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ARTICLE

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Response to therapy and prognosis according to molecular characterization in *CALR*-mutated essential thrombocythemia

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Abstract

Response to cytoreductive therapy according to *CALR* mutation type, *CALR* variant allele frequency (VAF), and additional mutations has not been previously studied in essential thrombocythemia (ET). The impact of the molecular profile on treatment response and the main clinical outcomes was analyzed in 557 *CALR* ET patients (*CALR* Type 1, *n* = 339, median VAF 36%; and *CALR* Type 2, *n* = 218, median VAF 35%). NGS data on additional mutations were available for 257 patients. *CALR* Type 2 showed a significantly higher rate of complete hematological response (CHR) to first-line cytoreduction. However, in the multivariate analysis, the effect of *CALR* mutation type in response rates was no longer significant once *CALR* VAF was considered, whereas high VAF remained independently associated with a lower likelihood of achieving CHR (hazard ratio [HR] = 0.482, 95% confidence interval (CI): 0.297–0.781; P = 0.003). Moreover, high VAF was also associated with an increased risk of arterial thrombosis (HR = 4.135, 95% CI: 1.093–15.645; P = 0.037), and progression to MF (HR = 2.631, 95% CI: 1.004–6.890; P = 0.049). Although allele burden affects overall survival (OS) in the entire population, its impact was surpassed by the presence of high molecular risk (HMR) mutations (HR = 2.114, 95% CI: 1.070–4.176; P = 0.031). Furthermore, the HMR profile was also associated with a higher risk of progression to acute leukemia, while it did not influence the probability of CHR or progression to MF. In conclusion, *CALR* VAF and HMR profile appear to be more important than *CALR* mutation type regarding treatment response and major clinical outcomes in ET.

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INTRODUCTION

Mutations in *CALR* are detected in approximately 20% of patients with essential thrombocythemia (ET), making it the second most common genetic alteration. These mutations consist of deletions or insertions affecting the last exon of the gene (Exon 9) and leading to premature truncation of the protein.[1] Several types of mutations have been described, with two main variants representing more than 80% of patients with mutated *CALR* ET: Type 1 (a 52-bp deletion, c.1099_1150del; L367fs*46) and Type 2 (a 5-bp insertion, c.1154_1155insTTGTC; K385fs*47). Other variants, distinct from Type 1 and Type 2, have been classified as Type 1-like or Type 2-like based on structural similarities in the protein caused by the mutation.[2,3]

In comparison with the *JAK2* V617F genotype, patients with the *CALR* mutation show different phenotypic and prognostic characteristics, including younger age, higher platelet count, lower rate of thrombosis, poor response to cytoreductive treatment, and increased propensity for myelofibrosis (MF) transformation.[4] In addition, differential clinical effects of *CALR* mutation subtypes have been reported, including a higher platelet count and lower probability of thrombosis and MF progression in patients with *CALR* Type 2 in comparison with those carrying the Type 1 mutation.[3–5] Despite these observations, current treatment recommendations do not take into consideration the specific *CALR* mutation subtype. Moreover, it is unknown whether *CALR* mutation variant allele frequency (VAF) or the presence of additional mutations modifies the prognostic value or the response to cytoreductive therapy according to the mutation type.

In the present study, we aim to analyze first-line treatment response and the main clinical outcomes according to genetic characterization, including *CALR* mutation type, *CALR* VAF, and additional mutations, in a large series of *CALR*-mutated ET patients enrolled in the Spanish Registry of Essential Thrombocythemia (RETE).

PATIENTS AND METHODS

The Spanish Registry of Essential Thrombocythemia is a nationwide, non-interventional prospective study started in 2015 by the Spanish Group of Ph-negative Myeloproliferative Neoplasms (GEMFIN). Patients diagnosed with ET after the year 2000 were eligible for inclusion. ET diagnosis was established locally at the treating hospital, according to the prevailing World Health Organization (WHO)/International Consensus Classification (ICC) criteria at the time of diagnosis. Patients with prefibrotic MF were not included in the registry. When ET diagnosis was previous to the inclusion in the registry, clinical data were collected retrospectively and prospectively updated for therapy changes and complications. All methods were performed in accordance with the relevant guidelines and regulations including Spanish Medicines Agency approval (September 9, 2015) and Hospital del Mar Ethic Committee approval (CEIC-Parc de Salut Mar resolution on November 10, 2015, Protocol number GEE-AAS-2015-01). Approval from local institutional review boards (IRBs) was also obtained in each participating center following Spanish guidelines for prospective studies. Informed consent for participation in the registry was obtained from all patients.

Molecular analysis of *CALR* mutation status and next-generation sequencing (NGS) studies were performed locally. Additionally, centralized NGS was offered to all study participants. *CALR* mutations were classified as Type 1/Type 1-like (CALR1) or Type 2/Type 2-like (CALR2) according to previous definitions.[2,3] *CALR* VAF was taken from NGS data; in the absence of NGS results, VAF was calculated as the percentage of mutated alleles relative to the number of total alleles (mutant + wild type) determined by polymerase chain reaction (PCR), followed by fragment analysis. We carried out a cross-comparison between the two methods, and they showed a moderate, statistically significant correlation (Spearman's $\rho = 0.555$, $P < 0.001$), indicating that both tests follow a similar trend in VAF quantification. Similar concordance between NGS and fragment analysis has also been reported by other groups.[6] The mean VAF in our cohort was 34.6%. For the purposes of this study, we defined a high mutant allele burden as a VAF at or above the 95% confidence interval (CI) of the populations' VAF, which corresponded to a threshold of 36%. Patients with CALR VAF ≥ 35% were therefore classified as having high VAF. Molecular data from NGS panels were available in 257 patients. Among patients with available NGS studies, those carrying mutations in *TP53* or in chromatin/spliceosome genes (*EZH2*, *IDH1*, *IDH2*, *ASXL1*, *PHF6*, *CUX1*, *ZRSR2*, *SF3B1*, *SRSF2*, *U2AF1*, *KRAS*, *NRAS*, *GNAS*, *CBL*, *RUNX1*, *STAG2*, or *BCOR*) were considered as high molecular risk (HMR), whereas the remaining were considered as low molecular risk (LMR), according to the genomic classification proposed by Grinfeld et al.[7]

Response to cytoreductive therapy was evaluated using the European Leukemia-Net (ELN) criteria.[8,9] Complete hematological response (CHR) was defined as the normalization of the platelet count ($<400 \times 10^9$/L), a leukocyte count $<10 \times 10^9$/L, absence of disease-related symptoms, and a normal spleen size. Any other response that did not meet these criteria was classified as no response.

CHR, thrombosis, bleeding, disease progression, and survival were assessed according to type of *CALR* mutation, *CALR* VAF, and the presence of additional mutations. Assuming that cytoreduction has no influence on the natural evolution of the disease, time at risk for survival and disease progression were assessed from the date of diagnosis until last contact or death. Since the majority of patients received cytoreduction, thrombosis and bleeding probabilities were calculated after the start of cytoreduction. Time to event curves were calculated using the Kaplan–Meier method with the log-rank test for comparisons. Multivariate analyses of the factors predicting the main outcomes were performed by Cox regression. P values less than or equal to 0.05 were considered statistically significant in all tests. Statistical analysis was performed using the SPSS 23.0 package (SPSS, Chicago, IL, USA).

RESULTS

Patient characteristics

A total of 557 patients were included in the study, corresponding to 339 and 218 cases with CALR1 and CALR2 mutations, respectively.

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No significant differences in age, sex, cardiovascular risk factors, microvascular symptoms, or history of thrombosis were observed between CALR1 and CALR2 groups (Table 1). Thrombosis at ET presentation was uncommon in both groups, without a significant difference. Revised IPSET-thrombosis stratification was similar according to the type of mutation, with 43%, 49%, and 8% of the patients corresponding to the very low risk, intermediate-risk, and high-risk categories, respectively. A higher leukocyte count was observed in CALR1 ET patients (8.4×10^9/L vs. 7.6×10^9/L, P = 0.013), and a non-significant trend for higher platelet count in CALR2 (814×10^9/L vs. 860×10^9/L, P = 0.157).

CALR mutant allele burden was available for 342 cases. The median VAF of the *CALR* mutation was 36% and 35% for CALR1 and CALR2, respectively (P = 0.714). A total of 113 patients in CALR1 and 67 patients in CALR2 had a VAF ≥ 35% (P = 0.911) (Table 1). Among the 257 patients with available NGS, 35 (14%) patients were classified as HMR due to the presence of mutations in *TP53* (*n* = 10) or chromatin/spliceosome genes (*n* = 25), whereas the remaining (*n* = 222) were classified as *CALR*-mutated ET with a LMR profile. There were no statistically significant differences in the frequency of HMR or in VAF of the *CALR* mutation by NGS between groups (Table 2).

Clinico-hematological response to cytoreduction

A total of 470 (84%) patients initiated cytoreduction. Indications for starting cytoreductive therapy and treatment modalities according to the type of *CALR* mutations are shown in Table 3. Seventy-four

TABLE 1 Main clinical characteristics at diagnosis in 557 *CALR*-essential thrombocythemia (ET) patients included in the Spanish Registry of Essential Thrombocythemia.

	Total (*N* = 557)	CALR1 (*n* = 339)	CALR2 (*n* = 218)	P value
Age, median (interquartile range)	62 (47–72)	62 (48–72)	60 (45–72)	0.266
Female sex, *n* (%)	284 (51)	172 (51)	112 (51)	0.931
Bleeding before diagnosis, *n* (%)	9 (2)	4 (1)	5 (2)	0.324
Thrombosis before diagnosis, *n* (%)	45 (8)	31 (9)	14 (6)	0.269
Cardiovascular risk factors				
Diabetes, *n* (%)	65 (12)	37 (11)	28 (13)	0.586
Therapy for hypertension, *n* (%)	203 (36)	132 (39)	71 (32)	0.130
Cholesterol, *n* (%)	113 (20)	68 (20)	45 (21)	0.913
Smoking, *n* (%)	58 (10)	32 (9)	26 (12)	0.390
Symptoms at diagnosis				
Microvascular disturbances, *n* (%)	74 (13)	49 (14)	25 (11)	0.848
Arterial thrombosis, *n* (%)	15 (3)	10 (3)	5 (2)	0.791
Venous thrombosis, *n* (%)	8 (1)	7 (2)	1 (0)	0.158
Bleeding, *n* (%)	0 (0)	0 (0)	0 (0)	-
Hemoglobin g/L, median (interquartile range)	139 (130–148)	140 (129–148)	138 (130–149)	0.706
Leukocyte count $\times 10^9$/L, median (interquartile range)	8.2 (6.9–9.8)	8.4 (7.0–10.0)	7.6 (6.5–9.6)	0.013
Platelet count $\times 10^9$/L, median (interquartile range)	828 (698–995)	814 (698–963)	860 (701–1016)	0.157
Classical risk stratification				
Low, *n* (%)	239 (43)	138 (41)	101 (46)	0.220
High, *n* (%)	316 (57)	199 (59)	117 (54)	
Revised IPSET-thrombosis stratification				
Very low, *n* (%)	239 (43)	138 (41)	101 (46)	0.312
Low, *n* (%)	0 (0)	0 (0)	0 (0)	
Intermediate, *n* (%)	271 (49)	168 (49)	103 (47)	
High, *n* (%)	45 (8)	31 (9)	14 (7)	
Missing, *n* (%)	2 (0)	2 (1)	0 (0)	
CALR VAF, median (interquartile range)[a]	36 (24-43)	36 (24-43)	35 (25-41)	0.714
High *CALR* VAF,[b] *n* (%)	180 (53)	113 (53)	67 (52)	0.911

Abbreviation: VAF, variant allele frequency.

[a]Available in 342 (61%) cases (CALR1 *n* = 213 [63%], CALR2 *n* = 129 [59%]).

[b]High *CALR* VAF defined as a mutant allele burden ≥ 35%.

patients did not initiate any cytoreductive treatment during the entire follow-up, corresponding to 44 (13%) and 30 (14%) in CALR1 and CALR2 groups, respectively (P = 0.798).

Treatment response was available for 349 out of 470 (74%) patients receiving cytoreduction. With a median treatment duration of 4 years, CHR was achieved by 176 patients (50%). CALR2 was associated with a higher rate of CHR: 1-year probability of 33% and 43% for CALR1 and CALR2, respectively (P = 0.030) (Figure 1). When CHR rates were analyzed according to *CALR* VAF, high VAF was associated with a lower probability of CHR (Figure 2). The presence of HMR mutations was not associated with CHR probability (data not shown). In the multivariate analysis, high VAF was associated with a lower probability of achieving CHR (hazard ratio [HR] = 0.482, 95% CI: 0.297–0.781; P = 0.003), after adjusting for *CALR* mutation type and the presence of HMR mutations. In contrast, the association with *CALR* mutation type was no longer significant (HR = 1.372, 95% CI: 0.837–2.248; P = 0.209). On modeling as a continuous variable, higher VAF remained as an independent predictor of lower CHR probability after adjustment for the same covariates (HMR status and *CALR* mutation type): for every 10% increase in VAF, the probability of achieving a CHR decreased by approximately 18% (HR = 0.817, 95% CI: 0.680–0.980; P = 0.028).

TABLE 2 Molecular characterization by next-generation sequencing (NGS) in 257 *CALR*-mutated essential thrombocythemia (ET) patients included in the Spanish Registry of Essential Thrombocythemia.

	Total (*n* = 257)	CALR1 (*n* = 156)	CALR2 (*n* = 101)	P value
HMR, *n* (%)	35 (14)	23 (15)	12 (12)	0.579
TP53 mutation	10 (4)	6 (4)	4 (4)	0.951
Chrom/splice mutation	25 (10)	17 (11)	8 (8)	0.521
CALR VAF, median (interquartile range)	36 (23–42)	34 (22–42)	37 (26–44)	0.107
High *CALR* VAF, *n* (%)	130 (51)	75 (48)	55 (54)	0.308

Abbreviations: chrom/splice, chromatin/spliceosome genes (*EZH2*, *IDH1*, *IDH2*, *ASXL1*, *PHF6*, *CUX1*, *ZRSR2*, *SF3B1*, *SRSF2*, *U2AF1*, *KRAS*, *NRAS*, *GNAS*, *CBL*, *RUNX1*, *STAG2*, or *BCOR*); high *CALR* VAF, allele mutant burden ≥ 35%; HMR, high molecular risk; VAF, variant allele frequency.

Thrombosis and bleeding

Nineteen patients presented an arterial thrombotic event after cytoreduction initiation; three of them had a history of prior thrombosis. According to the revised IPSET-thrombosis stratification, arterial thrombosis occurred in 3%, 4%, and 10% of patients categorized as very low risk, intermediate risk, and high risk, respectively (P = 0.221). The probability of arterial thrombosis at 10 years was 8% in both CALR1 and CALR2 groups (P = 0.708). Achievement of CHR was not associated with the probability of arterial thrombosis. Patients with high *CALR* VAF had a significantly higher probability of presenting an arterial thrombotic event under cytoreduction (10-year probability of 2% and 15% in low and high *CALR* VAF, respectively, P = 0.026). No association was found between HMR and arterial thrombosis. High *CALR* VAF was associated with a higher risk of arterial thrombosis (HR = 4.135; 95% CI: 1.093–15.645; P = 0.037) after adjustment for age (HR = 0.988; 95% CI: 0.955–1.023; P = 0.493), previous history of thrombosis (HR = 2.715, 95% CI: 0.708–10.403; P = 0.145), and cardiovascular risk factors (HR = 1.506, 95% CI: 0.400–5.671; P = 0.545). Modeling VAF as a continuous variable did not retain statistical significance (HR = 1.010, 95% CI: 0.977–1.045; P = 0.550). Twelve

TABLE 3 Characteristics of first-line cytoreductive treatment received by the 470 *CALR*-essential thrombocythemia (ET) patients.

	Total (*n* = 470)	CALR1 (*n* = 289)	CALR2 (*n* = 181)	P value
Age, median (interquartile range)	66 (55–75)	67 (57–75)	64 (52–75)	0.253
Revised IPSET-thrombosis stratification				
Very low, *n* (%)	135 (29)	76 (26)	59 (33)	0.265
Low, *n* (%)	0 (0)	0 (0)	0 (0)	
Intermediate, *n* (%)	284 (60)	179 (62)	105 (58)	
High, *n* (%)	44 (9)	30 (10)	14 (8)	
Missing, *n* (%)	7 (2)	4 (1)	3 (1)	
Cytoreductive drug				
HU, *n* (%)	395 (84)	243 (84)	152 (84)	0.976
Anagrelide, *n* (%)	60 (13)	35 (12)	25 (14)	0.670
Interferon, *n* (%)	12 (2)	8 (3)	4 (2)	0.774
HU + anagrelide, *n* (%)	3 (1)	3 (1)	0 (0)	0.288
Treatment indication				
Age (>60 years), *n* (%)	244 (52)	153 (53)	91 (50)	0.942
Extreme thrombocytosis, *n* (%)	110 (24)	66 (23)	44 (24)	0.567
Thrombosis or bleeding event, *n* (%)	43 (9)	31 (11)	12 (7)	0.188
Microvascular symptoms, *n* (%)	15 (3)	10 (3)	5 (3)	0.738
Other/not specified, *n* (%)	58 (12)	29 (10)	29 (16)	0.187
Median duration of treatment (years)	4	4	4	0.226

Abbreviation: HU, hydroxyurea.

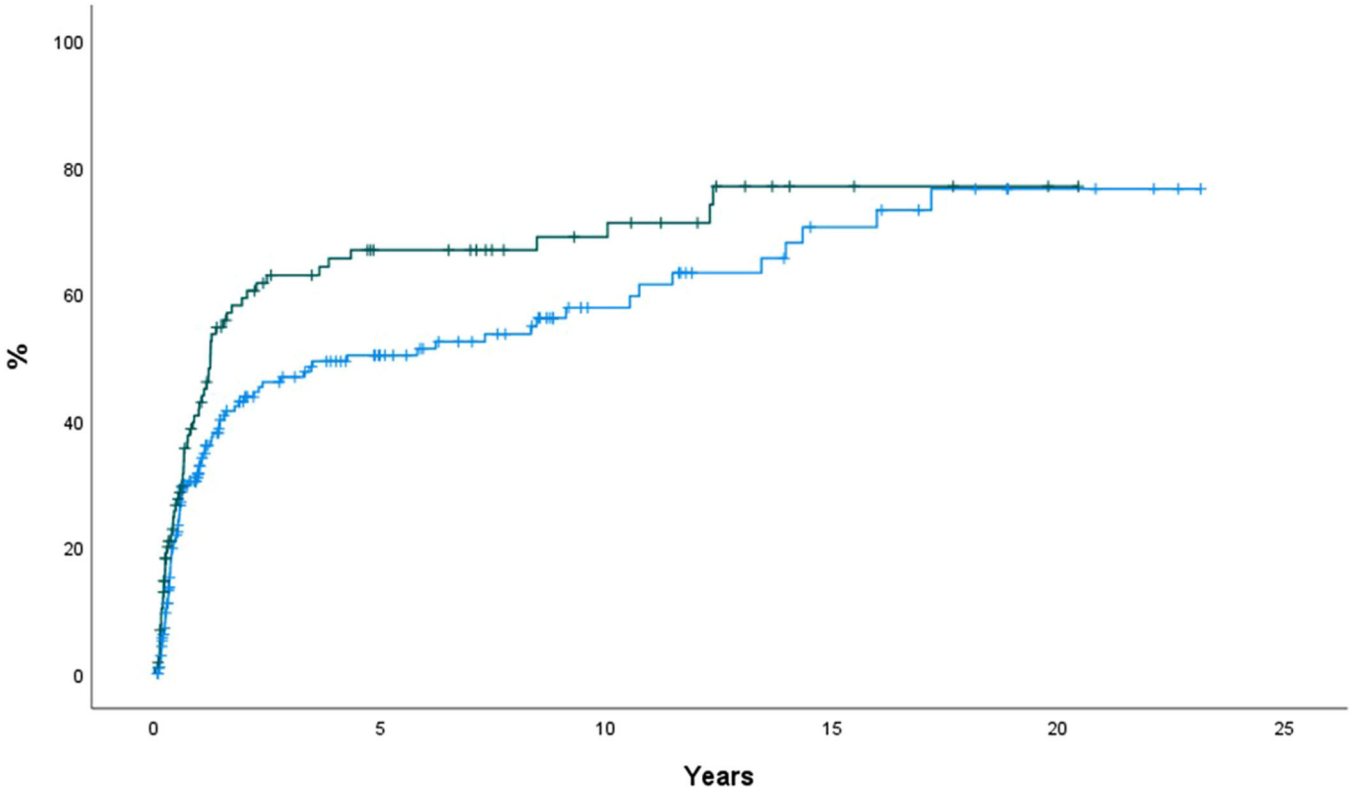

FIGURE 1 **Probability of complete hematological response (CHR) according to** the ***CALR*-mutation type in 349 patients included in the Spanish Registry of Essential Thrombocythemia.** Type 2 *CALR* was associated with a higher rate of CHR: 1-year probability of 33% and 43% for CALR1 and CALR2, respectively, P value 0.030. The blue solid line corresponds to Type 1 *CALR*, and the green solid line corresponds to Type 2 *CALR*.

patients presented a venous thrombotic event, with the probability of venous thrombosis at 10 years of 4% in both CALR1 and CALR2 groups (P = 0.992). CHR, high *CALR* VAF, and HMR were not associated with the risk of venous thrombosis.

Fourteen patients presented a major hemorrhagic event during follow-up, and no differences were observed between the study groups: 10-year probability of major bleeding of 7% in CALR1 and 4% in CALR2 (P = 0.916). No association was found between CHR, *CALR* VAF, or HMR with the probability of presenting major bleeding under cytoreduction.

Disease progression

Fifty-six patients progressed to MF, resulting in a cumulative incidence of MF of 15% after 10 years from diagnosis. CALR1 was associated with a higher, though non-statistically significant, probability of disease progression to MF: 10-year probability of 17% versus 12% in the CALR2 group (P = 0.168). Patients with high VAF showed a higher probability of MF transformation: 10-year probability of 10% in the *CALR* low-VAF group versus 29% in the *CALR* high-VAF group (P = 0.002). Achieving CHR was associated with a lower probability of MF progression (10-year probability of 20% and 10% for non-responders and responders, respectively, P = 0.012), whereas HMR did not have any impact on MF probability. In the multivariate analysis, only high VAF remained significantly associated with MF progression (HR = 2.631, 95% CI: 1.004–6.891; P = 0.049), after adjusting for CALR mutation type, presence of HMR additional mutations, type of response, and age. Modeling VAF as a continuous variable did not retain statistical significance (HR = 1.021, 95% CI: 0.997–1.045; P = 0.090). The probability of MF progression according to *CALR* type and its VAF is shown in Figure 3.

Twelve patients progressed to acute myeloid leukemia (AML), resulting in an incidence of 2% after 10 years from diagnosis. Most of the patients who progressed to AML were in the CALR1 group (*n* = 9) and presented an HMR profile (*n* = 9). HMR had a significant impact on AML-free survival rates: 10-year probability of 5% in the LMR group versus 22% in the HMR group (P < 0.001), whereas types of *CALR* and *CALR* VAF were not associated with AML progression.

Survival

With a median follow-up of 7 years, 99 patients deceased, resulting in a median projected survival of 20.9 years post-diagnosis. There were no significant differences in survival according to the *CALR* mutation type. Survival was significantly different according to *CALR* VAF (10-year probability of survival: 87% and 82% for low and high *CALR* VAF, respectively, P = 0.007) and HMR (10-year probability of survival: 91% and 77% for LMR and HMR, respectively, P < 0.001). In the entire study population, high *CALR* VAF was associated with an increased risk of death (HR = 2.129, 95% CI: 1.232–3.681; P = 0.007), after adjustment by age at diagnosis (HR = 1.115, 95% CI: 1.085–1.145; P < 0.001). Modeling VAF as a continuous variable retained statistical significance: for every 10% increase in VAF, the probability of death increased by 24% (HR = 1.243 [95% CI: 1.080–1.520; P < 0.001]). In the subset of patients with available NGS, older age at diagnosis and the presence of HMR additional

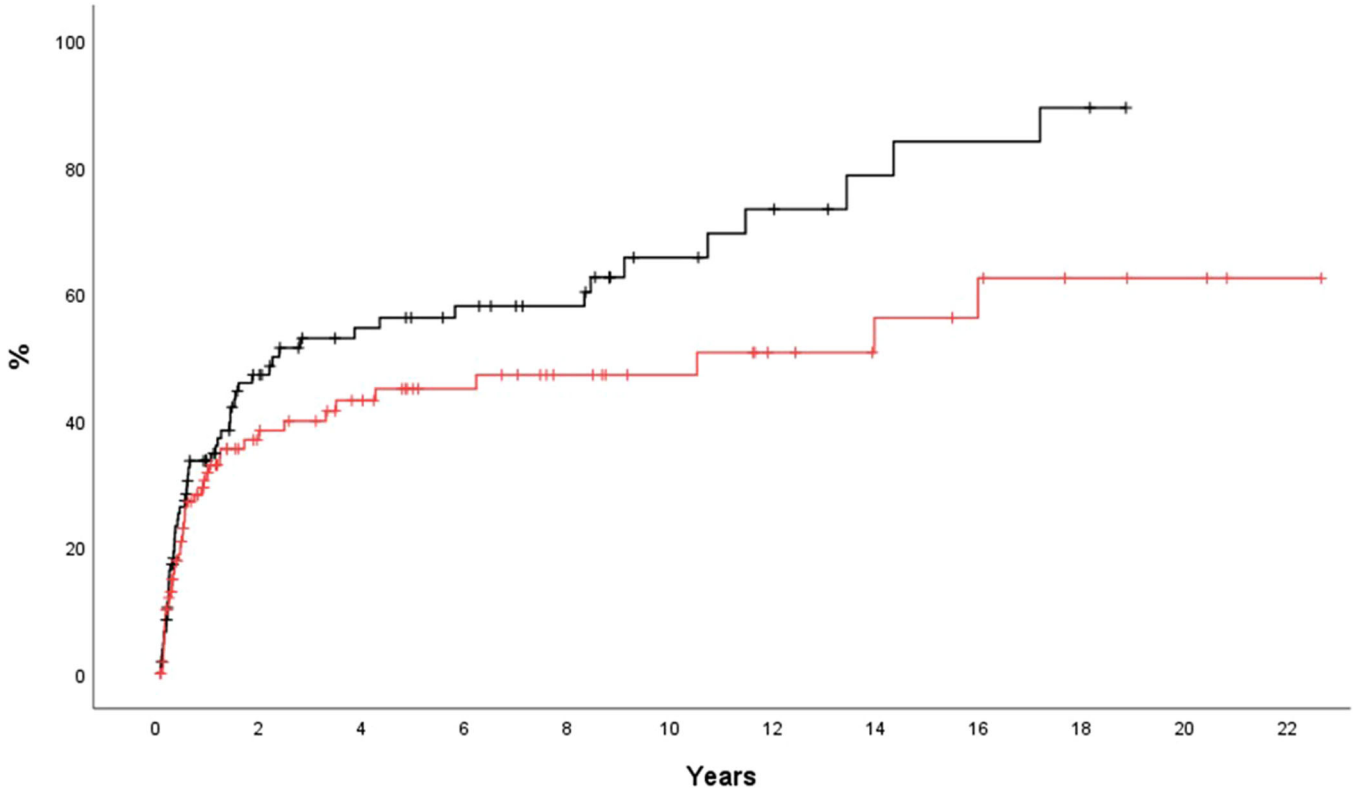

FIGURE 2 **Probability of complete hematological response (CHR) according to *CALR* allele burden.** Patients with high variant allele frequency (VAF) (considered as equal or greater than 35%) had a lower probability of CHR responses to first-line cytoreductive therapy: 1- and 2-year probability of 34% and 47% in low-VAF patients and 32% and 38% in high-VAF patients (P = 0.050). The black solid line corresponds to low-VAF patients, and the red solid line corresponds to high-VAF patients.

mutations were independently associated with worse overall survival (OS) in the multivariate analyses, whereas having high VAF was no longer associated with worse OS (Table 4). The presence of HMR additional mutations had a stronger impact on mortality risk than high VAF (Figure 4).

DISCUSSION

In the present study, we analyzed the impact of the mutational profile in *CALR*-mutated ET patients on their response to first-line cytoreductive treatment and the incidence of complications during follow-up. To the best of our knowledge, it constitutes the largest series reported to date on the topic and is the first to evaluate associations with hematological response. Notably, we found that CHR and the main clinical outcomes are not related to *CALR* mutation type, but are predominantly influenced by *CALR* VAF and HMR additional mutations. These findings support a uniform therapeutic approach for patients with Type 1 and Type 2 *CALR* mutations and highlight allele burden and the presence of HMR mutations as relevant factors to consider in therapeutic decision-making.

In a collaborative study involving 114 *CALR*-mutated patients from the Mayo Clinic, Tefferi et al. observed increased thrombocytosis in patients with CALR2. These findings were later confirmed in another study by Pietra et al. in a cohort of 216 patients with *CALR*-mutated ET.[3,10] Furthermore, Pietra et al. also reported a higher probability of MF transformation in CALR1 patients, with this observation being confirmed by others.[11] In addition, CALR2 has been associated with a lower rate of thrombosis in two other studies.[3,12] It should be noted that none of these studies have taken into consideration *CALR* VAF or the presence of additional HMR mutations. In the present study, the influence of *CALR* mutation type on the main clinical outcomes was completely nullified after adjustment by allele burden.

The prognostic value of the *CALR* VAF was assessed in a previous study of 281 *CALR*-mutated ET patients (CALR1 $n = 152$, CALR2 $n = 101$, and indeterminate $n = 28$) carried out by Guglielmelli et al. High *CALR* VAF (defined as an allele burden greater than 60%) was associated with a higher risk of MF progression.[13] In line with these results, Aubin et al. reported better survival in patients with low *CALR* allele burden (defined as *CALR* VAF < 20%) on 334 *CALR*-mutated ET patients from the French registry. Although *CALR* VAF below 20% was not associated with lower risk of MF progression, they demonstrated a progressive increase of *CALR* VAF over time in 11 (42%) out of 26 assessable patients, with three of them progressing to MF.[14] In the present study, we have demonstrated a clear association between high *CALR* VAF and key clinical outcomes such as arterial thrombosis, MF progression, and survival. Our results, along with those of prior studies, highlight the importance of *CALR* VAF in the management of patients with *CALR*-mutated ET.

In a previous GEMFIN study, we observed an increased risk of MF progression and lower survival in patients with intermediate revised IPSET-thrombosis ET lacking CHR, a risk group mostly including patients with the *CALR* genotype.[15] These results indicated that the absence of CHR might be associated with biological characteristics linked to disease progression. The results of the present study,

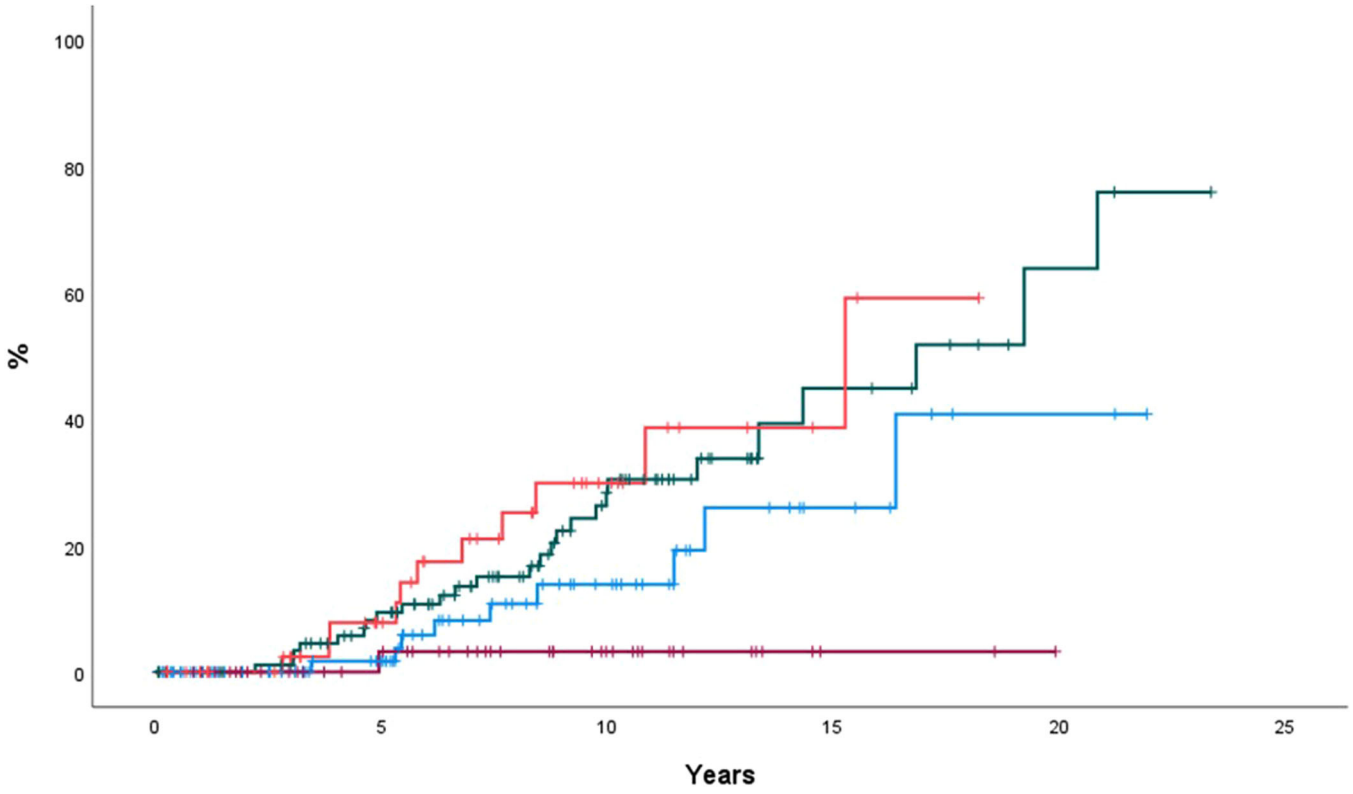

FIGURE 3 **Cumulative incidence of myelofibrosis according to type of *CALR* mutation and its allele burden.** Myelofibrosis (MF) probability at 10 years was 3%, 14%, 28%, and 30% for CALR2 low variant allele frequency (VAF), CALR1 low VAF, CALR1 high VAF, and *CALR2* high VAF, respectively (P = 0.009). Red line: *CALR2* with low VAF. Blue line: *CALR1* with low VAF. Green line: *CALR1* with high VAF. Orange line: *CALR2* with high VAF. High VAF was defined as a *CALR* VAF ≥ 35%.

TABLE 4 Multivariate COX regression analysis for overall survival in 257 patients with *CALR*-mutated essential thrombocythemia characterized by next-generation sequencing (NGS).

	HR (95% CI)	P value
Age	1.111 (1.072–1.150)	<0.001
HMR	2.114 (1.070–4.176)	0.031
High VAF	1.474 (0.747–2.907)	0.263

Abbreviations: 95% CI, 95% confidence interval; age modeled as a continuous variable; high VAF, variant allele frequency ≥ 35%; HMR, high molecular risk mutations; HR, hazard ratio.

showing that *CALR* VAF is a key factor determining the probability of CHR, suggest that high *CALR* VAF may be one of the underlying mechanisms linking lack of CHR to MF progression. Since conventional therapies such as HU or anagrelide have limited impact on CHR and no significant effect on reducing *CALR* VAF, the development of newer therapies is a current medical need. In this aspect, progress in the understanding of the molecular mechanisms of *CALR*-mutated ET has led to the development of targeted therapy such as antibodies directed to mutant *CALR*.[16,17] Our results suggest that higher mutational burden identifies patients at increased risk, who may benefit from novel therapeutic approaches. Therefore, VAF could serve as a useful criterion for patient selection or inclusion in future clinical trials.

The prognostic value of the presence of additional mutations has been extensively studied; however, its impact on treatment response has been less explored.[6,18] The influence of additional mutations on CHR has been recently evaluated in a series of 121 ET patients, 26 of them *CALR*-mutated, showing that the presence of at least one additional mutation is associated with lower rates of CHR.[19] However, the low number of patients and no adjustment by *CALR* VAF in the aforementioned study limit the generalization of this observation to *CALR*-mutated ET. In our study, an HMR profile was associated with reduced OS and an increased risk of progression to AML. However, such mutations were not associated with the probability of achieving CHR, which is primarily dependent on the allelic load of the *CALR* mutation.

It must be mentioned that the techniques used to quantify *CALR* VAF and the timing of quantification in relation to the chronology of the disease may have varied among the patients included in our study. Nevertheless, in our series, the median *CALR* VAF was 36%, being compatible with *CALR* quantification at diagnosis or in the early chronic phase, as reported by Guglielmelli et al.[13] Taking into consideration the absence of standardization for *CALR* VAF quantification and the low number of patients with *CALR* VAF greater than 60%, we decided to stratify patients by the median value of *CALR* VAF observed in our series. The main objective of this study was to assess the clinical impact of the allele burden on disease characteristics and outcomes, rather than to define a specific VAF cut-off value. The choice of a specific threshold was made primarily to simplify the statistical analysis and to allow for clearer comparisons between two groups (low VAF and high VAF). Having a VAF ≥ 35% was associated with lower rates of CHR, higher risk of presenting an arterial thrombotic event, and higher risk of progression to MF. To further explore this relationship, we performed additional multivariate analysis to evaluate the effect of the progressively higher VAF levels

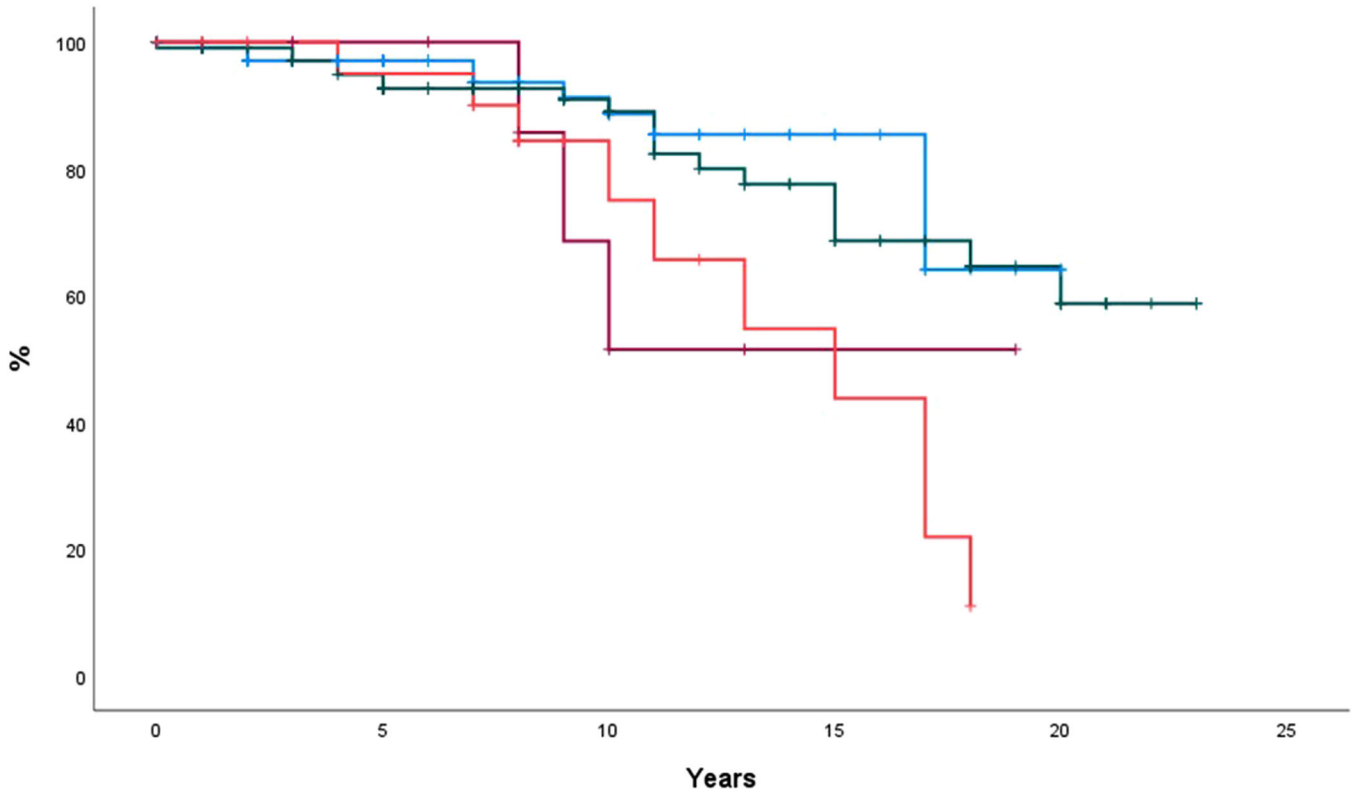

FIGURE 4 **Survival according to *CALR* variant allele frequency (VAF) and the presence of high-risk mutations.** Survival probability at 15 years was 85%, 78%, 51%, and 44% for low molecular risk (LMR) with low *CALR* VAF, LMR with high *CALR* VAF, high molecular risk (HMR) with low *CALR* VAF, and HMR with high *CALR* VAF, respectively (P = 0.014). Blue line: LMR with low *CALR* VAF. Green line: LMR with high *CALR* VAF. Red line: HMR with low *CALR* VAF. Orange line: HMR with high *CALR* VAF. High VAF was defined as a *CALR* VAF > 35%. HMR was defined by the presence of mutations in *TP53* or in chromatin/spliceosome genes (*EZH2*, *IDH1*, *IDH2*, *ASXL1*, *PHF6*, *CUX1*, *ZRSR2*, *SF3B1*, *SRSF2*, *U2AF1*, *KRAS*, *NRAS*, *GNAS*, *CBL*, *RUNX1*, *STAG2*, or *BCOR*).

on clinical outcomes. We found that for every 10% increase in VAF, the probability of achieving a CHR decreased by approximately 18%. However, when analyzed as a continuous variable, VAF did not retain statistical significance in the multivariate model for the risk of thrombosis or MF progression. This likely reflects a non-linear relationship between allele burden and the risk of these outcomes, with the effect being more pronounced above a certain threshold. Other limitations inherent to studies using real-world data include missing data regarding therapy response, *CALR* quantification, or availability of NGS studies. Since NGS studies are more prone to be requested in patients with advanced disease, centralized NGS was offered to all study participants in order to avoid bias by NGS indication.

In conclusion, the *CALR* mutation type has no influence on clinical response to conventional therapy or on major clinical outcomes, which are mainly affected by *CALR* VAF and the presence of additional HMR mutations. Patients with high *CALR* VAF or high-risk mutations constitute a clear unmet medical need and may be considered candidates for targeted therapies with disease-modifying potential.

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AUTHOR CONTRIBUTIONS

Marta Santaliestra: Writing—original draft; formal analysis; data curation; investigation; methodology; visualization. **Marta Garrote**: Writing—review and editing; formal analysis; investigation; resources; writing—original draft; methodology. **María Concepción Fernández-Rodríguez**: Writing—review and editing; investigation; resources. **Patricia Vélez**: Investigation; resources; writing—review and editing. **Alicia Senín**: Investigation; writing—review and editing; resources. **Eduardo Arellano-Rodrigo**: Investigation; resources; writing—review and editing. **Neus Garcia-Gisbert**: Investigation; resources; writing—review and editing. **Raquel Longarón**: Investigation; resources; writing—review and editing. **Manuel Pérez-Encinas**: Investigation; resources; writing—review and editing. **Gonzalo Caballero-Navarro**: Investigation; resources; writing—review and editing. **Maria Soledad Noya-Pereira**: Investigation; resources; writing—review and editing. **Beatriz Cuevas**: Investigation; resources; writing—review and editing. **María Teresa Gómez-Casares**: Investigation; resources; writing—review and editing. **Ruth Stuckey**: Investigation; resources; writing—review and editing. **Gonzalo Carreño-Tarragona**: Investigation; resources; writing—review and editing. **Francisca Ferrer-Marin**: Investigation; resources; writing—review and editing. **Miguel Ángel Cortés**: Investigation; resources; writing—review and editing. **Elena Magro**: Investigation; resources; writing—review and editing. **Isabel Mata**: Investigation; writing—review and editing; resources. **Anna Angona**: Investigation; writing—review and editing; resources. **Raúl Pérez**: Investigation; resources; writing—review and editing. **María Laura Fox**: Investigation; resources; writing—review and editing. **Irene Pastor-Galán**: Investigation; writing—review and editing; resources. **Paula Sastre**: Investigation; resources; writing—review and editing. **Ana Triguero**: Investigation; writing—review and editing; resources. **Valentín García-Gutierrez**: Investigation; resources; writing—review

and editing. **Juan Carlos Hernández-Boluda**: Investigation; resources; writing—review and editing; writing—original draft. **Beatriz Bellosillo**: Investigation; resources; writing—review and editing. **Alberto Alvarez-Larran**: Conceptualization; investigation; writing—original draft; writing—review and editing; formal analysis; resources; supervision; funding acquisition; methodology.

CONFLICT OF INTEREST STATEMENT

The Spanish registry of Essential thrombocythemia is financed by GEMFIN's own funds without direct collaboration from any pharmaceutical company.

DATA AVAILABILITY STATEMENT

The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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