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The prognostic contribution of *CBL*, *NRAS*, *KRAS*, *RUNX1*, and *TP53* mutations to mutation-enhanced international prognostic score systems (MIPSS70/plus/plus v2.0) for primary myelofibrosis

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Abstract

Contemporary risk models in primary myelofibrosis (PMF) include the mutation (MIPSS70) and mutation/karyotype enhanced (MIPSS70 plus/v2.0) international prognostic scoring systems. High molecular risk (HMR) mutations incorporated in one or both of these models include *ASXL1*, *SRSF2*, *EZH2*, *IDH1/2*, and *U2AF1*Q157; the current study examines additional prognostic contribution from more recently described HMR mutations, including *CBL*, *NRAS*, *KRAS*, *RUNX1*, and *TP53*. In a cohort of 363 informative cases (median age 58 years; 60% males), mutations included *JAK2* 61%, *CALR* 24%, *MPL* 6%, *ASXL1* 29%, *SRSF2* 10%, *U2AF1*Q157 5%, *EZH2* 10%, *IDH1/2* 4%, *TP53* 5%, *CBL* 5%, *NRAS* 7%, *KRAS* 4%, and *RUNX1* 4%. At a median follow-up of 4.6 years, 135 (37%) deaths and 42 (11.6%) leukemic transformations were recorded. Univariate analysis confirmed significant survival impact from the original MIPSS70/plus/v2.0 HMR mutations as well as *CBL* (HR 2.8; $p < .001$), *NRAS* (HR 2.4; $p < .001$), *KRAS* (HR 2.1; $p = .01$), and *TP53* (HR 2.4; $p = .004$), but not *RUNX1* mutations (HR 1.8; $p = .08$). Multivariate analysis (MVA) that included both the original and more recently described HMR mutations confirmed independent prognostic contribution from *ASXL1* (HR 1.8; $p = .007$), *SRSF2* (HR 4.3; $p < .001$), *U2AF1*Q157 (HR 2.9, $p = .004$), and *EZH2* (HR 2.4; $p < .001$), but not from *IDH1/2* ($p = .3$), *TP53* ($p = .2$), *CBL* ($p = .3$), *NRAS* ($p = .8$) or *KRAS* ($p = .2$) mutations. The lack of additional prognostic value from *CBL*, *NRAS*, *KRAS*, *RUNX1*, and *TP53* was further demonstrated in the setting of (i) MVA of mutations and karyotype, (ii) MVA of MIPSS70/plus/v2.0 composite scores and each one of the recently described HMR mutations, except *TP53*, and iii) modified MIPSS70/plus/plus v2.0 that included *CBL*, *NRAS*, *KRAS*, and *TP53* as part of the HMR constituency, operationally referred to as

"HMR+" category. Furthermore, "HMR+" enhancement of MIPSS70/plus/plus v2.0 did not result in improved model performance, as measured by C-statistics. We conclude that prognostic integrity of MIPSS70/plus/plus v2.0, as well as their genetic components, was sustained and their value not significantly upgraded by the inclusion of more recently described HMR mutations, including *CBL*, *NRAS*, *KRAS*, and *RUNX1*. Additional studies are needed to clarify the apparent additional prognostic value of *TP53* mutation and its allelic state.

1 | INTRODUCTION

Primary myelofibrosis (PMF), including prefibrotic and overt fibrotic PMF, is one of Philadelphia-negative myeloproliferative neoplasms (MPNs), which also enlist polycythemia vera (PV), essential thrombocythemia (ET), and MPN unclassifiable (MPN-U)/not otherwise specified (MPN-NOS) according to the latest International consensus (ICC)[1] and World Health Organization (WHO)[2] classification systems. Furthermore, ET or PV may develop a fibrotic phenotype over time, referred to as post-ET or post-PV myelofibrosis (MF).[1]

Main clinical manifestations of PMF include hepatosplenomegaly, anemia eventually leading to transfusion dependence, constitutional symptoms, cachexia, bone pain, pruritus, thrombosis, and bleeding. Causes of death are leukemic progression in approximately 20% of patients and comorbid conditions including cardiovascular events and consequences of cytopenias including infection or bleeding.[3]

Clinical heterogeneity of PMF reflects the complexity and heterogeneity of molecular landscape. Somatic mutations are operationally classified into "driver" and "other" mutations, mainly with a diagnostic and prognostic relevance, respectively. Driver mutations, which are usually mutually exclusive and responsible of constitutive activation of the JAK–STAT signaling, include *JAK2*V617F, exon 9 calreticulin (*CALR*) comprising type 1/1-like, type 2/2-like and noncanonical ones, and thrombopoietin receptor (*MPL* W515L/K/A) mutations, in approximately 60%, 20–25% and 5–8% of cases, respectively. Of note, a driver mutation is missing in 10–15% of PMF patients which are referred to as triple negative (TN).[4] Other mutations are described in more than 60% of PMF patients and include additional genomic abnormalities affecting genes involved in DNA methylation (*TET2*, *DNMT3A*, *IDH1*, and *IDH2*), histone modification (*ASXL1* and *EZH2*), mRNA splicing (*SF3B1*, *SRSF2*, *U2AF1*, and *ZRSR2*), signaling pathways (*LNK/SH2B3*, *CBL*, *NRAS*, *KRAS*, and *PTPN11*), and transcription factors (*RUNX1*, *NFE2*, *TP53*, and *PPM1D*).[5] Previous evidence supported prognostic contribution of type 1 (1-like) *CALR* mutation, which was associated with superior survival, whereas mutations in *ASXL1*, *SRSF2*, *EZH2*, and *IDH1/2*, formally defined as high molecular risk (HMR), were associated with an inferior survival, with more than one mutated gene harboring additional negative weight.[6] Moving from the consolidated dynamic international prognostic scoring system (DIPSS)[7] and plus version (DIPPS-plus),[8] but excluding age as risk factor, the aforementioned molecular variables along with clinical and laboratory variables as hemoglobin <10 g/dL, leukocyte count >25 × 10^9/L, circulating blasts ≥2%, constitutional symptoms, and marrow fibrosis grade ≥2, were included into the original mutation-enhanced international prognostic scoring system for transplant-age patients (MIPPS70)[9] and in plus version (including two cytogenetic risk categories)[9]; lately, MIPSS70 plus v2.0 was refined using three-tiered cytogenetic risk categories, *U2AF1*Q157 as an additional adverse mutation and revised sex-and severity-adjusted hemoglobin thresholds.[10]

The only potential curative treatment of PMF is allogeneic hematopoietic stem cell transplantation (allo-HSCT). Unfortunately, it is associated with 20–30% rate of transplant-related deaths or severe morbidity irrespective of the intensity of conditioning regimens and donor sources[11]; moreover, most patients are ineligible due to age and comorbidities. Accordingly, for the individual patient, the risk of allo-HSCT must be weighed against the expected survival without transplantation. DIPSS-plus and MIPPS70 plus/v2.0 scores are usually employed to risk-stratify patients and current treatment recommendations favor allo-HSCT for intermediate-2/high-risk disease, whereas a conservative therapeutic approach might be considered for intermediate-1/low-risk disease.[3,12]

More recently, mutations in *NRAS*, *KRAS*, *CBL*, *TP53*, and *RUNX1* were also associated with poor prognosis in PMF[13–15]; *RAS/CBL*-mutated patients also displayed poor symptom and spleen responses to JAK inhibitors.[16] Building upon these observations, the ICC authors suggested that, although there is no general consensus on how extensive the search for additional mutations by NGS should be in PMF patients, the inclusion of *TP53*, *NRAS/KRAS*, and *RUNX1* should be considered due to the impact on outcome and/or resistance to treatment.[17]

The aim of the current study was to elucidate the prognostic contribution of *CBL*, *NRAS*, *KRAS*, *RUNX1*, and *TP53* mutations on top of original MIPSS70/plus/v2.0 variables in a large PMF population of patients fully annotated for clinical and molecular data.

2 | MATERIALS AND METHODS

This retrospective study was approved by the Institutional review board of the Local Ethics Committee at of Azienda Ospedaliero-Universitaria Careggi (Florence, Italy; Mynerva project, #21267) and was conducted in accordance with the ethical guidelines of the Declaration of Helsinki. The study population consisted of PMF patients, both prefibrotic and overt fibrotic. The diagnosis of PMF and blast phase progression was confirmed according to the 2022 ICC and 5th

edition WHO classification criteria of myeloid neoplasms.[1,2] All the patients were followed until death or last follow-up, as assessed by medical records or through direct contact with patients or their physicians. All the patients underwent mutational analysis for driver mutations (*JAK2*V617F, *CALR* exon 9, and *MPL* W515) and targeted next generation sequencing (NGS) panel for at least 29 myeloid-relevant genes, including *ASXL1*, *EZH2*, *SRSF2*, *IDH1*, *IDH2* (defined as high-molecular risk-HMR) and *U2AF1*, *CBL*, *NRAS*, *KRAS*, *RUNX1*, and *TP53* in DNA from peripheral blood (PB) granulocytes as previously described.[9] Variants with a variant allele frequency (VAF) >5% were included. Functionally annotated variants were filtered based on the information retrieved from public databases (Single Nucleotide Polymorphism database [dbSNP], 1000 Genomes Project) and the potential pathogenetic role of filtered variants was assessed using available tools (SIFT, Polyphen, Catalogue of Somatic Mutations in Cancer [COSMIC]). Concerning *U2AF1* variants, only Q157 was considered for the purpose of the present study.[18] *TP53* mutations were categorized as single-hit or multihit in accordance with the ICC criteria.[1] Cytogenetic analysis and reporting were performed according to the International System for Human Cytogenetic Nomenclature criteria using standardized techniques.[19] Statistical analyses included clinical and laboratory parameters, obtained at diagnosis or first referral, that in 90% of cases coincided with sample collection for mutation analysis. Continuous variables were presented as the median (range) and categorical variables as the frequency (percentage). Differences in the distribution of continuous variables in the categories were compared by the Mann–Whitney *U* test. The χ^2 test was used for comparison of categorical variables. Cox proportional hazard regression model was used for univariate and multivariate analysis and to generate hazard ratios and 95% confidence intervals (CIs). Time to event analysis (overall [OS] and leukemia-free survival [LFS]) was performed using the method of Kaplan–Meier, with death (for OS), AML progression (for LFS), and allogeneic hematopoietic stem cell transplantation (both for OS and LFS) used as censors; survival curves were compared by the log-rank test. Comparison between the distinct preexisting scoring systems and the new enhanced proposed scores was done through the Harrell's' concordance index (C-index)[20] and 95% CIs, to evaluate the ability of the individual prognostic classifications to predict outcome. Statistical analyses were performed with SPSS software, version 27 (IBM-Corp), JMP Pro 15.1.0 software from SAS Institute (Cary, NC), and Statistical Package R version 4.1.1.

3 | RESULTS

3.1 | Patients' characteristics

The core of the study was constituted by 363 PMF patients followed at our Institution with clinical information concerning blood count at diagnosis, bone marrow histopathology, splenomegaly, presence of constitutional symptoms, and annotated for driver and additional myeloid mutations including classic high molecular risk (HMR) and *U2AF1*, *CBL*, *NRAS*, *KRAS*, *RUNX1*, and *TP53*. Cytogenetic information was available in 286 cases (78%); accordingly, the whole cohort was used for the validation of MIPSS70 score, whereas this subgroup was used for MIPSS70 plus/v2.0. Considering whole cohort of 363 patients, median age was 58 years (range18–89), and 60% were male. Median hemoglobin, leukocytes, and platelet count values were 12.3 g/dL, 8.7 $\times$ 10^9/L, and 413 $\times$ 10^9/L. Bone marrow fibrosis grade ≥2 was described in 62% of cases. A driver mutation was found in 89% of patients: *JAK2* V617F in 61%, exon 9 *CALR* in 24% (and *MPL* W515x in 6%; accordingly, 11% were triple negative (TN). A single HMR mutation was documented in 133 (37%) cases, whereas 56 (15%) cases had two or more HMR mutations. The most frequent nondriver-mutated genes were *ASXL1* ($n = 196$, 29%), *SRSF2* ($n = 37$, 10%), and *EZH2* ($n = 35$, 10%); among non-HMR genes, frequencies were as follows: *U2AF1* ($n = 19$, 5%) *CBL* ($n = 18$, 5%), *NRAS* ($n = 26$, 7%), *KRAS* ($n = 14$, 4%), *RUNX1* ($n = 15$, 4%), and *TP53* ($n = 17$, 5%). *TP53* mutations were single hit in 14/17 cases (82%).

Considering *TP53*-mutated patients, most ($n = 12$; 71%) were *JAK2*-mutated, 3 were *CALR*-mutated (18%), one *MPL* and one TN (6% each); the pattern of most frequent nondriver co-mutated genes included *ASXL1* ($n = 7$; 41%) followed by *U2AF1* ($n = 4$; 23%), *SRSF2* ($n = 3$; 18%), and *RUNX1* ($n = 2$; 12%). *NRAS*-mutated patients were mostly *CALR*-mutated ($n = 10$; 38%), followed by *JAK2* ($n = 8$; 31%), TN ($n = 7$; 27%), and *MPL* ($N = 1$; 4%); most frequent nondriver co-muted genes comprised *ASXL1* ($n = 19$; 73%), *EZH2* and *SRSF2* ($n = 6$ each, 23%), and *KRAS* ($n = 5$; 19%). *KRAS*-mutated patients were mostly *JAK2*-mutated ($n = 8$; 57%), followed by *CALR* ($n = 5$; 36%) and TN ($n = 1$; 7%); in addition to *NRAS*, they were frequently co-mutated with *ASXL1* ($n = 9$; 64%) and *EZH2* ($n = 3$; 21%). *CBL*-mutated patients were mostly *JAK2*-mutated ($n = 13$; 72%), followed by TN ($n = 3$; 17%), *CALR* and *MPL* ($n = 1$ each, 5%); the most frequent nondriver co-mutated genes were *ASXL1* ($n = 13$; 72%), *SRSF2* ($n = 5$; 28%), and *EZH2* ($n = 4$; 22%). Driver mutations in *RUNX1*-mutated patients were as follows: *JAK2* ($n = 8$; 53%), *CALR* ($n = 4$; 27%), TN ($n = 2$; 13%), and *MPL* ($n = 1$; 7%); non driver co-mutation pattern was mainly represented by *ASXL1* ($n = 8$; 53%) and *SRSF2* ($n = 4$; 27%) mutations. Overall, none of these mutations significantly clustered with any driver mutation, except for *CBL* ($p = .02$) and *NRAS* ($p < .001$) that clustered with TN category. Considering 286 evaluable cases, 19% ($n = 57$) had an abnormal karyotype defined as unfavorable or at very high-risk (VHR). Leukemic transformations were 42 (11%), whereas 135 (37%) patients died. Overall, in comparison with the whole cohort, laboratory, clinical and molecular characteristics of the cytogenetic annotated subgroup were similar, particularly concerning median age, blood cell count, molecular driver and nondriver distributions, and follow-up time, as highlighted in Table 1.

3.2 | Survival analysis

In order to decipher the impact of additional mutations on top of MIPSS/plus/v2.0 scores variables, we explored the impact of each variable on overall survival by univariate analysis that confirmed the prognostic impact of Hb values <10 g/dL (HR 3.7, $p < .001$); the

lower the hemoglobin level, the more significantly reduced the survival, according to sex- and severity-adjusted hemoglobin thresholds defined as moderate anemia (hemoglobin levels between 8 and 9.9 g/dL in women and between 9 and 10.9 g/dL in men) and severe anemia (hemoglobin levels of 8 g/dL in women and 9 g/dL in men), the latter with a HR of 8.6 ($p < .001$). In addition, leukocytes >25 × 10^9/L (HR 6.4; $p < .001$), circulating blasts ≥2% (HR 4.7; $p < .001$), bone marrow fibrosis grade ≥2 (HR 3.5; $p < .001$), and presence of constitutional symptoms (HR 2.7; $p < .001$) were confirmed as risk variables. Among molecular variables, the absence of type *CALR* type1/1-like mutations (HR 2.6; $p < .001$) and the presence of HMR mutations, with a HR between 2.5 and 5.6, were confirmed to have worst prognosis ($p \leq .01$ in all instances). Of interest, mutations in *CBL* (HR 2.8; $p < .001$), *NRAS* (HR 2.4; $p < .001$), *KRAS* (HR 2.1; $p = .01$), and *TP53* (HR 2.4; $p = .004$) were significantly associated with inferior survival, whereas borderline not significant value of *RUNX1* mutations (HR 1.8; $p = .08$) was reported. Considering adverse mutations under HMR (+/− *U2AF1*) and "HMR+" (including also *CBL*, *NRAS*, *KRAS*, *TP53*) categories, the unfavorable impact was incremental with higher number of mutated genes. In univariate analysis, the presence of two or more mutated genes under "HMR+" category (HR 4.9; $p < .001$) was significantly more detrimental for overall survival than a single "HMR+"-mutated gene (HR 4; $p < .001$). Moreover, the presence of an abnormal karyotype was confirmed to associate with worst prognosis, in particular for very high-risk category (HR 4.8; $p < .001$).

Multivariate analysis using each of the clinical and molecular variables of MIPSS70/plus/v2.0 scores confirmed their independent prognostic value, as reported in Table 2. In addition, in univariate analysis of genetic risk factors, a reduced leukemia-free survival (LFS) was predicted by the presence of *ASXL1* (HR 2.8; 95% CI 1.5–5.2; $p < .001$), *SRSF2* (HR 6.4; 95% CI 3.2–12.9; $p < .001$), *EZH2* (HR 2.6; 95% CI 1.2–5.7; $p = .02$), *CBL* (HR 4.1; 95% CI 1.7–9.7; $p < .001$), *NRAS*

TABLE 1 Characteristics of the patients included in the study considering cohorts adopted for MIPSS70 and MIPSS70 plus/v2.0.

Clinical and laboratory variables	MIPSS70 ($N = 363$)	MIPSS70 plus/v2.0 ($N = 286$)
Male sex; *n* (%)	217 (60)	166 (58)
Age at diagnosis, years; median (range)	58 (18–89)	57 (18–88)
Age >65 years; *n* (%)	113 (31)	88 (31)
Age ≤70 years; *n* (%)	296 (81)	235 (82)
Leukocytes, ×10^9/L; median (range)	8.7 (1.5–120)	8.7 (1.5–110)
Leukocytes >25 × 10^9/L; *n* (%)	34 (9)	27 (9)
Hemoglobin, g/dL; median (range)	12.3 (4.2–17.7)	12.5 (4.8–16.9)
Hemoglobin <10 g/dL; *n* (%)	66 (18)	39 (14)
Moderate anemia[a]; *n* (%)	52 (14)	33 (11)
Severe anemia[b]; *n* (%)	33 (9)	22 (8)
Platelets, ×10^9/L; median (range)	413 (10–1800)	472 (10–1720)
Platelets <100 × 10^9/L; *n* (%)	38 (10)	21 (8)
Circulating blasts ≥2%; *n* (%)	44 (12)	35 (12)
Bone marrow fibrosis grade ≥2; *n* (%)	224 (62)	142 (52)
Splenomegaly[c]; *n* (%)	240 (66)	182 (64)
Constitutional symptoms; *n* (%)	102 (28)	70 (25)
JAK2-mutated; *n* (%)	222 (61)	172 (60)
CALR-mutated; *n* (%)	89 (24)	71 (25)
MPL-mutated; *n* (%)	22 (6)	21 (7)
Double-mutated; *n* (%)	12 (3)	8 (3)
Triple negative; *n* (%)	39 (11)	30 (10)
ASXL1-mutated; *n* (%)	106 (29)	80 (28)
EZH2-mutated; *n* (%)	35 (10)	27 (9)
IDH1/2-mutated; *n* (%)	13 (4)	6 (2)
SRSF2-mutated; *n* (%)	37 (10)	28 (10)
U2AF1-mutated; *n* (%)	19 (5)	9 (3)
CBL-mutated; *n* (%)	18 (5)	12 (4)
NRAS-mutated; *n* (%)	26 (7)	23 (8)
KRAS-mutated; *n* (%)	14 (4)	10 (3)
RUNX1-mutated; *n* (%)	15 (4)	9 (3)
TP53-mutated; *n* (%)	17 (5)	13 (4)
Unfavorable karyotype[d] *n* (%); *n* evaluable = 286	57 (19)	57 (19)
Very high-risk karyotype[e]; *n* (%)	20 (7)	20 (7)
n evaluable = 286		
Unfavorable karyotype plus[f] *n* (%); *n* evaluable = 286	37 (13)	37 (13)
Deaths; *n* (%)	135 (37)	102 (36)
Leukemic transformation; *n* (%)	42 (11)	36 (12)
Follow-up years; median (range)	4.6 (0.1–36)	4.5 (0.1–35)

[a]Moderate anemia, defined by hemoglobin levels of 8 to 9.9 g/dL in women and 9 to 10.9 g/dL in men.
[b]Severe anemia defined by hemoglobin levels of 8 g/dL in women and 9 g/dL in men.
[c]Defined as >5 cm below the left costal margin.
[d]Unfavorable karyotype indicates any abnormal karyotype other than normal karyotype or sole abnormalities of 20q2, 13q2, +9, chromosome 1 translocation/duplication, 2Y, or sex chromosome abnormality other than −Y.
[e]Very high-risk (VHR): single/multiple abnormalities of −7, i(17q), inv (3)/3q21, 12p-/12p11.2, 11q-/11q23, or other autosomal trisomies not including +8/ + 9 (e.g., +21, +19).
[f]Unfavorable karyotype "plus" indicates any abnormal karyotype other than normal karyotype, sole abnormalities of 13q-, +9, 20q-, chromosome 1 translocation/duplication or sex chromosome including –Y and VHR abnormalities.

TABLE 2 Univariate and multivariate analysis for overall survival in PMF patients, considering MIPSS70/plus/v2.0 variables and additional detrimental mutations.

	Overall survival			
Clinical and laboratory variables	**Univariate analysis HR (95% CI) *p*[a]**	**Multivariate analysis HR (95% CI) *p*[a] (MIPSS variables)**	**Multivariate analysis HR (95% CI) *p*[a] (MIPSS plus variables)**	**Multivariate analysis HR (95% CI) *p*[a] (MIPSS plus v2.0 variables)**
Hemoglobin <10 g/dL	3.7 (2.6–5.4); **<0.001**	1.9 (1.3–2.8); **0.002**	2.2 (1.4–3.5); **0.002**	
Moderate anemia[a]	3.1 (1.9–5.3); **<0.001**			2.4 (1.4–4.1); **0.001**
Severe anemia[b]	8.6 (4.8–15.6); **<0.001**			4 (2.1–7.7); **<0.001**
Platelets <100 × 10^9/L	2.9 (1.9–4.4); **<0.001**	1.8 (1.1–2.8); **0.01**		
Leukocytes >25 × 10^9/L	6.4 (4–10.1); **<0.001**	3.9 (2.3–6.6); **<0.001**		
Circulating blasts ≥2%	4.7 (3.1–7.2); **<0.001**	1.7 (1.1–2.8); **0.02**	3 (1.7–5.1); **<0.001**	3.1 (1.8–5.2); **<0.001**
BM fibrosis grade ≥2	3.5 (2.2–5.7); **<0.001**	1.3 (1–1.9); **0.04**		
Constitutional symptoms	2.7 (1.9–3.8); **<0.001**	1.7 (1.1–2.4); **0.007**	1.4 (1.4–2.2); **0.04**	1.3 (1.1–1.9); **0.04**
Absence of type *CALR* type1/1-like mutations	2.6 (1.6–4.5); **<0.001**	2.6 (1.5–4.6); **0.001**	2 (1.1–3.8); **0.03**	1.9 (1–3.5); **0.04**
ASXL1 mutations	3.1 (2.2–4.3); **<0.001**			
EZH2 mutations	2.5 (1.6–3.9); **<0.001**			
IDH1/2 mutations	2.6 (1.2–5.2); **0.01**			
SRSF2 mutations	5.3 (3.4–8.2); **<0.001**			
U2AF1 mutations	3.4 (1.8–6.3); **<0.001**			
TP53 mutations	2.4 (1.3–4.4); **0.004**			
RUNX1 mutations	1.8 (0.9–3.9); 0.08			
CBL mutations	2.8 (1.6–5.1); **<0.001**			
NRAS mutations	2.4 (1.5–3.8); **<0.001**			
KRAS mutations	2.1 (1.2–3.9); **0.01**			
HMR mutation	3.6 (2.5–5.1); **<0.001**			
≥2 HMR mutations	4.8 (3.3–6.9); **<0.001**			
HMR + *U2AF1* mutations	3.8 (2.6–5-2); **<0.001**			
HMR + *U2AF1*+ other mutations[c]	4 (2.7–5.8); **<0.001**	2 (1.3–3.1); **0.002**	2.2 (1.4–4.2); **0.002**	2.3 (1.4–3.8); **0.002**
≥2 HMR + *U2AF1*+ other mutations[c]	4.9 (3.3–6.8); **<0.001**	3.3 (2.1–5.5); **<0.001**	4.3 (1.6–4.5); **<0.001**	3.9 (2.1–7.4); **<0.001**
Unfavorable karyotype[d]	3.1 (2.1–4.6); **<0.001**		2.7 (1.8–4.2); **<0.001**	
n evaluable = 286				
Very high-risk karyotype[e]	4.8 (2.9–7.8); **<0.001**			2.7 (1.5–5); **<0.001**
n evaluable = 286				
Unfavorable karyotype plus[f]	1.7 (1–2-7); **0.04**			1.9 (1.1–3.3); **0.03**
n evaluable = 286				

[a]Moderate anemia, defined by hemoglobin levels of 8 to 9.9 g/dL in women and 9 to 10.9 g/dL in men.
[b]Severe anemia defined by hemoglobin levels of 8 g/dL in women and 9 g/dL in men.
[c]Other mutations include *TP53*, *CBL*, *NRAS*, *KRAS* mutations.
[d]Unfavorable karyotype indicates any abnormal karyotype other than normal karyotype or sole abnormalities of 20q2, 13q2, +9, chromosome 1 translocation/duplication, 2Y, or sex chromosome abnormality other than –Y.
[e]Very high risk (VHR) includes single/multiple abnormalities of −7, i(17q), inv (3)/3q21, 12p-/12p11.2, 11q-/11q23, or other autosomal trisomies not including +8/ + 9 (e.g., +21, +19).
[f]Unfavorable karyotype "plus" indicates any abnormal karyotype other than normal karyotype, sole abnormalities of 13q-, +9, 20q-, chromosome 1 translocation/duplication or sex chromosome including –Y, and VHR abnormalities. Significant p values are highlighted in bold.

(HR 3.4; 95% CI 1.6–7.3; $p = .002$), and *RUNX1* (HR 7.1; 95% CI 3.7–17.6; $p < .001$) mutations. Conversely, *U2AF1* ($p = .2$) and *KRAS* ($p = .3$), *IDH1/2* ($p = .08$), and *TP53* ($p = .09$) mutations had no statistical significance. Overall, LFS was significantly affected by the presence of at least one mutated gene in the HMR category compared with no mutated gene (HR 3.5; 95% CI 1.9–6.7; $p < .001$); the

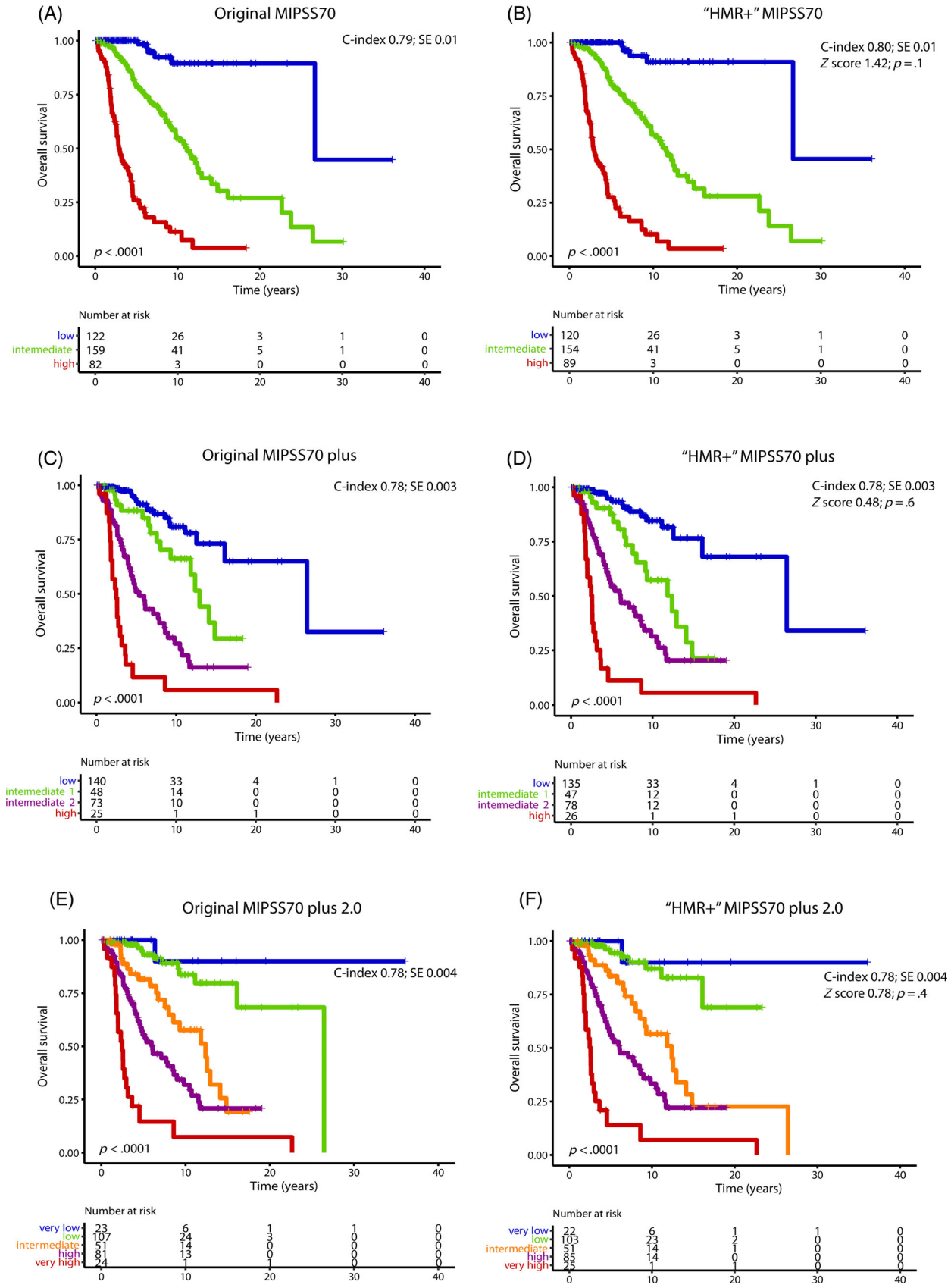

FIGURE 1 Overall survival (OS) by the original and "HMR+" MIPSS70 (A, B), MIPSS70 plus (C, D) and MIPSS70 plus v2.0 (E, F). Concordance (C) index was reported for each model and the results from the comparison of standard versus "HMR+" stratification were expressed by *Z*-score and *p* values at the right top part of B, D and F panels. [Color figure can be viewed at wileyonlinelibrary.com]

outcome was worst considering "HMR+" risk category (HR 6.3; 95% CI 2.9–13.4; $p < .001$). Moreover, similar to the OS, having ≥2 prognostically detrimental "HMR+"-mutated genes correlated with a more shortened LFS (HR 7.8; 95% CI 3.2–18.1; $p < .001$). All the above observations were confirmed also considering PMF patients ≤70 years aged only, representing 81% of the entire cohort (data not shown).

3.3 | Multivariate analysis for OS considering molecular and cytogenetic variables only

In multivariate analysis restricted to the presence of additional "HMR+" detrimental mutations, significance was retained for the presence of *ASXL1* (HR 1.8; 95% CI 1.2–2.8; $p = .007$), *EZH2* (HR 2.4; 95% CI 1.4–3.9; $p < .001$), *SRSF2* (HR 4.3, 95% CI 2.5–7.5; $p < .001$), and *U2AF1* (HR 2.9, 95% CI 1.4–6.1; $p = .004$) mutations only, unlike for *IDH1/2* ($p = .3$), *TP53* ($p = .2$), *CBL* ($p = .3$), *NRAS* ($p = .8$), and *KRAS* ($p = .2$). Adding cytogenetic to above analysis, we confirmed the independent strong significance especially for VHR category (HR 2.9; 95% CI 1.7–5; $p < .001$) and also for unfavorable karyotype (HR 2; 95% CI 1.2–3.6; $p = .01$); moreover, *ASXL1* (HR 1,9, 95% CI 1.1–3.1; $p = .01$), *EZH2* (HR 2.2; 95% CI 1.2–3.9, $p = .009$), *SRSF2* (HR 3.7; 95% CI 1.9–7; $p < .001$), *U2AF1* (HR 2.9; 95% CI 1.1–7.8; $p = .03$), and *TP53* (HR 2.2; 95% CI 1.1–4.7; $p = .03$) were confirmed as detrimental, unlike for *CBL* ($p = .6$), *NRAS* ($p = .8$), and *KRAS* ($p = .1$).

3.4 | Multivariate analysis by adding each new additional detrimental mutation to original MIPSS70 scores

Considering MIPSS70 scores as a variable, we added each new additional mutation, with the aim to decipher the individual prognostic contribution on OS. Starting from the original MIPSS70 score, the addition of *U2AF1* ($p = .08$) and *TP53* (0.07) mutations added a borderline significant detrimental value, whereas *CBL* ($p = .8$), *NRAS* ($p = .3$), and *KRAS* ($p = .6$) mutations were not significant. Concerning MIPSS70 plus, *CBL* ($p = .4$) and *NRAS* ($p = .9$) mutations were not significant, whereas *KRAS* ($p = .06$) had a trend of significance; on the contrary, the presence of *TP53* mutations (HR 2.4; 95% CI 1.2–4.6; $p = .01$) was clearly significant. Similar data were reported in MIPSS70 plus v2.0 with significance for *TP53* mutations (HR 2.4; 95% CI 1.2–4.5, $p = .01$), no impact documented for *CBL* ($p = .7$) and *NRAS* ($p = .8$), and a borderline significance for *KRAS* mutations ($p = .07$).

3.5 | "HMR+" category and MIPSS70 scores performances

Once established, the individual prognostic contribution of *U2AF1*, *CBL*, *NRAS*, *KRAS*, and *TP53* mutations in terms of reduced OS and LFS, we defined a new "HMR+" category aside the original HMR mutations (+/− *U2AF1*), with the aim to decipher the prognostic contribution on top of original MIPSS70/plus/v2.0 variables. To this end, we stratified the patients according to MIPSS70, MIPSS70 plus, and MIPSS70 plus v2.0, each of them in their original and "HMR+" version. While original version of MIPSS (Figure 1A), MIPSS plus (Figure 1C), and MIPPS plus v2.0 (Figure 1E) were confirmed to have

TABLE 3 Overall survival with hazard ratios and confidence intervals of PMF patients by original MIPSS70/plus/v2.0 and "HMR+" enhanced.

Original scores		
Category	**Median OS (range); years**	**HR (95% CI) p**
MIPSS		
Low	26.7 (2.4–50)	1
Intermediate	11.1 (9.2–13.1)	7.9 (3.4–18.4); **<0.001**
High	2.9 (2.3–3.4)	38.9 (16.6–90.9); **<0.001**
MIPSS plus		
Low	26.4 (11.8–41.1)	1
Intermediate-1	12.9 (11.1–14.7)	2.5 (1.3–4.9); **0.009**
Intermediate-2	5.4 (3.7–7)	6.9 (4–12.1); **<0.001**
High	2.5 (1.5–3.4)	18.1 (9.4–34.5); **<0.001**
MIPSS plus v2.0		
Very low	NR	1
Low	26.4 (18–32.2)	3 (0.4–24.3); 0.2
Intermediate	12.4 (7.5–17.3)	11.6 (1.5–92.1) **0.02**
High	6.1 (3.6–8.6)	21.7 (2.8–168); **0.003**
Very high	2.5 (1.6–3.4)	61 (7.6–420); **<0.001**
"HMR+" scores		
Category	**Median OS (range); years**	**HR (95% CI) p**
MIPSS		
Low	26.7 (2.4–50)	1
Intermediate	11.5 (9.7–13.4)	8.9 (3.6–22.3); **<0.001**
High	2.8 (2.2–3.3)	46.7 (18.6–117); **<0.001**
MIPSS plus		
Low	26.4 (11.8–41.1)	1
Intermediate-1	12.4 (7.6–17-1)	3.5 (1.7–7); **<0.001**
Intermediate-2	6.1 (3.5–8.7)	7.2 (4–13.2); **<0.001**
High	2.4 (1.4–3.2)	21.6 (10.9–42.6); **<0.001**
MIPSS plus v2.0		
Very low	NR	1
Low	25.1 (18–30)	2.1 (0.3–17); 0.2
Intermediate	12.3 (8.6–16.1)	9.1 (1.2–68); **0.03**
High	6.2 (3.3–8.8)	17.4 (2.4–127); **0.005**
Very high	2.6 (1.9–3.3)	51.7 (6.8–380); **<0.001**

Note: Significant *p* values are highlighted in bold.
Abbreviations: CI, confidence interval; HMR, high molecular risk; HR, hazard ratio, MIPSS, Molecular enhanced International Prognostic Scoring System; OS, overall survival; NR, not reached.

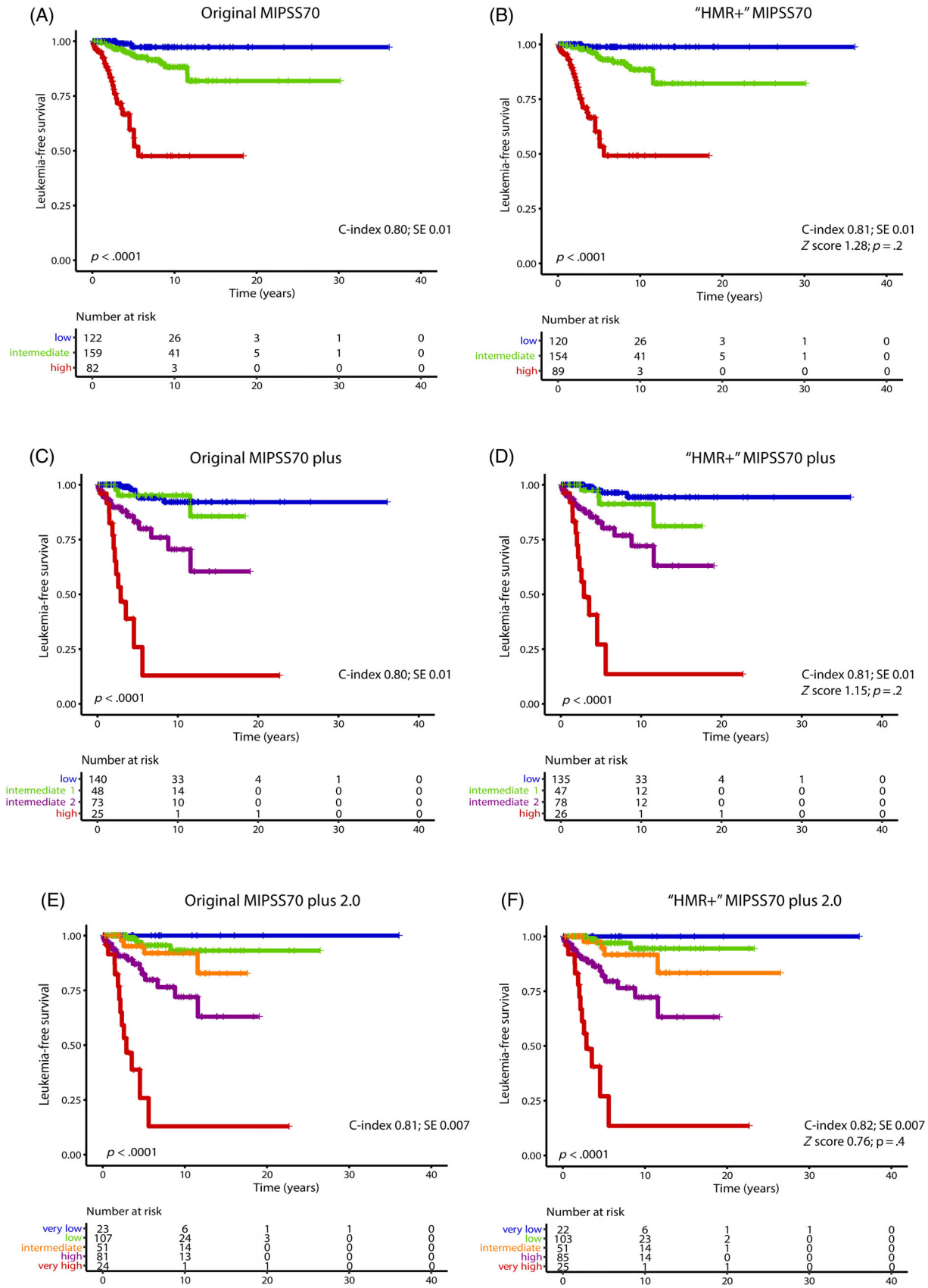

FIGURE 2 Leukemia free survival (LFS) by the original and "HMR+" MIPSS70 (A, B), MIPSS70 plus (C, D) and MIPSS70 plus v2.0 (E, F). Concordance (C) index was reported for each model and the results from the comparison of standard versus "HMR+" stratification were expressed by *Z*-score and *p* values in the bottom part of B, D and F panels. [Color figure can be viewed at wileyonlinelibrary.com]

a strong survival prediction with C-indexes of 0.79 and 0.78 (both for MIPPS70 plus and v2.0), their "HMR+" counterparts failed to statistically improve score performances; in particular, for "HMR+" MIPSS70, C index was 0.80 (*Z* score 1.42; $p = .1$, Figure 1B), whereas it was 0.78 both for "HMR+" MIPSS70 plus (*Z* score 0.48; $p = .6$, Figure 1D) and "HMR+" MIPSS70 plus v2.0 (*Z* score 0.78; $p = .4$, Figure 1F). In Table 3, the median overall survival and corresponding HRs (95% CI) for original and "HMR+" risk categories are summarized. Moreover, the original MIPSS70 scores were also confirmed to be effective in predicting LFS, without a statistically significant benefit by introducing "HMR+" category. In particular, "HMR+" MIPSS70 had a C index of 0.81 (vs. 0.80 of original counterpart, *Z* score 1.28; $p = .2$, Figure 2A, B), similarly to MIPSS70 plus (C index 0.81 vs. 0.80; Z score 1.15; $p = .2$, Figure 2C, D) and "HMR+" MIPSS70 plus v2.0 (C index of 0.82 vs. 0.81; *Z* score 0.76; $p = .4$, Figure 2E, F). Overall, these results reflect the low number of reclassified patients according to the "HMR+" models versus original ones. In particular, considering original MIPSS70 score, two low-risk patients were reclassified as intermediate and seven intermediate patients as high-risk. In MIPSS70 plus, four low-risk patients were reclassified as intermediate-1, one low-risk as intermediate-2, and five intermediate-1 as intermediate-2. Finally, in MIPSS plus v2.0, patients were reclassified as follows: one very low-risk to low-risk, four low-risk to intermediate, one low-risk to high-risk, four intermediate to high-risk, and one high-risk to very high-risk.

4 | DISCUSSION

Prognostic stratification in PMF is crucial for estimating the probability of disease progression and shortened survival, supporting main clinical decisions such as the patient's allocation to allo-HSCT. To this end, several prognostic models incorporating clinical and molecular variables were developed over the years.

The most recent models are MIPSS70,[9] MIPSS70 plus,[9] and MIPSS70 plus v2.0,[10] that are recommended also by the NCCN guidelines. These prognostic models included variables that highlighted the independent prognostic contribution of driver[21] and other mutations,[18,22,23] karyotype,[24] and hemoglobin levels.[25] In particular, MIPSS70 utilizes mutations and clinical variables, whereas MIPSS70 plus and MIPSS70 plus v2.0 include mutations, karyotype, and clinical variables. GIPSS[26] (the genetically inspired prognostic scoring system) is based exclusively on mutations (including *ASXL1*, *SRSF2*, and *U2AF1*) and karyotype. In addition, *RAS/CBL* mutations in PMF were associated with poor response to JAKi therapy, poor prognostic features, and inferior survival.[15,16] The significant prognostic contribution of *TP53* mutations is also documented.[13]

The current retrospective study, performed in a large, monocentric PMF patient cohort fully annotated for clinical and molecular information ($n = 363$) and partially for cytogenetic ($n = 286$) reports a comprehensive analysis on the prognostic contribution of *CBL*, *NRAS*, *KRAS*, *RUNX1*, and *TP53* mutations on top of original MIPSS70/plus/v2.0 variables. Starting from univariate analysis for OS, we confirmed the detrimental prognostic contribution of *CBL*, *NRAS*, *KRAS*, and *TP53* mutations (which we included in a "HMR+" category along with the established "HMR" mutations, i.e., *ASXL1*, *EZH2*, *SRSF2*, *IDH1/2*, and *U2AF1* mutations), whereas a borderline not significant effect of *RUNX1* mutations was documented, thereby leading to the exclusion of the latter from subsequent analyses. Once confirmed the significant prognostic value of all clinical, laboratory, and cytogenetic variables included in the original MIPSS70 scores, we sought to determine whether the new "HMR+" category would result in improved performance. To this end, we compared the performance of the different models through the calculation of relative C indexes. Overall, when we integrated MIPSS70 and MIPSS70 plus models with *CBL*, *NRAS*, *KRAS*, and *TP53* mutations, we did not observe any risk redistribution across the models or improvement of statistical power, suggesting that such mutations do not add relevant practical information to current systems. Overall, nine patients (2%) in MIPSS70, 10 (4%) in MIPSS70 plus, and 11 (4%) in MIPSS70 plus v2.0 were reassigned to different categories, being annotated to a higher risk category. The main reason is that the co-mutation pattern of *CBL*, *NRAS*, *KRAS*, and *TP53* mutations mainly included *ASXL1*, *EZH2*, and *SRSF2* mutations, already comprised in the HMR category. Of note, *ASXL1* and *SRSF2*, along with *U2AF1* mutations, have previously been independently associated with an inferior survival as highlighted by GIPSS score.[26]

Our study has some acknowledgeable limitations, primarily related to the retrospective design, spanning a relatively large enrolment period. This may have caused some biases, for instance, due to higher number of early diagnoses and changes in therapeutic approaches over time, possibly with an outcome influence. However, all considering, our study strongly confirms the validity and accuracy of MIPSS70 scores in risk-stratified PMF patients both for OS and LFS (C indexes from 0.78 to 0.82), highlighting the role of leukemic transformation in patients' outcome, particularly for those identified at a high or very high risk.

In conclusion, from a practical point of view, we suggest that current MIPSS70 scores are accurate enough to risk-stratify PMF patients and the inclusion of *CBL*, *NRAS*, and *KRAS* mutations does not improve their performance, largely due to co-mutational profile including mainly *ASXL1*, *EZH2*, and *SRSF2* mutations[15,16]; the *TP53* mutations, although not improving score performances by C index, should be including in MIPSS70 scores, owing to their independent role in predicting a dismal outcome. In this regard, *TP53* mutations were associated with a dismal outcome in PMF,[13] PV, and ET patients,[13,27] as in other myeloid neoplasms,[28,29] highlighting how in MPN, the *TP53* acquisition was a late event, leading to the multistep process of expansion over time of clone harboring *TP53* mutation.[13,30]

Recently, *TP53* allelic state (single-hit or multiple-hit events) was reported to be able in differentiating *TP53*-mutated MDS patients, with a high-risk presentation and poor outcomes specific to multihit patients only.[31] Similarly, in MF patients who are undergoing allo-HSCT, multihit *TP53* mutations represented a very high-risk group with a reduced survival and higher risk of AML progression[32]; conversely, single-hit *TP53* mutations showed similar outcome to patients

with wild-type *TP53*.[32] In the current study, the low number of cases prevented us by analyzing the individual prognostic contribution of different *TP53* mutations.

Overall, although larger studies are needed to decipher the weight of *TP53* allelic state in PMF patients, our results, along with previously noted observations, reinforce the needing to include *TP53* as HMR mutations in PMF, punctually evaluating over time the indication of allo-HSCT through MIPSS70 scores.

AUTHOR CONTRIBUTIONS

Giuseppe G. Loscocco, Alessandro M. Vannucchi, and Paola Guglielmelli designed the research and analyzed and interpreted data. Giuseppe G. Loscocco, Francesco Mannelli, Giacomo Coltro, Chiara Paoli, Maria Esposito, Alessandro M. Vannucchi, and Paola Guglielmelli provided direct patient care and data abstraction. Giada Rotunno, Francesca Gesullo, Fabiana Pancani, Leonardo Signori, and Chiara Maccari performed molecular analysis; Giuseppe G. Loscocco provided statistical analysis and prepared the tables and figures. Giuseppe G. Loscocco, Alessandro M. Vannucchi, and Paola Guglielmelli wrote the manuscript. All the authors checked and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

Giuseppe G. Loscocco and Paola Guglielmelli received personal fees for advisory board and/or lectures from Novartis. Alessandro M. Vannucchi received personal fees for advisory board and/or lectures from Novartis, AbbVie, AOP Pharmaceuticals, BMS and Incyte. All the other authors have no conflicts to report.

DATA AVAILABILITY STATEMENT

Non-identifiable data that support the findings of this study are available from the corresponding author with an appropriate request

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