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Prognostic relevance of variant allelic frequency for treatment outcomes in patients with acute myeloid leukemia: a study by the Spanish PETHEMA registry

Authors

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Running heads: "Prognostic relevance of variant allelic frequency for treatment outcomes in patients with AML"

Data-sharing statement

For data sharing please email to Pau Montesinos (montesinos_pau@gva.es) coordinator of the PETHEMA AML group

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Contributions

RC, NA, RA and PM conceived and designed the study. RC and RA wrote the paper. RC, NA, CMA, AAB and RA performed statistical analyses. All authors discussed the results, contributed to the final manuscript and approved the version to be published.

Disclosures

The authors declare no conflicts of interest.

Acute myeloid leukemia (AML) is a heterogeneous pathology in terms of its cytogenetic and molecular alterations, which are used for prognostic stratification and as therapeutic targets(1–3). Some studies have shown the negative impact of a high allelic burden at diagnosis regarding the mutations of some genes (*EZH2*, *SRSF2*, *TP53*) on the evolution of AML(4–6). The most studied gene is TP53; different variant allele frequencies (VAF) thresholds (i.e., 10% or 40%)) at diagnosis could have an impact on patient outcomes(7,8). Although the mutational burden, according to VAF measurements, has been associated with the prognosis of these patients, this parameter is not well established for risk stratification. In this study, we analyzed the impact of the mutational burdens for gene variants detected with a myeloid panel via NGS in a cohort of AML patients included in a large epidemiological registry of the "Programa Español para el Tratamiento de las Hemopatías Malignas" (Pethema) (ClinicalTrials.gov Identifier: NCT02607059), focusing on overall survival (OS).

This was a non-interventional, systematic, retrospective chart review of data from patients enrolled in the Pethema registry, which included patients diagnosed with AML, regardless of the treatment administered. This study was conducted in a cohort of 3,018 adult patients with AML who were diagnosed between 2003 and 2021 and underwent testing with an NGS panel; these patients were diagnosed in 108 centers belonging to Pethema cooperative group. The study was approved by a formally constituted review board. The samples were obtained at diagnosis, refractoriness, and relapse; the comprehensive mutational profile of this cohort was published previously (Sargas et al(3)). The patients were assigned to therapeutic groups based on the front-line approach: intensive chemotherapy (IC), non-intensive chemotherapy (non-IC) such as hypomethylating agents or low-dose cytarabine schemes; patients who received venetoclax-based schedules were excluded due to the low number of patients. The mutational profiles were determined in seven Spanish Pethema reference laboratories, which were instructed to use NGS to use NGS to assess the mutational status of genes that define diagnosis and prognosis as well as guide treatment options (*ASXL1*, *BCOR*, *CEBPA*, *EZH2*, *FLT3*, *IDH1*, *IDH2*, *NPM1*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, *ZRSR2*, and *TP53*). Moreover, there was a recommendation for the study of other genes with proven evidence for their relevance in AML pathogenesis (*ABL1*, *BRAF*, *CALR*, *CBL*, *CSF3R*, *DNMT3A*, *ETV6*, *GATA2*, *HRAS*, *JAK2*, *KIT*, *KRAS*, *MPL*, *NRAS*, *PTPN11*, *SETBP1*, *TET2*, and *WT1*). NGS methods were harmonized and periodically validated across centers (3,9). Using the single-nucleotide polymorphism database (NCBI, dbSNP150), variants with VAFs less than 0.01 in the general population were discarded. Other databases used to search the filtered variants were the Catalogue of Somatic Mutations in Cancer (COSMIC) and VarSome.

All the statistical analyses were performed using SPSS version 22 (IBM, Armonk, NY, USA) and Stata InterCooled for Windows version 16 (StataCorp LLC, 2019); statistical significance was considered at a p-value less than or equal to 0.05. A Chi-square test was used to assess the associations between categorical variables, and median test, Student's t-test, and ANOVA test were performed to compare differences in the median and mean values of continuous variables. The analysis was performed using the VAF as a continuous variable (in those genes without mutations, the resulting value of the variable is 0). The VAF was expressed as a percentage of one. The prognostic impacts of the mutational burdens of gene variants were analyzed with respect to the type of leukemia treatment received. Cox proportional hazard

models were used to assess the association of variables (clinical data and mutational load) with the patients LFS and OS. For multivariate analyses, we adjusted for patient age (continuous variable) and VAF gene mutations (1% increments). Mixed regression models combine fixed and random effects to analyze correlated data. In this study, we used mixed-effects ML (machine learning) regression to account for patient heterogeneity by treating patients as random factors and assess the impact of variant allele frequencies on survival, considering gene mutations, death, and relapse as fixed factors; this approach allowed for efficient analysis of multiple gene mutations per patient. The receiver operating characteristic (ROC) curve was constructed under the nonparametric assumption, and analysis was performed to identify the cutoff score that would assist in distinguishing between live and dead patients for each gene.

Among the 3,018 samples analyzed (Figure 1A), 2,464 samples were from patients at first AML diagnosis (81.6%), and the remaining 554 samples were from 473 patients at relapse/refractory episodes. The most frequently mutated gene was *DNMT3A* (24.3%), followed by *NPM1* (22.5%), *TET2* (21.2%), and *RUNX1* (18.8%).

In the diagnosis group (2,464 patients), the median age at first AML diagnosis was 67 years (range: 18-98). Patients received front-line intensive chemotherapy schemes (55.6%), hypomethylating treatment with a single agent (27.1%) or LDAC-based treatments (14.9%). In patients who received intensive chemotherapy schedules, 70.3% achieved CR and 36.2% received alloHSCT. The risk classification according to ELN2017 was favorable in 15.0% of cases, intermediate in 34.0%, and adverse in 51.1%. OS and LFS analyses were performed among 2,464 patients at initial diagnosis; the median OS (1,381 patients) was 12.6 months (95% CI: 11.4-13.7 months) and the median LFS (1,137 patients) was 10.1 months (95% CI: 9.3-10.9 months). The CR rate in the diagnosis group was 49.1% (487/991 patients).

The patient's age; leukocyte count, and low mutational loads for some genes, such as *ASXL1*, *FLT3*, *RUNX1* or *TP53*, or high mutational loads for *DNMT3A* or *NPM1* were associated with achieving a complete response (CR). In the multivariate logistic regression model obtained, a higher age of the patient (OR 0.935 (0.913-0.958), p<0.001) and higher mutational load for the *SRSF2* gene (OR 0.978 (0.967-0.990), p<0.001) were associated with a lower probability of achieving a CR. However, a higher mutational load for *NPM1* (OR 1.025 (1.007-1.043), p<0.001) was associated with a greater chance of achieving a CR.

To avoid negative cases impacting the analyses of the VAF effect on OS, we carried out a mixed-effects ML regression (Table S3). We observed that increased allelic loads for *ASXL1* (OR 1.317, 95% CI 0.084-2.550, p=0.036), *FLT3* (OR 1.382, 95% CI 0.148-2.615, p=0.028), *JAK2* (OR 1.400, 95% CI 0.167-2.633, p=0.026), *RUNX1* (OR 2.215, 95% CI 0.982-3.448, p<0.001), *SRSF2* (OR 3.263, 95% CI 2.030-4.496, p<0.001), *TET2* (OR 2.662, 95% CI 1.429-3.896, p<0.001), *TP53* (OR 4.712, 95% CI 3.479-5.946, p<0.012), and *U2AF1* (OR 1.270, 95% CI 0.036-2.503, p=0.044) were associated with an adverse prognosis for OS; however, an increase in *NPM1* burden conferred a good prognosis (OR -2.417, 95% CI -3.651--1.184, p<0.001). The results were obtained in comparison with those for the *ABL1* mutation load; any differences observed when compared with some previous results were associated with the comparative gene, but *SRSF2, TP53*, and *NPM1* were consistent in all analyses. This model for LFS was not significant.

To facilitate the application of results in clinical practice, we attempted to determine a cutoff for each gene to define changes in OS; different optimal cutoff points were obtained, namely *ASXL1* (VAF 0.475), *JAK2* (VAF 0.038), *RUNX1* (VAF 0.043), *SRSF2* (VAF 0.028), *TET2* (VAF 0.030), and *TP53* (VAF 0.024). This confirmed statistically significant differences, with a better OS associated with a low VAF for all genes (*ASXL1*: low VAF vs. high VAF, 15.84 vs. 13.51 months, p=0.025; *JAK2*: 15.87 vs. 10.10 months, p<0.001; *SRSF2*: 16.16 vs. 12.49 months, p<0.001; *TET2*: 17.02 vs. 10.69 months, p<0.001; *TP53*: 17.21 vs. 6.95 months, p<0.001), with the exception of *RUNX1* (15.41 vs. 16.03 months, p=0.789), for which the results were not significant.

We also evaluated the impact of 1% increases in the mutational load on the risk of death (OS) and relapse (LFS) in the group of patients treated with intensive regimens for OS (n=467 patients with complete data set) (Table 1 and Figure 1B). In a multivariate analysis, we observed a worse OS in older patients (HR 1.04, p<0.001) or patients with a higher leukocyte count (HR 1.04, p<0.001); in addition, we observed that higher VAFs for *BRAF* (HR 1.04, p=0.009), *EZH2* (HR 1.03, p=0.005), *KRAS* (HR 1.05, p<0.001), *SRSF2* (HR 1.02, p=0.006), *TP53* (HR 1.02, p<0.001), and *U2AF1* (HR 1.02, p=0.009) were associated with a worse OS, and a higher VAF for *IDH1* was associated with a better OS (HR 0.98, p=0.03). Regarding LFS (n=466 patients with complete data set) (Table 2), in the multivariate analysis, we observed a worse LFS with higher VAFs for *ASXL1* (HR 1.02, p=0.016) and *CALR* (HR 1.02, p=0.033), and a better LFS with a higher VAF for *IDH2* (HR 0.98, p=0.033). *EZH2* is a transcriptional regulation gene, and *U2AF1* is a splicing factor gene; both are related to dysplasia and are included in the adverse risk category in ELN2022 classification. An association between a higher *EZH2* clonal burden and a worse LFS has been reported previously(5); however, to our knowledge, the relationship between a high *U2AF1* VAF and worse outcome has not been reported before. To our knowledge, no study has shown that patients with a high *CALR* VAF have a worse OS or LFS; this could be related to acute leukemias secondary to chronic myeloproliferative neoplasms, which have a worse evolution than de novo AML.

In the LDAC group, regarding OS (n=158 patients with complete data set), a higher age (HR 1.06, p=0.002), higher leukocyte count (HR 1.01, p<0.001), and higher VAFs for *BRAF* (HR 1.10, p=0.008), *CBL* (HR 1.07, p=0.016), *DNMT3A* (HR 1.01, p=0.015), and *TP53* (HR 1.01, p<0.001) were associated with poor outcomes. In the hypomethylating agent group, regarding OS (n=227 patients with complete data set), higher VAFs of *CBL* (HR 1.01, p=0.03) and *TP53* (HR 1.01, p<0.001) were identified as poor risk factors for OS, as well as a higher blast count in bone marrow (HR 1.01, p=0.011). In patients receiving LDAC, splicing factors were not detected as having an impact on OS; in patients who received hypomethylating treatment, epigenetic factors were not detected as having a prognostic impact. These differences have not been previously described and could be related to the type of treatment received; in previous studies, adding venetoclax to LDAC may mitigate the poor prognosis of splicing mutations, or hypomethylating agents may eliminate the prognostic impact of genes involved in epigenetic pathways(10).

Our results are consistent with the already known results, with a negative impact of the *TP53* VAF on OS in the global cohort and in each one of the three treatment sub-groups. Previously, Short et al. established a 0.40 VAF threshold, showing a better OS in low-*TP53* VAF patients

treated with a cytarabine-based regimen(7); other studies have shown similar results although it is difficult to establish a threshold (5,8,11–13).

In summary, our results show that mutation allele burden of certain signaling (FLT3, JAK2), transcription factors (RUNX1), epigenetic (*ASXL1*, *TET2*), and splicing (*SRSF2* and *U2AF1*) genes, in addition to *TP53*, worsen OS survival in AML patients. Also, we determined a specific prognostic cut-off for each of those genes. More studies are needed to confirm our results and further establish the prognostic or predictive value of the allele burden in AML patients.

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Tables

Table 1. Overall survival: multivariate analyses patients at diagnosis in each group. Biomarkers identified with an adjusted cox regression analysis (model; P≤0.05), were included in the table. Cox regression model was adjusted for age, gender and gene VAF detected in the panel. Age and VAFs were analyzed by continuous variable. CI: confidence interval. HR: hazard ratio. OS: overall survival. VAF: variant allele frequency.

OS (PATIENTS AT DIAGNOSIS, INTENSIVE CHEMOTHERAPY), N=671				
	N	**HR**	**95% CI**	**p-value**
AGE	467	1.04	1.02, 1.05	**<0.001**
LEUKOCYTE COUNT	467	1	1.00, 1.01	**0.014**
***BRAF* VAF**	467	1.04	1.01, 1.06	**0.009**
***EZH2* VAF**	467	1.03	1.01, 1.05	**0.005**
***IDH1* VAF**	467	0.98	0.96, 1.00	**0.03**
***KRAS* VAF**	467	1.05	1.02, 1.07	**<0.001**
***SRSF2* VAF**	467	1.02	1.00, 1.03	**0.006**
***TP53* VAF**	467	1.02	1.02, 1.03	**<0.001**
***U2AF1* VAF**	467	1.02	1.01, 1.04	**0.002**
OS (PATIENTS AT DIAGNOSIS, HYPOMETHYLATING AGENTS), N=327				
	N	**HR**	**95% CI**	**p-value**
BM BLAST %	227	1.01	1.00, 1.02	**0.011**
***CBL* VAF**	227	1.01	1.00, 1.03	**0.03**
***TP53* VAF**	227	1.01	1.01, 1.02	**<0.001**
OS (PATIENTS AT DIAGNOSIS, LDAC), N=181				
	N	**HR**	**95% CI**	**p-value**
AGE	158	1.06	1.02, 1.09	**0.002**
LEUKOCYTE COUNT	158	1.01	1.01, 1.02	**<0.001**
***BRAF* VAF**	158	1.10	1.03, 1.18	**0.008**
***CBL* VAF**	158	1.07	1.01, 1.13	**0.016**
***DNMT3A* VAF**	158	1.01	1.00, 1.02	**0.015**
***TP53* VAF**	158	1.01	1.01, 1.02	**<0.001**

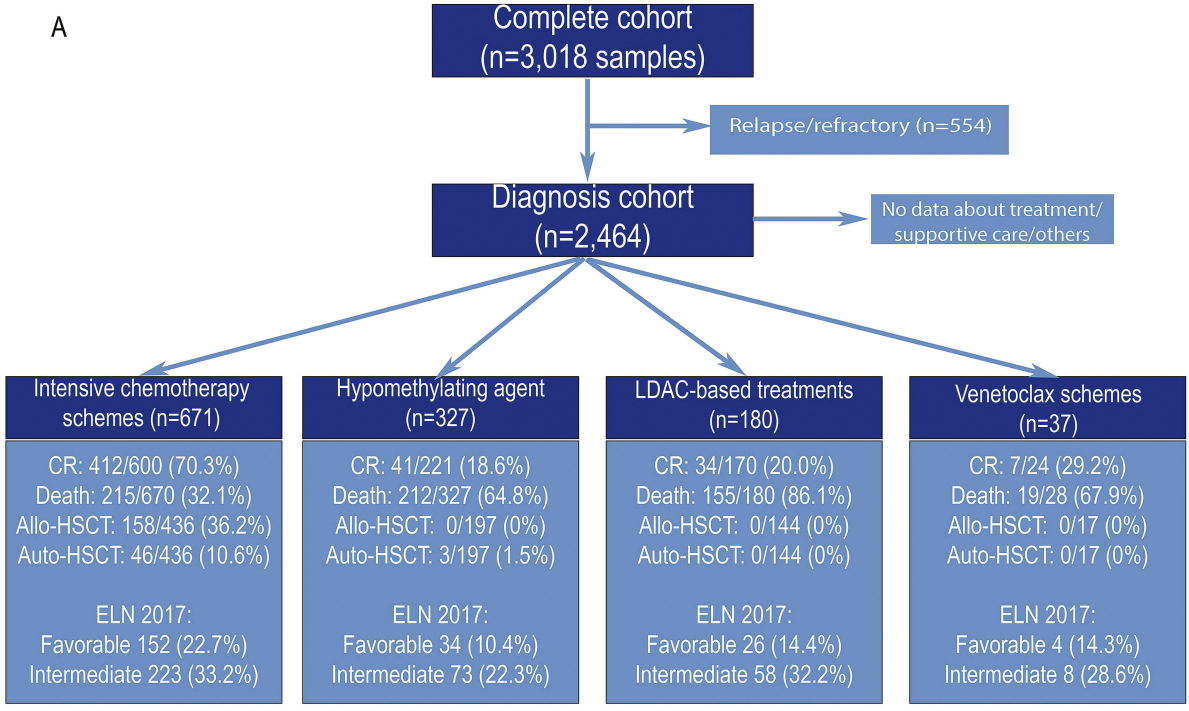
Table 2. Leukemia Free Survival: multivariate analyses patients at diagnosis in each group. Biomarkers identified with an adjusted cox regression analysis (model; P≤0.05), were included in the table. Cox regression model was adjusted for age, gender and gene VAF detected in the panel. Age and VAFs were analyzed by continuous variable. CI: confidence interval. HR: hazard ratio. LFS: leukemia-free survival. VAF: variant allele frequency.

LFS (PATIENTS AT DIAGNOSIS, INTENSIVE CHEMOTHERAPY), N=671				
	N	**HR**	**95% IC**	**p-value**
ASXL1 **VAF**	466	1.02	1.00, 1.03	**0.016**
CALR **VAF**	466	1.02	1.00, 1.05	**0.033**
IDH2 **VAF**	466	0.98	0.97, 1.00	**0.033**
LFS (PATIENTS AT DIAGNOSIS, HYPOMETHYLATING AGENTS), N=327				
	N	**HR**	**95% IC**	**p-value**
BM BLAST %	227	0.98	0.97, 0.99	**<0.001**
CBL **VAF**	227	1.04	1.02, 1.07	**<0.001**
DNMT3A **VAF**	227	1.01	1.00, 1.03	**0.049**
EZH2 **VAF**	227	1.02	1.01, 1.04	**0.002**
NPM1 **VAF**	227	1.04	1.03, 1.06	**<0.001**
TP53 **VAF**	227	1.01	1.00, 1.02	**0.004**
LFS (PATIENTS AT DIAGNOSIS, LDAC), N=181				
	N	**HR**	**95% IC**	**p-value**
LEUKOCYTE COUNT	158	1.01	1.00, 1.02	**0.006**
DNMT3A **VAF**	158	1.02	1.00, 1.04	**0.038**
JAK2 **VAF**	158	1.03	1.00, 1.05	**0.033**
SRSF2 **VAF**	158	1.02	1.01, 1.03	**0.007**
WT1 **VAF**	158	1.03	1.00, 1.05	**0.024**

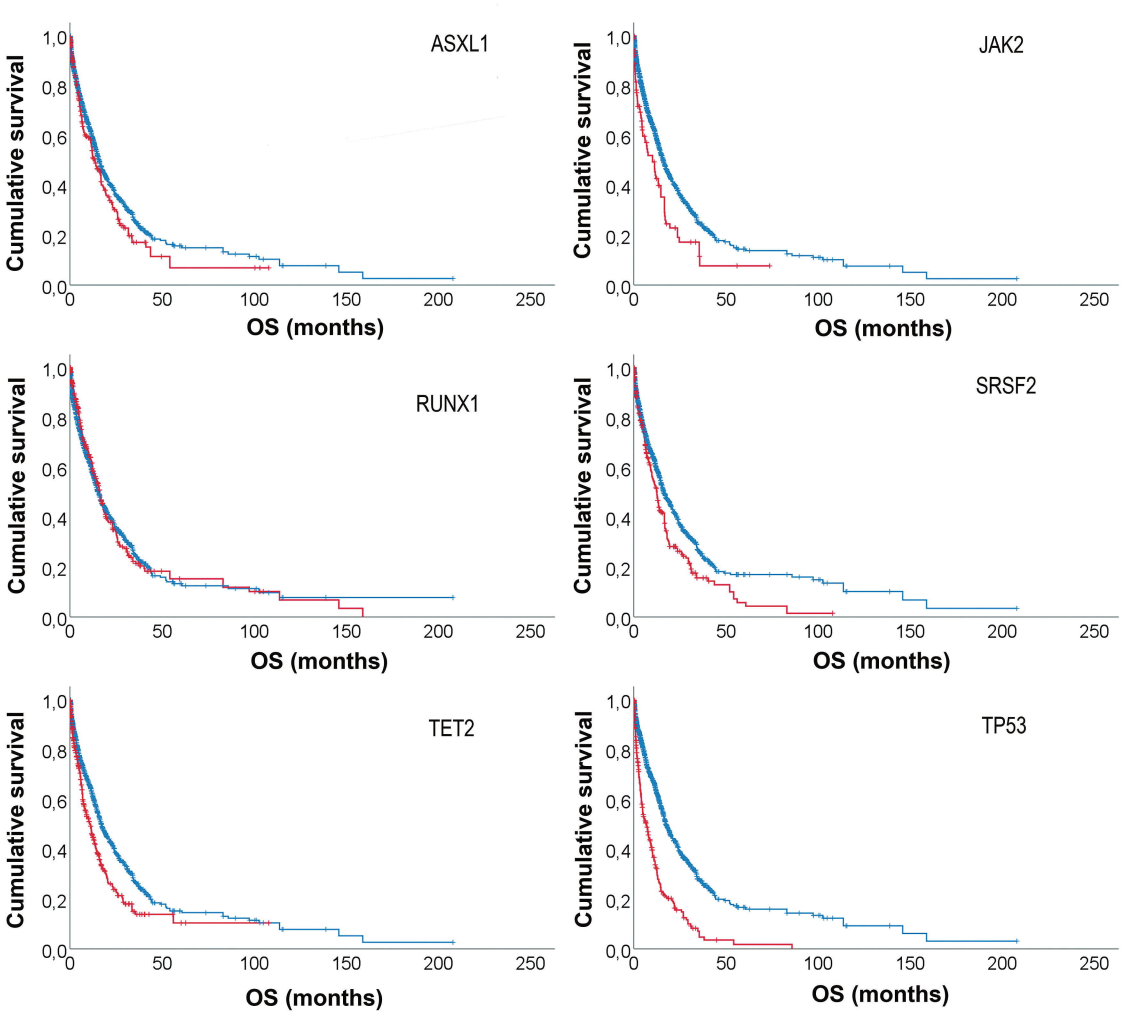
Figure

Figure 1. Study design and Kaplan-Meier curve of overall survival depending on the cut-off of some genes. A. Diagram showing the study design. Allo-SCT: allogeneic hematopoietic stem-cell transplantation. Auto-HCST: Autologous hematopoietic stem-cell transplantation. CR: complete remission. HSCT: European LeukemiaNet. **B. Kaplan-Meier curve of overall survival, depending on the cut-off of *ASXL1*, *JAK2*, *RUNX1*, *SRSF2*, *TET2* and *TP53*.** The entire diagnostic cohort is represented. Blue shows patients with VAF (variant allele frequency) below cutoff and red shows patients with VAF above cutoff.

A


Complete cohort
(n=3,018 samples)
Relapse/refractory (n=554)
Diagnosis cohort
(n=2,464)
No data about treatment/
supportive care/others
Intensive chemotherapy schemes (n=671)
CR: 412/600 (70.3%)
Death: 215/670 (32.1%)
Allo-HSCT: 158/436 (36.2%)
Auto-HSCT: 46/436 (10.6%)
ELN 2017:
Favorable 152 (22.7%)
Intermediate 223 (33.2%)
Hypomethylating agent (n=327)
CR: 41/221 (18.6%)
Death: 212/327 (64.8%)
Allo-HSCT: 0/197 (0%)
Auto-HSCT: 3/197 (1.5%)
ELN 2017:
Favorable 34 (10.4%)
Intermediate 73 (22.3%)
LDAC-based treatments (n=180)
CR: 34/170 (20.0%)
Death: 155/180 (86.1%)
Allo-HSCT: 0/144 (0%)
Auto-HSCT: 0/144 (0%)
ELN 2017:
Favorable 26 (14.4%)
Intermediate 58 (32.2%)
Venetoclax schemes (n=37)
CR: 7/24 (29.2%)
Death: 19/28 (67.9%)
Allo-HSCT: 0/17 (0%)
Auto-HSCT: 0/17 (0%)
ELN 2017:
Favorable 4 (14.3%)
Intermediate 8 (28.6%)

B


ASXL1
JAK2
RUNX1
SRSF2
TET2
TP53
Cumulative survival
OS (months)
0,0
0,2
0,4
0,6
0,8
1,0
0
50
100
150
200
250

Supplemental material

Prognostic relevance of variant allelic frequency for treatment outcomes in patients with acute myeloid leukemia: a study by the Spanish PETHEMA registry

R Colmenares et al.

Table S1: Main characteristics of the diagnosis cohort. ECOG: Eastern Cooperative Oncology Group performance status. ELN2017: European LeukemiaNet. LDAC: low-dose cytarabine. Intensive treatments included '7+3' schemes. Low-dose cytarabine treatments contained cytarabine 70-200 mg/m2 daily as a bolus or infusion for 2-5 days, sometimes in combination with fludarabine in the FLUGA scheme. Patients treated in the hypomethylating group received it as monotherapy, without venetoclax.The type of induction treatment received was only available in 1,178 cases.

		TOTAL	INTENSIVE CHEMOTHERAPY	HYPOMETHYLATING AGENT	LDAC
		N=2,464	N=671	N=327	N=180
GENDER (MEN, (%)) N=2,464		1,399 (56.8%)	365 (54.4%)	101 (56.1%)	188 (57.5%)
AGE AT DIAGNOSIS (YEARS, MEDIAN (RANGE)) (N=2,464)		67 (18-98)	58 (18-78)	76 (64-96)	75 (45-98)
ECOG (N=1,299)	0	529 (40.7%)	271 (49.9%)	77 (26.6%)	67 (39.4%)
	1	527 (40.6%)	218 (40.1%)	132 (45.7%)	67 (39.4%)
	2	160 (12.3%)	36 (6.6%)	60 (20.8%)	25 (14.7%)
	3	63 (4.8%)	12 (2.2%)	20 (6.9%)	8 (4.7%)
	4	20 (1.5%)	6 (1.1%)	0 (0.0%)	3 (1.8%)
BLAST AT DIAGNOSIS IN BONE MARROW (%, MEDIAN (RANGE)) N=990		35 (3-100)	37 (3-100)	30 (5-96)	26,5 (4-98)
LEUKOCYTE COUNT AT DIAGNOSIS (10^9/L, MEDIAN (RANGE)) N=1,319		8.1 (0.3-374)	8.9 (0.3-374)	12.7 (0.4-284)	4.9 (0.6-219)
GENETIC RISK (ELN 2017) N=2,464	Favorable	369 (15.0%)	152 (22.7%)	34 (10.4%)	26 (14.4%)
	Intermediate	837 (34.0%)	223 (33.2%)	73 (22.3%)	58 (32.2%)
	Adverse	1,258 (51.1%)	296 (44.1%)	220 (67.3%)	96 (53.3%)
PRIMARY OR SECONDARY AML	Primary	905 (64.7%)	450 (74.9%)	174 (61.3%)	113 (63.5%)
	Secondary	494 (35.3%)	151 (25.1%)	110 (38.7%)	65 (36.5%)

Table S2. Mutational frequency according to diagnosis, age, gender, and ELN 2017 classification. These results correspond to all samples (diagnosis, refractoriness, and relapse). ELN: European leukemia-net. VAF: variant allele frequency.

						VAF BY AGE			GENDER			PRIMARY OR SECONDARY			VAF BY ELN2017 CLASSIFICATION			
	Mutated patients		Median	Mean	Range VAF	≤ 65 years	> 65 years	P-value	Male	Female	P-value	Primary	Secondary	P-value	Favorable	Intermediate	Adverse	P-value
ABL1	12	0.40%	0.49	0.48	0.35-0.53	0.5	0.47	0.164	0.5	0.47	0.183	0.5	0.49	0.308	0.48	0.48	0.51	0.236
ASXL1	440	14.60%	0.43	0.38	0.01-0.90	0.35	0.39	**0.011**	0.38	0.37	0.575	0.38	0.39	0.426	0.39	0.38	0.36	0.635
BRAF	18	0.60%	0.46	0.37	0.03-0.55	0.38	0.37	0.884	0.41	0.34	0.493	0.43	0.24	0.797	0.03	0.46	0.49	**0.029**
CALR	55	1.80%	0.49	0.49	0.04-0.96	0.48	0.49	0.713	0.49	0.48	0.776	0.5	0.52	**0.014**		0.48	0.49	0.921
CBL	109	3.60%	0.34	0.35	0.01-0.97	0.37	0.33	0.331	0.33	0.38	0.398	0.34	0.34	0.101	0.18	0.4	0.32	0.199
CEBPA	184	6.10%	0.4	0.34	0.01-0.97	0.38	0.31	**0.018**	0.35	0.34	0.812	0.38	0.27	0.871	0.22	0.34	0.39	0.381
CSF3R	87	2.90%	0.46	0.38	0.02-0.89	0.39	0.37	0.578	0.42	0.34	**0.045**	0.36	0.44	0.341	0.49	0.42	0.4	0.886
DNMT3A	734	24.30%	0.44	0.41	0.01-0.98	0.41	0.41	0.836	0.42	0.41	0.174	0.4	0.42	0.465	0.42	0.4	0.42	0.484
ETV6	96	3.20%	0.44	0.38	0.03-0.97	0.38	0.38	0.943	0.39	0.35	0.348	0.4	0.36	**0.003**	0.39	0.35	-	0.375
EZH2	161	5.30%	0.46	0.45	0.01-0.99	0.34	0.49	**0.004**	0.46	0.44	0.786	0.41	0.51	0.835	0.07	0.43	0.46	0.453
FLT3	51	1.70%	0.31	0.32	0.02-0.76	-	0.32	-	-	0.32	0.713	0.31	0.35	0.818	0.32	0.3	-	0.199
FLT3-TKD	166	5.50%	0.16	0.2	0.01-0.62	0.21	0.2	0.765	0.2	0.21	0.934	0.21	0.18	0.927	0.06	0.26	0.14	**0.002**
FLT3-ITD	452	15.00%	0.26	0.28	0.01-0.99	0.3	0.26	0.069	0.27	0.29	0.228	0.3	0.26	0.194	0.26	0.31	0.29	0.814
GATA2	92	3.00%	0.34	0.31	0.01-0.98	0.3	0.31	0.86	0.3	0.31	0.759	0.3	0.33	0.976	-	0.32	0.35	0.616
IDH1	313	10.40%	0.41	0.34	0.01-0.76	0.35	0.33	0.424	0.34	0.34	0.715	0.35	0.32	**0.037**	0.46	0.33	0.34	0.251
IDH2	449	14.90%	0.43	0.4	0.01-0.96	0.37	0.42	**0.001**	0.39	0.4	0.539	0.39	0.42	0.412	0.42	0.39	0.41	0.701
JAK2	158	5.20%	0.46	0.38	0.01-0.99	0.41	0.37	0.324	0.39	0.37	0.632	0.35	0.43	0.306	0.51	0.39	0.33	0.623
KIT	102	3.40%	0.26	0.26	0.01-0.60	0.26	0.25	0.844	0.28	0.23	0.138	0.26	0.29	0.971	0.21	0.26	0.25	0.688
KRAS	222	7.40%	0.16	0.19	0.01-0.70	0.19	0.19	0.948	0.19	0.19	0.969	0.19	0.17	0.335	0.23	0.15	0.26	**0.003**
MPL	52	1.70%	0.46	0.37	0.01-0.91	0.38	0.35	0.529	0.32	0.4	0.194	0.37	0.44	0.554	0.48	0.33	0.47	0.169
NPM1	678	22.50%	0.37	0.35	0.01-0.69	0.34	0.36	**0.009**	0.35	0.35	0.653	0.35	0.34	0.122	0.34	0.34	0.36	0.06
NRAS	464	15.40%	0.21	0.23	0.01-0.94	0.23	0.25	0.145	0.24	0.22	0.16	0.23	0.26	0.498	0.24	0.25	0.26	0.9
PTPN11	170	5.60%	0.18	0.21	0.01-0.51	0.23	0.19	0.109	0.21	0.21	0.995	0.23	0.19	0.687	0.05	0.23	0.19	0.356
RUNX1	566	18.80%	0.41	0.39	0.02-0.99	0.38	0.4	0.2	0.4	0.37	0.173	0.41	0.39	0.826	0.29	0.4	0.4	0.674
SETBP1	98	3.20%	0.41	0.34	0.01-0.72	0.31	0.35	0.21	0.34	0.33	0.823	0.35	0.29	0.644	-	0.31	0.34	0.608
SF3B1	168	5.60%	0.41	0.35	0.02-0.51	0.33	0.36	0.214	0.35	0.34	0.43	0.34	0.36	0.811	0.48	0.33	0.38	0.241
SRSF2	486	16.10%	0.46	0.42	0.01-0.96	0.39	0.42	**0.008**	0.41	0.42	0.894	0.41	0.43	0.618	0.42	0.41	0.44	0.37
TET2	640	21.20%	0.46	0.43	0.01-0.99	0.4	0.44	**0.014**	0.42	0.44	0.098	0.43	0.44	0.217	0.27	0.43	0.42	0.262
TP53	509	16.90%	0.5	0.52	0.01-0.99	0.48	0.54	**0.029**	0.52	0.53	0.555	0.52	0.52	**0.036**	0.02	0.5	0.53	0.169
U2AF1	191	6.30%	0.43	0.37	0.02-0.53	0.36	0.38	0.28	0.38	0.36	0.438	0.38	0.36	0.614	0.45	0.36	0.38	0.747
WT1	177	5.90%	0.31	0.3	0.01-0.98	0.3	0.3	0.93	0.29	0.31	0.614	0.30	0.32	**0.039**	0.2	0.28	0.34	0.255
SH2B3	4	0.10%	0.09	0.1	0.01-0.22	0.1	0.09	0.919	0.11	0.09	0.812	-	-	-	0.05	0.22	0.11	0.247
ZRSR2	2	0.10%	0.1	0.1	0.02-0.17	0.17	0.02	-	1	-	-	-	-	-	-	0.02	0.17	-

Table S3. Mixed-effects ML regression for OS in global AML cohort. To avoid the impact of negative cases on the analyses of the VAF effect, we carried out a mixed-effects ML regression. OR: odds ratio. CI: confidence interval. Effects regression was used to analyze data where there are fixed effects and random effects. This approach is commonly used in studies with hierarchical or nested data, where replications or variability between different levels can be observed. In retrospective studies, where data have already been collected and are analyzed with the aim of finding associations, mixed-effects regression allows modelling both fixed effects (GEN, relapse and death) and random effects (unobserved variability between groups, such as individual differences not explained by the measured variables). Our aim was to control for variability not explained by the observed variables, as there were differences between analysis groups, heterogeneity, which we tried to correct for with random effects. We used the bootstrap cross-validation technique to verify that the model is not over-fitted and that the results are robust and generalizable. This technique allowed us to generate multiple subsets of the data by sampling with replacement. The model is then trained and evaluated on each subset generated. In addition, an assessment of the variance explained by the fixed and random effects was carried out at each iteration of cross-validation, to ensure that the model generalizes well and does not overfit the specific characteristics of the training data. Mixed-effects regression is a useful and common tool in retrospective studies, especially when there is repeated or nested data. However, as with any statistical model, it is important to employ techniques to avoid overestimating effects or artificially increasing statistical significance.

GENE	OR	P-VALUE	95% CI LOW	95% CI HIGH
ASXL1	1.317	**0.036**	0.084	2.550
BRAF	0.582	0.355	-0.651	1.816
CALR	0.132	0.834	-1.101	1.365
CBL	0.201	0.749	-1.032	1.434
CEBPA	-0.363	0.564	-1.597	0.870
CSF3R	0.191	0.762	-1.042	1.424
DNMT3A	-1.156	0.066	-2.389	0.077
ETV6	0.521	0.408	-0.713	1.754
EZH2	0.943	0.134	-0.290	2.176
FLT3	1.382	**0.028**	0.148	2.615
***FLT3*-TKD**	-0.373	0.554	-1.606	0.860
***FLT3*-OTHER**	-0.306	0.626	-1.539	0.927
***FLT3*-ITD**	-0.902	0.152	-2.135	0.331
GATA2	0.107	0.865	-1.126	1.340
HRAS	0.077	0.902	-1.156	1.311
IDH1	-0.279	0.657	-1.513	0.954
IDH2	-0.113	0.858	-1.346	1.120
JAK2	1.400	**0.026**	0.167	2.633
KIT	0.173	0.783	-1.060	1.407
KRAS	-0.096	0.879	-1.329	1.137
MPL	-0.288	0.647	-1.521	0.945
NPM1	-2.417	**<0.001**	-3.651	-1.184
NRAS	0.587	0.351	-0.646	1.821

PTPN11	-0.125	0.842	-1.358	1.108
RUNX1	2.215	**<0.001**	0.982	3.448
SETBP1	0.501	0.426	-0.732	1.734
SF3B1	0.284	0.657	-0.969	1.537
SRSF2	3.263	**<0.001**	2.030	4.496
TET2	2.662	**<0.001**	1.429	3.896
TP53	4.712	**<0.001**	3.479	5.946
U2AF1	1.270	**0.044**	0.036	2.503
WT1	0.662	0.292	-0.571	1.896

Table S4. Empirical cut point estimation for VAF regarding OS, using Youden method. CI: confidence interval. NPV: negative predict. PPV: positive predictive value. VAF: variant allele frequency. Genes with the highest predictive ability have been highlighted, based on Youden index and area under the curve. FLT3 (which includes DIT and SNV) has not been highlighted, because the laboratories that perform amplicon enrichment underestimate the VAF of FLT3-ITD, compared to capture methods; therefore, the distribution of VAF of FLT3-ITD is not homogeneous.

GEN	EMPIRICAL OPTIMAL CUTPOINT (VAF)	YOUDEN INDEX	SENSITIVITY AT CUTPOINT	SPECIFICITY AT CUTPOINT	AREA UNDER ROC	PPV	PPV (95% CI)		NPV	NPV (95 % CI)	
ABL1	0.353	0	0.00	1.00	0.50	25.0%	5.5%	57.2%	66.8%	65.0%	68.5%
ASXL1	0.475	0.031	0.06	0.97	0.52	49.2%	40.3%	58.2%	67.5%	65.8%	69.2%
BRAF	0.114	0.011	0.01	1.00	0.51	85.7%	57.2%	98.2%	67.0%	65.3%	68.7%
CALR	0.533	0.003	0.00	1.00	0.50	66.7%	22.3%	95.7%	66.9%	65.1%	68.6%
CBL	0.118	0.004	0.03	0.97	0.50	36.7%	26.1%	48.3%	66.9%	65.1%	68.6%
CEBPA	0.628	0.002	0.00	1.00	0.50	44.4%	13.7%	78.8%	66.8%	65.1%	68.5%
CSF3R	0.080	0.006	0.03	0.98	0.50	38.6%	27.2%	51.0%	66.9%	65.2%	68.6%
DNMT3A	0.478	0.022	0.07	0.95	0.51	42.0%	34.3%	50.0%	67.3%	65.5%	69.0%
ETV6	0.460	0.013	0.02	0.99	0.51	53.7%	37.4%	69.3%	67.1%	65.3%	68.8%
EZH2	0.181	0.02	0.05	0.97	0.51	44.1%	34.9%	53.5%	67.2%	65.5%	69.0%
FLT3	0.025	0.042	0.04	1.00	0.52	88.0%	75.7%	95.5%	67.7%	66.0%	69.4%
FLT3-ITD	0.772	0.003	0.01	0.99	0.50	44.4%	21.5%	69.2%	66.9%	65.1%	68.6%
GATA2	0.472	0.006	0.01	1.00	0.50	35.4%	23.9%	48.2%	66.8%	65.1%	68.6%
IDH1	0.477	0.014	0.03	0.99	0.51	50.0%	36.3%	63.7%	67.1%	65.4%	68.8%
IDH2	0.463	0.013	0.05	0.96	0.51	40.7%	31.7%	50.1%	67.1%	65.3%	68.8%
JAK2	0.038	0.03	0.07	0.96	0.52	47.5%	39.0%	56.1%	67.5%	65.7%	69.2%
KIT	0.454	0.009	0.01	1.00	0.50	59.1%	36.4%	79.3%	67.0%	65.3%	68.7%
KRAS	0.029	0.005	0.06	0.94	0.50	35.2%	28.2%	42.7%	66.9%	65.1%	68.7%
MPL	0.503	0.001	0.00	1.00	0.50	36.4%	10.9%	69.2%	66.8%	65.1%	68.5%
NPM1	0.468	0.016	0.04	0.98	0.51	47.3%	35.6%	59.3%	67.2%	65.4%	68.9%
NRAS	0.408	0.019	0.04	0.98	0.51	47.3%	36.7%	58.0%	67.2%	65.5%	69.0%
PTPN11	0.480	0.004	0.01	1.00	0.50	71.4%	29.0%	96.3%	66.9%	65.1%	68.6%
RUNX1	0.043	0.042	0.21	0.83	0.52	38.4%	34.2%	42.6%	67.9%	66.0%	69.8%
SETBP1	0.005	0.015	0.04	0.97	0.51	43.3%	33.3%	53.7%	67.1%	65.4%	68.9%
SF3B1	0.468	0.011	0.02	0.99	0.51	52.9%	35.1%	70.2%	67.1%	65.3%	68.8%
SRSF2	0.028	0.073	0.21	0.87	0.54	43.4%	38.9%	48.0%	68.7%	66.9%	70.5%
TET2	0.030	0.054	0.24	0.81	0.53	39.0%	35.1%	42.9%	68.3%	66.4%	70.2%
TP53	0.024	0.079	0.22	0.86	0.54	43.9%	39.4%	48.4%	68.9%	67.0%	70.7%
U2AF1	0.011	0.028	0.08	0.95	0.51	43.2%	36.0%	50.7%	67.5%	65.7%	69.2%
WT1	0.035	0.019	0.07	0.95	0.51	40.9%	33.2%	48.9%	67.2%	65.5%	69.0%

Table S5. Univariate analysis, comparing VAF average between responder and no responder patients. VAF: variant allele frequency. SD: standard deviation.

	RESPONDER		**NO RESPONDER**		
	Mean	SD	Mean	SD	p-value
AGE	57.58	13.95	68.23	12.86	0.009
LEUKOCYTE COUNT	37.3	56.83	29.62	53.38	0.012
***ASXL1* VAF**	**3.31**	**0.1188**	**0.0729**	**0.1764**	**<0.001**
***BRAF* VAF**	**0.0001**	**0.0016**	**0.0051**	**0.049**	**<0.001**
***CSF3R* VAF**	0.0101	0.0656	0.0062	0.0577	0.047
***DNMT3A* VAF**	**0.1248**	**0.2028**	**0.0913**	**0.1873**	**<0.001**
***EZH2* VAF**	0.0185	0.0994	0.0317	0.1477	0.001
***FLT3* VAF**	**0.0057**	**0.0471**	**0.0201**	**0.09**	**<0.001**
***FLT3*-TKD VAF**	**0.0178**	**0.0727**	**0.0039**	**0.0296**	**<0.001**
***FLT3*-ITD VAF**	**0.0531**	**0.1447**	**0.0302**	**0.1122**	**<0.001**
***IDH2* VAF**	**0.0506**	**0.1439**	**0.0748**	**0.1828**	**<0.001**
***JAK2* VAF**	**0.0122**	**0.0745**	**0.0316**	**0.1295**	**<0.001**
MPL VAF	0.0039	0.0427	0.0096	0.0717	0.003
***NPM1* VAF**	**0.1369**	**0.183**	**0.0603**	**0.1491**	**<0.001**
***PTPN11* VAF**	**0.0212**	**0.0809**	**0.0115**	**0.065**	**<0.001**
***RUNX1* VAF**	**0.0648**	**0.1682**	**0.099**	**0.2182**	**<0.001**
***SETBP1* VAF**	**0.0082**	**0.0614**	**0.0169**	**0.0867**	**<0.001**
***SRSF2* VAF**	**0.0365**	**0.1248**	**0.1046**	**0.1984**	**<0.001**
***TET2* VAF**	**0.0768**	**0.1758**	**0.1166**	**0.2226**	**<0.001**
***TP53* VAF**	**0.0394**	**0.1527**	**0.126**	**0.2809**	**<0.001**
***U2AF1* VAF**	**0.0114**	**0.0662**	**0.0331**	**0.1166**	**<0.001**