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

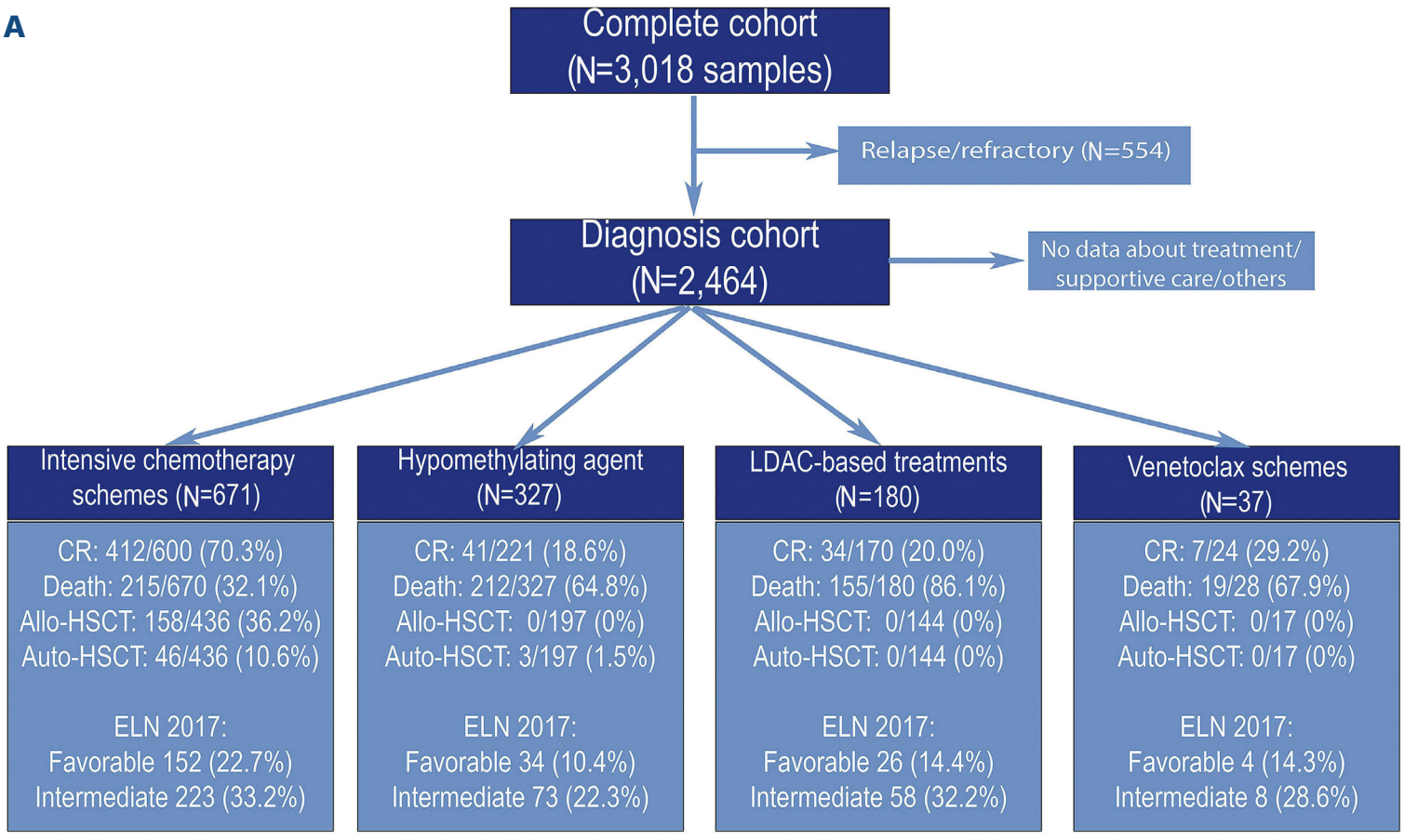
Acute myeloid leukemia (AML) is a heterogeneous pathology in terms of its cytogenetic and molecular alterations, which are used for prognostic stratification and as therapeutic targets.[1-3] Some studies have shown the negative impact of a high allelic burden at diagnosis regarding the mutations of some genes (*EZH2*, *SRSF2*, *TP53*) on the evolution of AML.[4-6] The most studied gene is *TP53*; different variant allele frequency (VAF) thresholds (i.e., 10% or 40%) at diagnosis could have an impact on patients' outcomes.[7,8] Although the mutational burden, according to VAF measurements, has been associated with the prognosis of patients, this parameter is not well established for risk stratification. In this study, we analyzed the impact of the mutational burdens of gene variants detected with a myeloid panel via next-generation sequencing 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 a next-generation sequencing 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, during refractoriness, and at relapse; the comprehensive mutational profile of this cohort was published previously.[3] The patients were assigned to therapeutic groups based on the front-line approach: intensive chemotherapy, non-intensive chemotherapy such as hypomethylating agents, or low-dose cytarabine schemes; patients who received venetoclax-based schedules were excluded because of the low number of such patients. The mutational profiles were determined in seven Spanish PETHEMA reference laboratories, which were instructed to use next-generation sequencing 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 to study other genes for which there is proven evidence of their relevance in AML pathogenesis (*ABL1*, *BRAF*, *CALR*, *CBL*, *CSF3R*, *DNMT3A*, *ETV6*, *GATA2*, *HRAS*, *JAK2*, *KIT*, *KRAS*, *MPL*, *NRAS*, *PTPN11*, *SETBP1*, *TET2*, and *WT1*). The next-generation sequencing methods were harmonized and periodically validated across centers.[3,9] Using the single-nucleotide polymorphism database (NCBI, dbSNP150), variants with a VAF 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, College Station, TX, USA); statistical significance was considered at a *P* value less than or equal to 0.05. A χ^2 test was used to assess the associations between categorical variables, and a median test, Student *t* test, and analysis of variance were performed to compare differences in the median and mean values of continuous variables. The analysis was performed using VAF as a continuous variable (for those genes without mutations, the value of the variable was 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' leukemia-free survival (LFS) and OS. For multivariate analyses, we adjusted for patients' age (continuous variable) and VAF of gene mutations (1% increments). Mixed regression models combine fixed and random effects to analyze correlated data. In this study, we used mixed-effects machine-learning regression to account for patients' heterogeneity by treating patients as random factors and assess the impact of VAF 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 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 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 re-



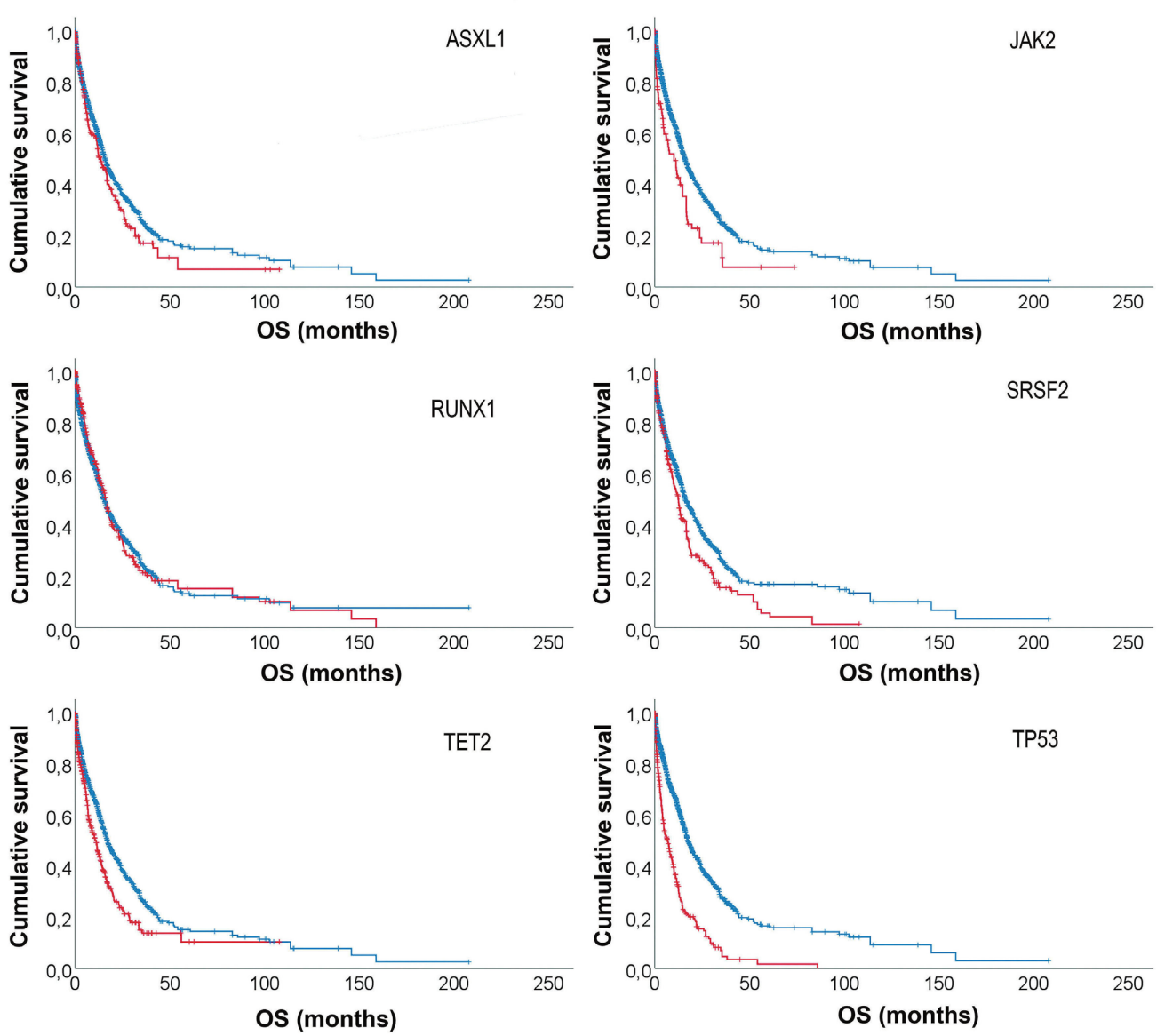


Figure 1. Study design and Kaplan-Meier curve of overall survival depending on the cutoff of variant allele frequencies of some genes. (A) Study design. (B) Kaplan-Meier curve of overall survival, depending on the cutoff of the variant allele frequencies (VAF) of *ASXL1*, *JAK2*, *RUNX1*, *SRSF2*, *TET2* and *TP53*. The entire diagnostic cohort is represented. Blue represents patients with VAF below the cutoff and red represents patients with VAF above the cutoff. CR: complete remission; Allo-HSCT: allogeneic hematopoietic stem-cell transplantation; Auto-HCST: autologous hematopoietic stem-cell transplantation; ELN: European LeukemiaNet; LDAC: low-dose cytarabine; OS: overall survival.

ceived front-line intensive chemotherapy schemes (55.6%), hypomethylating treatment with a single agent (27.1%) or low-dose cytarabine-based treatments (14.9%). In patients who received intensive chemotherapy schedules, 70.3% achieved complete remission and 36.2% underwent allogeneic hematopoietic stem cell transplantation. The risk group according to the European LeukemiaNet (ELN) 2017 classification 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% confidence interval [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 complete response 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. In the multivariate logistic regression model, a higher age of the patient (odds ratio [OR]=0.935, 95% CI: 0.913-0.958, *P*<0.001) and higher mutational load for the *SRSF2* gene (OR=0.978, 95% CI: 0.967-0.990, *P*<0.001) were associated with a lower probability of achieving a complete response. However, a higher mutational load for *NPM1* (OR=1.025, 95% CI: 1.007-1.043, *P*<0.001) was associated with a greater chance of achieving a complete response.

To avoid negative cases impacting the analyses of the VAF effect on OS, we carried out a mixed-effects machine learning regression (*Online Supplementary 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 to -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 comparator 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) for some. 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 statistically 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 (N=467 patients with a complete data set) (Table 1, Figure 1B). In a multivariate analysis, we observed a worse OS in older patients (hazard ratio [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 VAF 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 a complete data set) (Table 2), in the multivariate analysis, we observed a worse LFS with higher VAF 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 the ELN2022 classification. An association between a higher *EZH2* clonal burden and

Table 1. Overall survival: multivariate analyses of factors at diagnosis in patients in each treatment group.

Factor	N	HR	95% CI	*P*
Group treated with intensive chemotherapy, N=671				
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
Group treated with hypomethylating agents, N=327				
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
Group treated with low-dose cytarabine, N=181				
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

Biomarkers identified with an adjusted Cox regression analysis model (*P*≤0.05) were included in the table. The Cox regression model was adjusted for age, gender and gene variant allele frequency detected in the panel. Age and variant allele frequencies were analyzed as continuous variables. HR: hazard ratio; 95% CI: 95% confidence interval; VAF: variant allele frequency; BM: bone marrow.

Table 2. Leukemia-free survival: multivariate analyses of factors at diagnosis in patients in each treatment group.

Factor	N	HR	95% CI	*P*
Group treated with intensive chemotherapy, N=671				
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
Group treated with hypomethylating agents, N=327				
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
Group treated with low-dose cytarabine, N=181				
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

Biomarkers identified with an adjusted Cox regression analysis model ($P \leq 0.05$) were included in the table. The Cox regression model was adjusted for age, gender and gene variant allele frequency detected in the panel. Age and variant allele frequencies were analyzed as continuous variables. HR: hazard ratio; 95% CI: 95% confidence interval; VAF: variant allele frequency; BM: bone marrow.

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 that of *de novo* AML.

In the 'low-dose cytarabine group', regarding OS (N=158 patients with a complete data set), a higher age (HR=1.06, P=0.002), higher leukocyte count (HR=1.01, P<0.001), and higher VAF 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 a complete data set), higher VAF 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 low-dose cytarabine, 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; adding venetoclax to low-dose cytarabine 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 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 subgroups. Previously, Short *et al*. established a VAF threshold of 0.40, showing a better OS in patients with a low *TP53* VAF 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 factor (*RUNX1*), epigenetic (*ASXL1*, *TET2*), and splicing (*SRSF2, U2AF1*) genes, in addition to *TP53*, worsen OS survival in AML patients. We also determined a specific prognostic cutoff 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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Disclosures

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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 the statistical analyses. All authors discussed the results, contributed to the final manuscript and approved the version to be published.

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Data-sharing statement

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