RESEARCH ARTICLE

Clinical and molecular predictors of fibrotic progression in essential thrombocythemia: A multicenter study involving 1607 patients

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Abstract

The current retrospective study involving a total of 1607 patients was designed to identify clinical and molecular variables that were predictive of inferior myelofibrosis-free survival (MFS) in WHO-defined essential thrombocythemia (ET), utilizing three independent patient cohorts: University of Florence, Italy ($n = 718$); Mayo Clinic, USA ($n = 479$) and Policlinico Gemelli, Catholic University, Rome, Italy ($n = 410$). The Florence patient cohort was first examined to identify independent risk factors for MFS, which included age > 60 years (HR 2.5, 95% CI 1.3–4.9), male sex (2.1, 1.2–3.9), palpable splenomegaly (2.1, 1.2–3.9), *CALR* 1/1-like or *MPL* mutation (3.4, 1.9–6.1) and *JAK2*V617F variant allele frequency > 35% (4.2, 1.6–10.8). Subsequently, an operational molecular risk category was developed and validated in the other two cohorts from Mayo Clinic and Rome: "high molecular risk" category included patients with *JAK2*V617F VAF >35%, *CALR* type 1/1-like or *MPL* mutations; all other driver mutation profiles were assigned to "low molecular risk" category. The former, compared to the latter molecular risk category, displayed significantly higher risk of fibrotic transformation: Florence cohort with respective fibrotic transformation risk rates of 8% vs. 1.2% at 10 years and 33% vs. 8% at 20 years ($p < 0.001$; HR 6.1; 95% CI 3.2–11.7); Mayo Cohort, 16% vs. 7% at 10 years and 44% vs. 25% at 20 years ($p < 0.001$; HR 2.5; 95% CI 1.6–4.1); and Rome cohort 7.8% vs. 4.6% at 10 years and 31.2% vs. 7.1% at 20 years ($p = 0.007$, HR 2.7; 95% CI 1.3–5.8). The present study provides practically useful risk signals for fibrotic transformation in ET and facilitates identification of patients who require close monitoring and appropriate counseling.

1 | INTRODUCTION

Essential thrombocythemia (ET) is one of Ph-negative myeloproliferative neoplasms (MPN) along with polycythemia vera (PV), overt and pre-fibrotic myelofibrosis (MF), as defined by the latest World Health Organization (WHO) classification.[1] Essential thrombocythemia is characterized by clonal thrombocytosis associated with an increased risk of thrombo-hemorrhagic complications, progression to MF and, to a lesser extent, transformation to acute myeloid leukemia.[2] Approximately 90% of ET patients harbor one of three MPN-specific clonal markers, including *JAK2*V617F, *CALR* (type 1/1-like and type 2/2-like) and *MPL* mutations that induce constitutive activation of JAK-STAT signaling pathway; the remaining 10% are referred to as triple-negative (TN).[3] Current risk stratification in ET targets likelihood of thrombosis, with important implications on therapy, and relies on International prognostic score in essential thrombocythemia (IPSET) for thrombosis[4] that includes age > 60 years, thrombosis history, cardiovascular risk factors and *JAK2*V617F mutation, as risk variables. The score was re-analyzed and led to a refined four-tiered version,[5] which excluded cardiovascular risk factors evaluation; subsequently this score was validated in a large independent cohort.[6] Classically, survival prediction in ET was based on clinically-derived risk variables, including age, thrombosis history and leukocyte count.[7] More recently, collaborative studies between the Mayo Clinic, Rochester, USA and University of Florence, Italy, established an integrated clinical and genetic survival risk model for ET (MIPSS-ET) identifying the presence of *SF3B1* and *SRSF2* mutations (occurring in ~10% of patients), age > 60 years, male sex, and leukocytosis (≥ 11×10^9/L) as independent risk factors for reduced overall survival[8]; this collaborative effort also identified *SF3B1 and U2AF1* mutations as risk factors for fibrotic progression and highlighted the protective role on arterial thrombosis of *ASXL1, RUNX1* and *EZH2* mutations.[9] Myelofibrotic transformation was reported to be significantly higher among *CALR*-mutated ET patients than *JAK2*V617F patients in one initial report.[10] Follow-up studies were inconsistent, some showing no increased risk of transformation[11,12] but one large-scale study again found a higher risk of transformation to MF.[13] Interestingly, the increase in the incidence of MF transformation among *CALR* mutated ET patients appears to be restricted to type 1/1-like mutations while patients with type 2/2-like mutations displayed no differences when compared to *JAK2*V617F patients.[14] This is in agreement with a recent study on post-ET myelofibrosis showing a time to MF progression significantly longer in type 2/2-like than in type 1/1-like *CALR* and *JAK2* V617F mutations.[15] An increased risk for MF progression was also reported for patients with *MPL* mutations in two studies.[12,16] Moreover, although an impact of *JAK2*V617F variant allele frequency (VAF) on risk of thrombosis and MF progression was suggested in PV,[17,18] no information is available in large cohorts of ET patients. The aim of the current study was to retrospectively identify clinical and molecular variables that were predictive of inferior myelofibrosis-free survival (MFS) in WHO-defined ET, utilizing three independent patient cohorts: University of Florence, Italy ($n = 718$); Mayo Clinic, USA ($n = 479$) and Policlinico Gemelli, Catholic University, Rome, Italy ($n = 410$).

2 | METHODS

Study patients were selected from institutional databases and the study was approved by the respective local ethics committees of the University of Florence, Mayo Clinic and Policlinico Gemelli, Catholic University, and was conducted in accordance with the Declaration of Helsinki. Diagnostic criteria for ET and leukemic transformation were according to the 2016 WHO classification.[1] Diagnostic criteria for post-ET MF were according to the international working group for MPN research and treatment (IWG-MRT).[19] Treatment approaches were in accordance with what was considered the standard of care at the time of initial diagnosis. The *JAK2*V617F mutated patients were annotated for VAF in samples collected within 5 years of diagnosis, according to published methods.[12] In order to minimize the inadvertent inclusion of patients with masked PV, *JAK2* mutated cases with hemoglobin (Hb) level > 16 g/dl in women and 16.5 g/dl in men were excluded; similarly, cases with anemia defined by sex adjusted Hb level of < 11 g/dl in women and 12.5 g/dl in men were excluded, in order to minimize the chance of inadvertent inclusion of patients with pre-fibrotic MF. All statistical analyses considered clinical and laboratory parameters obtained at time of initial diagnosis. Differences in the distribution of continuous variables between categories were analyzed by either Mann–Whitney (for comparison of two groups) or Kruskal–Wallis (comparison of three or more groups) test. Patient groups with nominal variables were compared by chi-square test. Survival analysis was considered from the date of diagnosis to date of death or last contact. Leukemia-free and myelofibrosis-free survival calculations considered the transformation event as the uncensored variable. Survival curves were prepared by the Kaplan–Meier method and compared by the log-rank test. Cox proportional hazard regression model was used for multivariable analysis. A receiver operating characteristic curve (ROC) was used to determine the best thresholds of VAF using area under curve (AUC) estimate. The p values less than 0.05 were considered significant. All the analyses were performed with SPSS software, version 27 (IBM-Corp) and JMP Pro 14.1.0 software from SAS Institute (Cary, NC).

3 | RESULTS

The primary study population included 718 patients from the University of Florence and the observations in this cohort were subsequently validated in two independent cohorts of 479 and 410 patients from the Mayo Clinic, Rochester, MN, USA and Policlinico Gemelli, Catholic University, Rome, Italy, respectively. Main clinical and laboratory characteristics of the University of Florence cohort are highlighted in Table 1. Median age at diagnosis was 57.9 years (range, 12.9–92.9), women 64.8%. Driver mutation distribution was 65.6% *JAK2*, 12.8% *CALR* type1/1like, 7.1% *CALR* type2/2like, 3.6% *MPL* and 10.9% TN. The median follow-up was 106.4 months (6.1–421.6), during which 53 patients (7.4%) progressed to MF (23 *JAK2*V617F, 20 *CALR* 1/1-like, four *CALR* 2/2-like, five *MPL*, one TN, $p < 0.001$) after a median of 146.2 months (8.6–362.9) and 106 patients (14.8%) died.

TABLE 1 Clinical and laboratory variables of 718 patients with essential thrombocythemia from University of Florence cohort, stratified by their driver mutational status

Clinical and laboratory variables	All patients (n = 718)	JAK2 (n = 471; 65.6%)	CALR type 1/1 like (n = 92; 12.8%)	CALR type 2/2 like (n = 51; 7.1%)	MPL (n = 26; 3.6%)	TN (n = 78; 10.9%)	p values[a]
Age in year; median (range)	57.9 (12.9–92.9)	62.0 (14.9–92.9)	52.6 (12.9–85.1)	48.9 (20.5–84.4)	54.8 (37.3–89.2)	49.2 (15.2–81.3)	**<0.001**
Age ≥ 60 years; n (%)	333 (46.4)	253 (53.7)	30 (32.6)	13 (25.5)	11 (42.3)	26 (33.3)	**<0.001**
Sex, females; n (%)	465 (64.8)	318 (67.5)	39 (42.6)	25 (49.0)	20 (76.9)	63 (80.8)	**<0.001**
White blood cells × 10^9/L; median (range) N evaluable = 695	8.3 (3.7–18.2)	8.7 (3.7–18.2)	8.2 (3.9–18.2)	7.2 (3.8–14.7)	6.8 (4.0–16.7)	7.6 (6.4–13.3)	**<0.001**
White blood cells ≥11 × 10^9/L; n (%) N evaluable = 695	114 (16.4)	90 (19.6)	11 (12.8)	2 (4.2)	5 (20.0)	6 (7.7)	**0.01**
Hemoglobin, g/dl; median (range)	14.1 (11.5–16.5)	14.4 (11.8–16.5)	13.8 (11.8–15.9)	13.7 (11.9–16.3)	13.6 (11.9–16.1)	13.5 (11.5–16.4)	**<0.001**
Hematocrit, %; median (range)	42.9 (35.1–51.0)	43.9 (36.0–50.0)	41.6 (36.7–47.9)	41.2 (35.6–49.4)	41.3 (38.0–47.0)	40.9 (35.1–51.0)	**<0.001**
Platelets, ×10^9/L; median (range)	739 (455–2348)	698 (455–1881)	823 (504–2000)	886 (495–2348)	851.5 (467–1742)	794 (460–1700)	**<0.001**
Platelets ≥ 1000 ×10^9/L; n (%)	126 (17.5)	61 (12.9)	24 (26.1)	16 (31.4)	7 (26.9)	18 (23.1)	**0.001**
Palpable splenomegaly; n (%)	97 (13.5)	67 (14.2)	15 (16.3)	6 (11.8)	4 (15.4)	5 (6.4)	0.3
Constitutional symptoms; n (%)	43 (6.0)	27 (5.7)	5 (5.4)	3 (5.9)	2 (7.7)	6 (7.7)	0.9
Major thrombosis before diagnosis; n (%) N evaluable = 633	39 (6.2)	35 (8.6)	1 (1.2)	0 (0)	0 (0)	3 (3.8)	**0.01**
Major thrombosis at diagnosis; n (%) N evaluable = 631	42 (6.7)	31 (7.8)	2 (2.4)	1(2)	4 (16.0)	4 (5.1)	0.07
Overall thrombosis at follow-up; n (%) N evaluable = 715	99 (13.8)	69 (14.7)	14 (15.4)	7 (14)	7 (26.9)	2 (2.6)	**0.01**
Overall major bleeding; n (%) N evaluable = 713	59 (8.3)	41 (8.7)	9 (10.0)	2 (3.9)	3 (11.5)	4 (5.1)	0.5
Overall minor bleeding; n (%) N evaluable = 709	90 (12.7)	54 (11.6)	13 (14.4)	7 (14.3)	7 (26.9)	9 (11.5)	0.2
Cardiovascular risk factors; n (%) N evaluable = 618	327 (52.9)	235 (59.5)	34 (44.7)	15 (34.1)	12 (48.0)	31 (39.7)	**<0.001**
Microcirculatory symptoms; n (%) N evaluable = 709	235 (33.1)	149 (32.0)	25 (28.1)	18 (36)	16 (61.5)	27 (34.6)	**0.03**
Median follow up; (months), range	106.4 (6.1–421.6)	105.9 (6.1–421.6)	104.9 (7.3–417.9)	101.1 (12.1–403.5)	140.3 (17.4–275.1)	106.6 (8.6–390.9)	0.7
MF progression; n (%)	53 (7.4)	23 (4.9)	20 (21.7)	4 (7.9)	5 (19.2)	1 (1.3)	**<0.001**
AML progression; n (%)	12 (1.7)	9 (1.9)	1 (0.7)	0 (0.0)	2 (7.7)	0 (0.0)	0.08
Death; n (%)	106 (14.8)	77 (16.3)	15 (16.3)	3 (5.9)	6 (23.1)	5 (6.4)	**0.04**

Abbreviations: TN, triple negative.

[a]Significant p values are highlighted in bold.

Leukocytosis (≥ 11×10^9/L) was documented in 16.4% of patients and extreme thrombocytosis (≥1000×10^9/L) in 17.5%. Palpable splenomegaly was present in 97 (13.5%) patients; 43 (6%) and 235 (33.1%) patients had constitutional and microcirculatory symptoms. Differences in overall, thrombosis-free, myelofibrosis-free (MFS), and leukemia-free survival among patients stratified by their mutational status are reported in Figure S1. Univariate analysis for MFS identified age > 60 years ($p = 0.02$; HR 1.9; 95%CI 1.1–3.9), male sex ($p = 0.001$; HR 2.4; 95%CI 1.4–4.2), palpable splenomegaly ($p = 0.002$; HR 2.5; 95%CI 1.4–4.5), constitutional symptoms ($p = 0.01$; HR 2.6; 95%CI 1.2–5.3) along with CALR mutation ($p = 0.01$; HR 2.1; 95%CI 1.2–3.6) and MPL mutation ($p = 0.008$; HR 3.2; 95%CI 1.4–4.7) as risk factors for fibrotic progression. Type 1/1-like CALR patients were more prone to fibrotic transformation compared with type 2/2-like ($p = 0.04$; HR 3.2; 95% CI 1.1–9.3), while

the risk was lower in JAK2V617F mutated patients ($p = 0.01$; HR 0.5; 95% CI 0.3–0.9). Multivariable analysis confirmed male sex, splenomegaly, constitutional symptoms, and mutational status (CALR type 1/1-like and MPL) as independent prognostic factors for MF progression (Table 2). We also found JAK2V617F VAF, as a continuous variable, to be correlated with MF progression ($p = 0.002$; HR 1; 95% CI 1–1.1).

A receiver operating characteristic (ROC) curve, used to determine the best cut-off level for JAK2V617F VAF predicting fibrotic evolution, showed an area under curve (AUC) of 0.76 (Figure S2) and best VAF value was 35%. Accordingly, we divided JAK2V617F mutated patients in those with VAF ≤ 35% (364; 77.3%) and >35% (107; 22.7%) (Table 3); the latter showed a significantly higher risk for MF progression ($p < 0.001$; HR 5.9; 95% CI 2.4–14.4) (Figure 1(A)), that remained significant ($p = 0.003$; HR 4.2; 95% CI 1.6–10.8) in

TABLE 2 Univariate and multivariable analysis of clinical and molecular risk factors for myelofibrosis-free survival (MFS) among 718 patients with essential thrombocythemia from University of Florence cohort, Italy

Clinical and laboratory variables	Univariate analysis p^a (HR; 95% CI)	Multivariable analysis^b p^a (HR; 95% CI)	Multivariable analysis^c p^a (HR; 95% CI)	Multivariable analysis^d p^a (HR; 95% CI)
White blood cells ($\times 10^9$/L) (continuous variable)	0.05 (0.9; 0.8–1)			
White blood cells ≥11×10^9/L	0.07 (0.4; 0.1–1.1)			
Hb (g/dl) (continuous variable)	0.4 (0.9; 0.7–1.1)			
Hct levels (%) (continuous variable)	0.5 (0.9; 0.8–1.1)			
Platelet count ($\times 10^9$/L) (continuous variable)	0.05 (0.9; 0.9–1)			
Platelet count ≥1000×10^9/L	0.05 (0.5; 0.2–1)			
Age >60 years	**0.02** (1.9; 1.1–3.9)	**0.005** (2.5; 1.3–4.9)	**0.02** (3.8; 1.3–11.4)	0.05 (1.9; 0.9–3.5)
Age (continuous variable)	**0.03** (1; 1–1.1)			
Male sex	**0.001** (2.4; 1.4–4.2)	**0.02** (2.1; 1.2–3.9)	0.3 (1.7; 0.7–4.2)	**0.01** (2; 1.2–3.5)
Constitutional symptoms	**0.01** (2.6; 1.2–5.3)	0.06 2.1 (0.9; 4.7)	0.3 (2.6; 0.5–12.1)	**0.04** (2.2; 1–4.8)
Palpable splenomegaly	**0.002** (2.5; 1.4–4.5)	**0.01** (2.1; 1.2–3.9)	**0.003** (4.3; 1.7–11)	**0.02** (2.1; 1.1–3.8)
Microcirculatory symptoms	0.5 (1.2; 0.7–2.2)			
JAK2 mut	**0.01** (0.5; 0.3–0.9)			
CALR 1/1-like and 2/2-like	**0.01** (2.1; 1.2–3.6)			
MPL mut	**0.008** 3.2 (1.4–7.7)			
TN	0.09 0.2 (0.02–1.3)			
CALR 1/1-like vs. 2/2-like	**0.04** (3.2; 1.1–9.3)			
CALR 1/1-like/MPL	**<0.001** (3.8; 2.2–6.6)	**<0.001** (3.4; 1.9–6.1)		
JAK2V617F VAF >35% vs. ≤35%	**<0.001** (5.9; 2.4–14.4)		**0.003** (4.2; 1.6–10.8)	
JAK2V617F > 35% vs. all the others	**0.009** (2.2; 1.2–4)			
CALR type 1/1-like + MPL + JAK2V617F > 35% vs. all the others	**<0.001** (6.1; 3.2–11.7)			**<0.0001** (5.2; 2.7–10)

^{a}Significant p values are highlighted in bold.
^{b}Considering all ET cohort ($n = 718$) with CALR type 1/1-like/MPL as molecular risk variable.
^{c}Considering only JAK2 patients ($n = 471$) with VAF >35% as molecular risk variable.
^{d}Considering all ET cohort ($n = 718$) with CALR type 1/1-like/MPL/ JAK2 VAF >35% as molecular risk variable.

TABLE 3 Clinical characteristics of JAK2V617F positive ET patients from University of Florence cohort stratified by their variant allele frequency (VAF, ≤ 35% vs. > 35%)

| Clinical and laboratory variables	All JAK2 patients (n = 471)	JAK2 V617F VAF ≤35% (n = 364; 77.3%)	JAK2 V617F VAF > 35% (n = 107; 22.7%)	p valuesa
Age in year; median (range)	62.0 (14.9–92.9)	60.1 (14.9–92.9)	66.5 (17.5–85.3)	**0.02**
Age ≥ 60 years; n (%)	253 (53.7)	183 (50.3)	70 (65.4)	**0.01**
Sex females; n (%)	318 (67.5)	258 (70.9)	60 (56.1)	**0.008**
White blood cells × 10^9/L; median (range) N evaluable = 458	8.7 (3.7–18.2)	8.6 (4.0–18.2)	9.2 (3.7–18.2)	0.1
White blood cells ≥11 × 10^9/L; n (%) N evaluable = 458	90 (19.6)	67 (18.9)	23 (22.3)	0.3
Hemoglobin, g/dl; median (range)	14.2 (11.8–16.5)	14.2 (11.8–16.5)	14.6 (11.8–16.5)	**0.02**
Hematocrit, % median (range)	43.8 (36.0–50.0)	43.4 (36.0–50.0)	45 (37.0–50.0)	**0.003**
Platelets × 10^9/L; median (range)	698 (455–1881)	694 (455–1867)	728 (455–1881)	**0.03**
Platelets ≥1000 × 10^9/L; n (%)	61 (12.9)	43 (11.8)	18 (16.8)	0.3
Palpable splenomegaly; n (%)	67 (14.2)	40 (11.0)	27 (25.2)	**0.0003**
Constitutional symptoms; n (%)	27 (5.7)	15 (4.1)	12 (11.2)	**0.009**
Major thrombosis before diagnosis; n (%) N evaluable = 405	35 (8.6)	25 (8.0)	10 (11.0)	0.4
Major thrombosis at diagnosis; n (%) N evaluable = 397	31 (7.8)	21 (6.8)	10 (11.2)	0.09
Overall thrombosis at follow-up; n (%) N evaluable = 470	69 (14.7)	52 (14.3)	17 (15.9)	0.7
Overall major bleeding; n (%) N evaluable = 470	41 (8.7)	34 (9.4)	7 (6.5)	0.4
Overall minor bleeding; n (%) N evaluable = 467	54 (11.6)	40 (11.1)	14 (13.1)	0.6
Cardiovascular risk factors; n (%) N evaluable = 395	235 (59.5)	186 (60.0)	49 (57.6)	0.7
Microcirculatory symptoms; n (%) N evaluable = 466	149 (32.0)	113 (31.4)	36 (34.0)	0.6
MF progression; n (%)	23 (4.9)	7 (1.9)	16 (14.9)	**<0.001**
AML progression: n (%)	9 (1.9)	5 (1.4)	4 (3.7)	0.1
Death; n (%)	77 (16.3)	51 (13.9)	28 (25.9)	**0.003**

Abbreviations: VAF, variant allele frequency.
aSignificant p values are highlighted in bold.

multivariable analysis including age, sex, splenomegaly, and constitutional symptoms. Among the latter covariates, only age > 60 years (p = 0.02; HR 3.8; 95% CI 1.3–11.4) and splenomegaly (p = 0.003; HR 4.3; 95% CI 1.7–11) remained significant (Table 2). Patients with JAK2V617F VAF > 35% were older (p = 0.02), more likely to be male (p = 0.008), and displayed higher hemoglobin (p = 0.02), platelet counts (p = 0.03), palpable splenomegaly (p < 0.001) and constitutional symptoms (p = 0.009). Moreover, patients with VAF > 35% showed a trend for reduced overall survival (p = 0.08; HR 1.5; 95% CI 0.9–2.4) without differences in thrombosis risk (p = 0.9; HR 0.9; 95% CI 0.5–1.6) and leukemic progression (p = 0.3; HR 2; 95% CI 0.5–7.7) (Figure S3). Multivariable analysis, including age, sex, palpable spleno-megaly, and constitutional symptoms, confirmed the independent contribution of JAK2V617F VAF > 35% (p < 0.001; HR 4.6; 95% CI

2.1–9.8) and presence of CALR type 1/1-like and MPL mutations (p < 0.001; HR 6; 95% CI 2.9–12.2) in predicting MF progression.The above noted observations were corroborated by analysis of two separate cohorts of patients with 2016 WHO-defined ET, according to the above reported criteria to avoid including incidentally cases of masked PV and pre-fibrotic MF: Mayo Clinic, USA, and Policlinico Gemelli, Catholic University, Rome, Italy. In the Mayo Clinic ET database, 604 patients (median age 58 years, range 18–90; females 63%) fully annotated for driver mutations were identified; information on JAK2V617F VAF was available in 206 of 347 JAK2V617F mutated patients, including 34 (17%) with VAF >35%. Accordingly, the total number of patients that were fully annotated for driver mutations and JAK2V617F VAF were 479: 199 (42%) JAK2V617F, 101 (21%) CALR type 1/1-like, 87 (18%) CALR type 2/2-like, 19 (4%) MPL and 73 (15%)
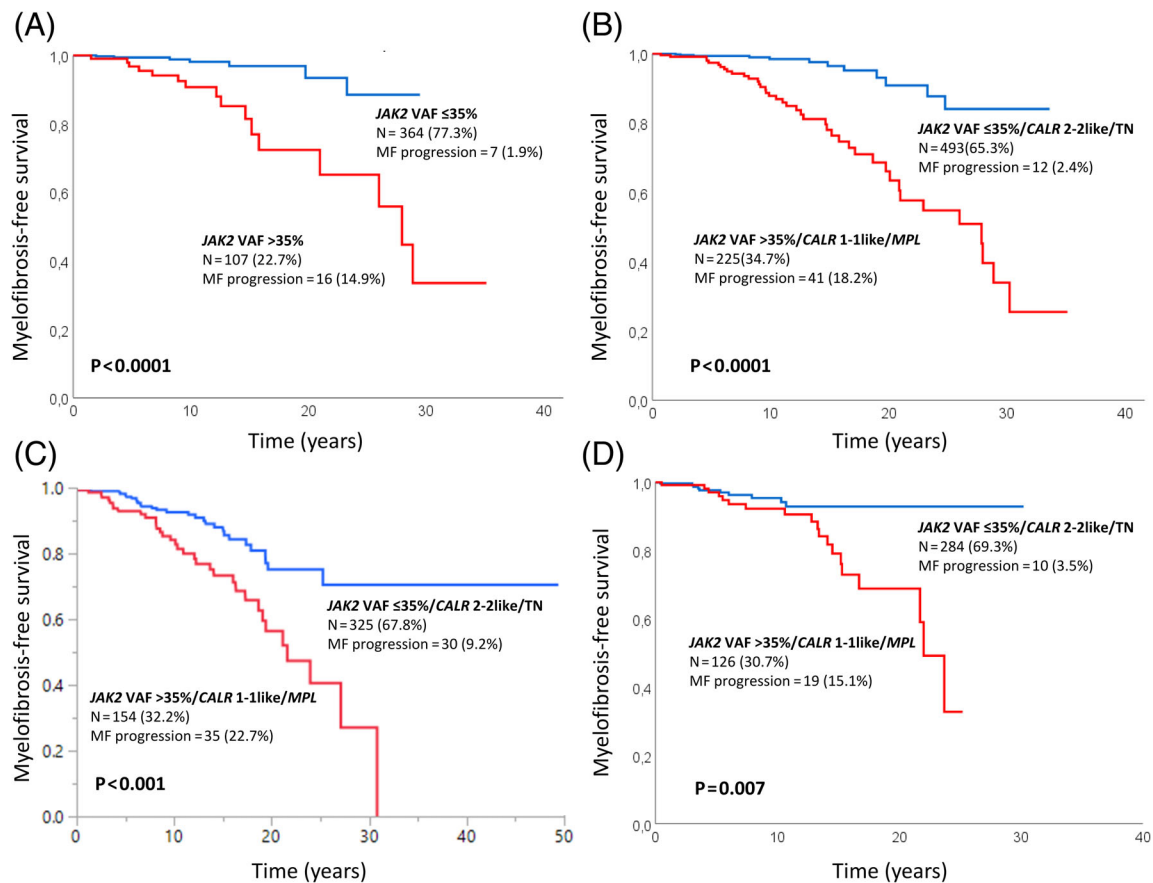
FIGURE 1 (A) Myelofibrosis-free survival (MFS) among 471 *JAK2*V617F mutated 2016-WHO essential thrombocythemia (ET) patients from University of Florence cohort, stratified by their VAF, ≤ 35% vs. >35%; (B) MFS among 7 182 016-WHO ET patients from University of Florence cohort, fully annotated for driver mutation type and *JAK2*V617F VAF, stratified by high-risk (*JAK2*V617F VAF >35%, *CALR* type 1/1-like and *MPL*) and low-risk (*JAK2*V617F VAF ≤35%, *CALR* type 2/2-like and TN) for MF progression. (C) External validation of the molecular model in 479 ET patients from Mayo Clinic, USA and (D) 410 from Policlinico Gemelli, Catholic University, Italy [Color figure can be viewed at wileyonlinelibrary.com]

TN. Median time of follow-up was 110 months (4–588). Multivariable analysis confirmed the independent contribution of *JAK2*V617F VAF >35% (*p* < 0.01; HR 4.0; 95% CI 1.9–8.2) and presence of *CALR* type 1/1-like and *MPL* mutations (*p* = 0.003; HR 2.2; 95% CI 1.3–3.8) in predicting MF progression; these risk factors remained significant when analysis was adjusted for age, sex, or palpable splenomegaly; among the latter, only splenomegaly displayed additional borderline significance (*p* = 0.07). The Policlinico Gemelli, Catholic University database, contained 454 patients (median age 54 years, range 16–92 years; females 67%) who were fully annotated for driver mutations and in 227 *JAK2*V617F patients VAF was available, including 62 (27%) with VAF >35%. Accordingly, the total number of patients that were fully annotated for driver mutations and *JAK2*V617F VAF were 410: 227 (55.4%) *JAK2*V617F, 45 (11%) *CALR* type 1/1-like, 33 (8%) *CALR* type2/2-like, 19 (4.6%) *MPL* and 86 (21%) TN. Median time of follow-up was 75.6 months (4–362.4). Multivariable analysis confirmed the independent contribution of *JAK2*V617F VAF >35% in predicting transformation to myelofibrosis (*p* = 0.01; HR, 2.9, 95%CI 1.2–7.0), that remained significant after adjusting for age, sex, and palpable splenomegaly. The latter covariate displayed a borderline significance

(*p* = 0.07); conversely, *CALR* type 1/1-like and *MPL* mutations were not statistically significant.

Subsequently, using the University of Florence patient cohort, we developed a two-tiered, driver mutation-based, model for risk of MF progression in ET identifying a high molecular risk (*JAK2*V617F VAF > 35%, *CALR* type 1/1-like and *MPL*) and low molecular risk (*JAK2*V617F VAF ≤ 35%, *CALR* type 2/2-like and TN) category. Kaplan-Meier analysis documented a significantly different MFS (*p* < 0.001; HR 6.1; 95% CI 3.2–11.7) with respective risk rates of MF evolution of 1.2% (population at risk, *n* = 195) and 8% (*n* = 100) at 10 years and 8% (*n* = 40) and 33% (*n* = 26) at 20 years, respectively for the low and high-risk category (Figure 1 (B)). The model remained statistically significant (*p* < 0.001; HR 5.2; 95%CI 2.7–10) in multivariable analysis including age, sex, splenomegaly, and constitutional symptoms; among the latter covariates, male sex (*p* = 0.01; HR 2; 95% CI 1.2–3.5), splenomegaly (*p* = 0.02; HR 2.1; 95% CI 1.1–3.8) and constitutional symptoms (*p* = 0.04; HR 2.2; 95% CI 1–4.8) remained independently significant whereas age > 60 years displayed a borderline significance (*p* = 0.05), (Table 2).

This operational molecular model developed in University of Florence cohort was validated in the other two independent cohorts. In the Mayo Clinic cohort, the rate of progression to MF was 9% (events = 30) and 23% (events = 35) in the low and high-risk category, with respective risk rates of 7% (population at risk; $n = 124$) and 16% ($n = 65$) at 10 years, and 25% ($n = 30$) and 44% ($n = 14$) at 20 years. Figure 1(C) illustrates application of the Florence molecular model for MF progression in ET, in the Mayo Clinic validation cohort ($p < 0.001$; HR 2.5; 95% CI 1.6–4.1). In the Catholic University, Policlinico Gemelli cohort, the rate of progression to MF was 3.5% (events $n = 10$) and 15% (events $n = 19$) in the low and high-risk category, with respective risk rates of 4.6% (population at risk; $n = 80$) and 7.8% ($n = 59$) at 10 years, and 7.1% ($n = 6$) and 31.2% ($n = 9$) at 20 years. Application of the molecular model, using the Policlinico Gemelli cohort, is shown in Figure 1(D) ($p = 0.007$, HR 2.7; 95% CI 1.3–5.8).

4 | DISCUSSION

To date, while many studies have investigated the presence of risk factors impacting on survival and an increased risk of thrombosis in ET, few data are available on the risk of progression to MF. With limitations imposed by the retrospective nature of analysis, the current study included a remarkable series of 1607 patients from three independent institutions, identified a category of patients at high risk for MF progression among 2016 WHO-defined ET patients. Firstly, Florence patient cohort was examined to identify independent risk factors for MFS, which included age > 60 years (HR 2.5, 95% CI 1.3–4.9), male sex (2.1, 1.2–3.9), palpable splenomegaly (2.1, 1.2–3.9), CALR 1/1-like or MPL mutation (3.4, 1.9–6.1) and JAK2V617F variant allele frequency > 35% (4.2, 1.6–10.8). Subsequently, an operational molecular risk category was developed and validated in the other two independent cohorts from Mayo Clinic and Rome. "High molecular risk" category included patients with JAK2V617F VAF > 35%, CALR type 1/1-like or MPL mutations whereas all other driver mutation profiles were assigned to "low molecular risk" category. All the clinical and molecular variables analyzed in the present study are routinely collected at diagnosis, including the VAF of JAK2V617F that is provided by the majority of assays employed in diagnostic laboratories. From a practical standpoint, the findings provided by the current study may be useful for patients' counseling and, prospectively, stratification in clinical trials. Most recently, targeted next generation sequencing (NGS) has revealed the presence of other mutations in many ET patients, with the most frequent being TET2 (16%) and ASXL1 (11%). In the same study, SH2B3, SF3B1, U2AF1, TP53, IDH2, and EZH2 mutations were associated with inferior overall, leukemia-free or myelofibrosis-free survival.[20] Consequently, these notions were incorporated in a molecular-clinical integrated prognostic model for survival (MIPSS-ET).[8] Future collaborative efforts will investigate possible correlations between the VAF of JAK2V617F and the presence/absence of additional mutations. Moreover, we have found a significantly higher risk of fibrotic progression in patients carrying type 1/1-like CALR mutations than in those with type 2/2-like. This result was highlighted in a previous study including a large cohort of ET patients, but without a multivariable model.[14] Interestingly, our observations are in according with a recent experimental paper,[21] in which the authors generated heterozygous and homozygous conditional inducible knock-in (KI) mice expressing a chimeric murine CALR del-52 (type 1) or ins-5 (type 2) with the human mutated C-terminal tail to investigate their pathogenic effects on hematopoiesis. Thus, Del52 induces greater phenotypic changes than ins5 including thrombocytosis, leucocytosis, splenomegaly, bone marrow hypocellularity, megakaryocytic lineage amplification. Moreover, homozygous del52 KI mice displayed features of a penetrant myelofibrosis-like disorder with extramedullary hematopoiesis linked to splenomegaly, megakaryocyte hyperplasia and the presence of reticulin fibers. In this regard, it would be interesting to evaluate in future studies the in vivo impact, if any, of VAF both for CALR type 1/1 like and type 2/2-like in large cohorts of ET patients.

We acknowledge potential limitations in the interpretation of findings reported herein. First, the retrospective nature of the study that may harbor intrinsic selection biases, and second, the absence of a centralized histopathological review of BM biopsies. In this respect, it is reasonable to entertain the possibility that some of the cases in the current study population might have represented occult cases of early/prefibrotic MF or masked PV. Moreover, as recently reported,[22,23] we recognize that a definite diagnosis of early cases of PV may require red cell mass (RCM) determination by isotopic methods. However, this is really far from a routine clinical scenario, therefore we made our best to adhere to the latest WHO criteria that uses BM histopathology along with subnormal erythropoietin levels as a potential surrogates. Finally, to minimize the inadvertent inclusion of cases with masked-PV we excluded those cases that exceeded the Hb/Hct threshold recommended by WHO, whereas cases with anemia were excluded as potential misdiagnosis of pre-fibrotic MF. On the other hand, it should be emphasized that histopathology analysis was performed, and clinical and hematological information were collected and evaluated, in centers of huge experience in MPNs, that may add to the quality and cleanness of the series reported in this manuscript.

Finally, the possibility that the current model merely selects morphologically bypassed pre-fibrotic MF cases or, in other words, the fact that VAF of JAK2V617F may help, together with clinical and histopathological picture, in discriminating true ET from pre-MF cases is an intriguing issue that might be studied in large cohorts of patients with a centralized histopathological review by a panel of experts.[24] However, from a practical standpoint, this possibility by itself does not undermine the main objective of the current study, which is to identify patients who require close monitoring and appropriate counseling.

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