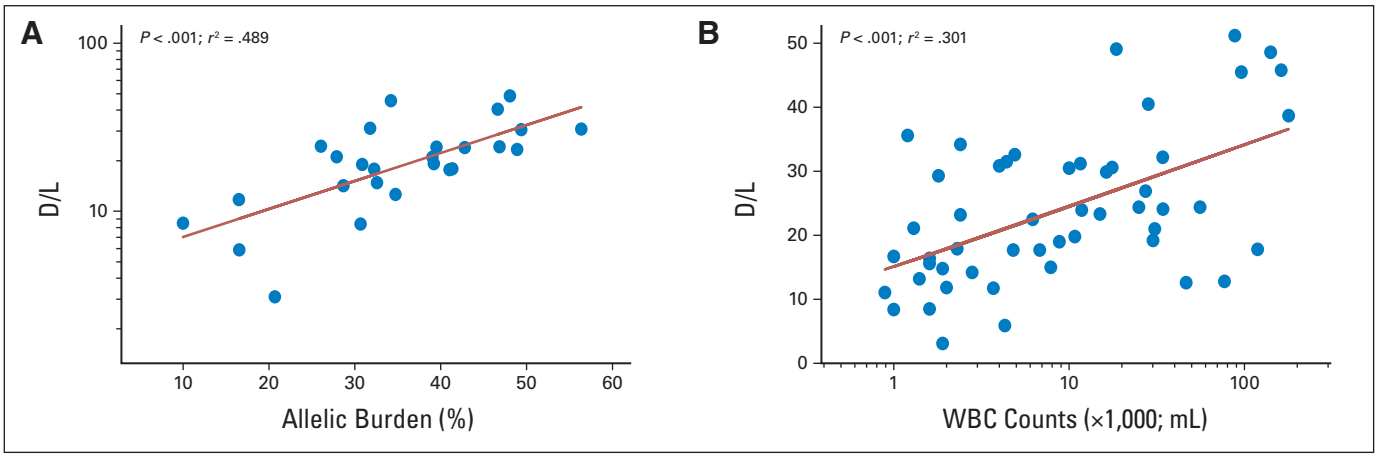

Fig A2. Correlation of D to L ratio with (A) mutation burden and (B) WBC count. Regression lines, associated *P* values, and r^2 values are indicated.

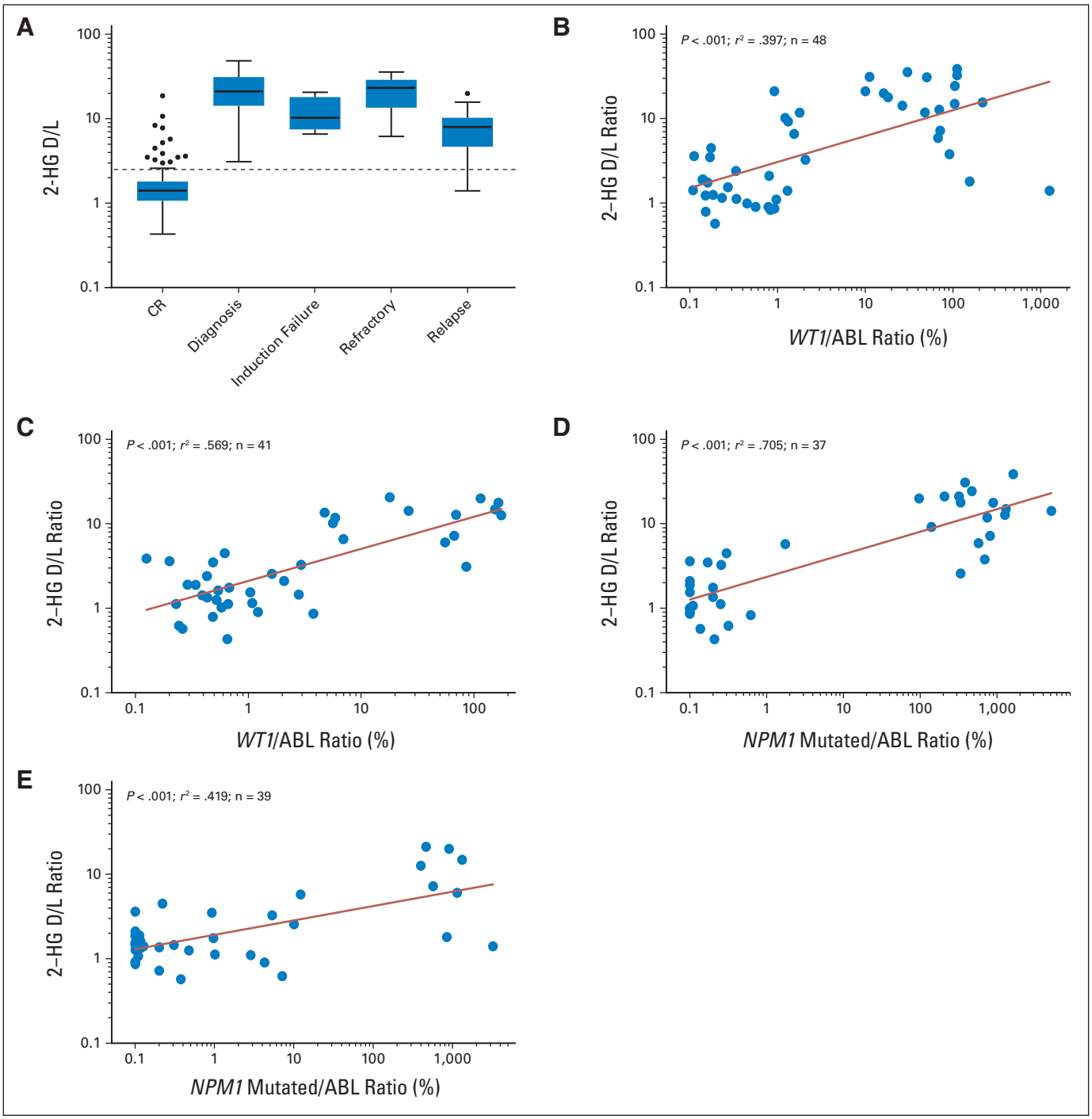

Fig A3. Correlation of D to L level with (A) clinical status and (B) minimal residual disease markers. Real-time quantitative polymerase chain reaction was used to estimate *NPM1* mutation copies (relative to *ABL1* copies) and monitor *WT1* expression level (relative to *ABL1* expression level) in blood and bone marrow (BM) samples. Regression lines, associated *P* values, and r^2 values are indicated. Induction failure indicates no achievement of complete remission (CR) after one induction course. (A) Clinical status. *WT1* expression in (B) blood or (C) BM. *NPM1* copy numbers in (D) blood and (E) BM.

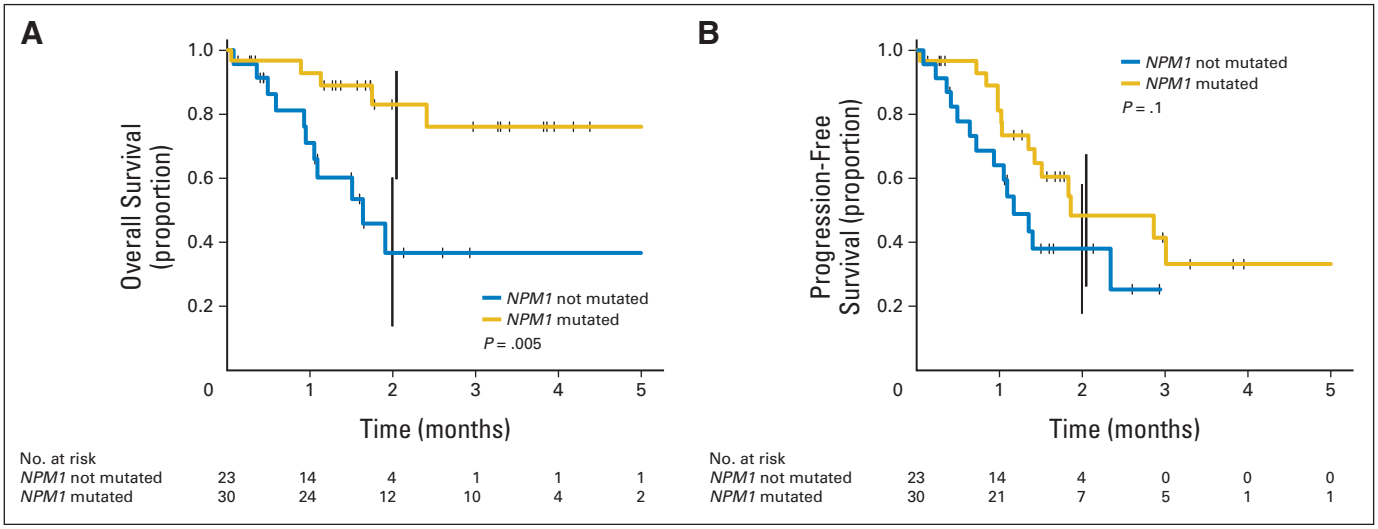

Fig A4. Kaplan-Meier survival curves for 53 patients with acute myeloid leukemia with *IDH* mutations with (n = 30) or without (n = 23) *NPM1* mutations (gold *v* blue lines, respectively). (A) Overall survival; (B) progression-free survival.