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CLINICAL TRIALS AND OBSERVATIONS

Clinical effect of driver mutations of *JAK2*, *CALR*, or *MPL* in primary myelofibrosis

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Key Points

- Patients with PMF may carry *JAK2* (V617F), a *CALR* exon 9 indel, an *MPL* exon 10 mutation, or none of these genetic lesions.
- The genetic subtypes of PMF differ substantially as regards clinical course, disease progression, and overall survival.

We studied the impact of driver mutations of *JAK2*, *CALR*, (calreticulin gene) or *MPL* on clinical course, leukemic transformation, and survival of patients with primary myelofibrosis (PMF). Of the 617 subjects studied, 399 (64.7%) carried *JAK2* (V617F), 140 (22.7%) had a *CALR* exon 9 indel, 25 (4.0%) carried an *MPL* (W515) mutation, and 53 (8.6%) had nonmutated *JAK2*, *CALR*, and *MPL* (so-called triple-negative PMF). Patients with *CALR* mutation had a lower risk of developing anemia, thrombocytopenia, and marked leukocytosis compared with other subtypes. They also had a lower risk of thrombosis compared with patients carrying *JAK2* (V617F). At the opposite, triple-negative patients had higher incidence of leukemic transformation compared with either *CALR*-mutant or *JAK2*-mutant patients. Median overall survival was 17.7 years in *CALR*-mutant, 9.2 years in *JAK2*-mutant, 9.1 years in *MPL*-mutant, and 3.2 years in triple-negative patients. In multivariate analysis corrected for age, *CALR*-mutant patients had better overall survival than either *JAK2*-mutant or triple-negative patients. The impact of genetic lesions on survival was independent of current prognostic scoring systems. These observations indicate that driver mutations define distinct disease entities within PMF. Accounting for them is not only relevant to clinical decision-making, but should also be considered in designing clinical trials. (*Blood*. 2014;124(7):1062-1069)

Introduction

Primary myelofibrosis (PMF) is a Philadelphia-negative myeloproliferative neoplasm (MPN) characterized by abnormal proliferation of megakaryocytes, deposition of fibrous connective tissues in the bone marrow, abnormal stem cell trafficking, and extramedullary hematopoiesis (myeloid metaplasia).[1,2] In an international study of 1054 patients with PMF, the overall median survival was found to be 5.8 years, but considerable variability was observed.[3] This study identified age >65 years, presence of constitutional symptoms, hemoglobin level <10 g/dL, leukocyte count >25 × 10^9/L, and circulating blast cells 1% or greater as independent predictors of shortened survival at diagnosis. The use of these parameters led to the definition of the International Prognostic Scoring System (IPSS), which identifies 4 prognostic groups with substantially different survival in PMF.[3] A subsequent study investigated whether the acquisition of the above factors during follow-up predicted survival of PMF patients, and eventually led to the development of the dynamic IPSS (DIPSS).[4]

The 2008 World Health Organization (WHO) definition of PMF includes *JAK2* (V617F) or *MPL* (W515) mutations as a major diagnostic criterion that unequivocally proves the clonal nature of the disease.[1] However, the genomic landscape of PMF has changed considerably since then.[5] In 2013, somatic mutations of *CALR*, the gene encoding calreticulin, have been found in 20% to 25% of patients with essential thrombocythemia (ET) or PMF.[6,7] Like *JAK2* and *MPL* mutations, somatic mutations of *CALR* behave as driver mutations responsible for the myeloproliferative phenotype.[5] Recent studies have also identified subclonal mutations in genes like *ASXL1*, *SRSF2*, *EZH2*, *IDH1*, and *IDH2*, which are commonly associated with disease progression and identify PMF patients at high risk for leukemic transformation or premature death.[8,9]

In the original study on the identification of calreticulin mutations in patients with ET or PMF, a multivariate Cox regression analysis of overall survival (OS) showed that patients with a *CALR* mutation had a lower risk of death than those with *JAK2* (V617F) or an *MPL*

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Table 1. Demographic and clinical features at diagnosis of 617 patients with PMF subdivided according to their genotype (*JAK2*, *CALR*, and *MPL* mutation status)

	JAK2 (V617F)-mutant patients	*CALR*-mutant patients	*MPL*-mutant patients	Patients with nonmutated *JAK2*, *CALR*, and *MPL* (triple-negative subjects)	*P*
No. (%)	399 (64.7%)	140 (22.7%)	25 (4.0%)	53 (8.6%)	
Sex (male/female)	266/133	77/63	17/8	34/19	.101
Age at onset, median (range), y	63 (18-91)	50 (26-83)	64 (31-84)	67 (31-88)	<.001
Hemoglobin, median (range), g/dL	12 (3-19.6)	11.7 (7.1-15.9)	11 (6.5-15)	9.9 (5-19)	<.001
WBC count, median (range), $\times 10^9$/L	10 (1.6-106.2)	8.2 (2.2-45)	8.4 (2.1-20.3)	8.4 (2.4-90.8)	.002
PLT count, median (range), $\times 10^9$/L	310 (25-1963)	509 (46-1563)	307 (53-958)	175 (19-3279)	<.001
Circulating blasts, median (range), %	0 (0-20)	0 (0-10)	0 (0-4)	0 (0-16)	<.001
Lactate dehydrogenase, median (range), mU/mL	553 (149-3440)	692 (203-3610)	580 (183-2291)	531 (160-3173)	.208
Circulating $CD34^+$ cells, median (range), $\times 10^6$/L	16.2 (0.8-1190)	34.2 (1.7-1902)	100 (6.3-506.3)	45.3 (1.6-485.5)	.022
IPSS risk group, %					
Low	31	51	28	10	<.001
Intermediate 1	31	23	36	26	
Intermediate 2	22	18	24	17	
High	16	8	12	47	

mutation.[6] In the current work, we studied a large population of patients with PMF followed at 4 different centers and analyzed the impact of driver mutations of *JAK2*, *CALR*, or *MPL* on clinical course, risk of leukemic transformation, and OS.

Patients and methods

This study was approved by the institutional ethics committee (Comitato di Bioetica, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico [IRCCS] Policlinico, San Matteo, Pavia, Italy), and by the institutional review boards of the remaining centers. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000, and samples were obtained after patients had provided written informed consent.

Study population and definitions

Inclusion in the current study required the availability of demographic, clinical, and hematologic data at diagnosis (age, evaluation of constitutional symptoms, hemoglobin level, white blood cell count, and percentage of blasts in peripheral blood) that allow calculation of IPSS, and at least 1 DNA sample to assess mutation status of the 3 driver genes: *JAK2*, *MPL*, and *CALR*. A total of 617 patients with PMF were recruited from 4 centers: 187 from the Department of Hematology Oncology and 171 from the Center for the Study of Myelofibrosis (Pavia, Italy), 163 from the University Hospital (Florence, Italy), and 96 from the Hospital Clínic (Barcelona, Spain).

Diagnoses of PMF and that of leukemic transformation (blast phase) were performed in accordance with the 2008 WHO criteria.[1] For the assessment of bone marrow fibrosis, paraffin sections were stained with Gomori silver impregnation technique, and fibrosis was assessed semiquantitatively following the European consensus guidelines.[10] Thrombotic events were defined as described in detail by the CYTO-PV Collaborative Group.[11] IPSS and DIPSS risks were estimated as previously described.[3,4]

JAK2, *CALR*, and *MPL* mutation analysis

Granulocyte *JAK2* (V617F) mutation status and mutant allele burden were assessed using a quantitative polymerase chain reaction–based allelic discrimination assay on a Rotor-Gene 6000 real-time analyzer (Qiagen), as previously described.[12-14] Patients without *JAK2* (V617F) were evaluated for *MPL* exon 10 mutations using a high-resolution melt assay or Sanger sequencing.[15,16] Patients with nonmutated *JAK2* and *MPL* were studied for *CALR* exon 9 mutations as reported in our original article[6] or by Sanger sequencing, as described elsewhere.[16]

Statistical analysis

Numerical variables have been summarized by their median and range, and categorical variables by count and relative frequency (%) of each category. Comparisons of quantitative variables between groups of patients were carried out by the nonparametric Wilcoxon rank-sum test. The Wilcoxon signed-rank test was applied to compare measures of quantitative variables repeated in different phases of the disease. Association between categorical variables (2-way tables) was tested by the Fisher exact test. The cumulative incidence of anemia, thrombocytopenia, marked leukocytosis, thrombotic events, and leukemic transformation was estimated with a competing risk approach, considering death for any cause as a competing event.[17] The comparison of cumulative incidence curves in different groups of patients was carried out using the Pepe-Mori test,[18] whereas the effect of quantitative covariates was estimated by applying the Fine-Gray regression model.[19] OS was estimated using the Kaplan-Meier product limit method, and survival curves of different subgroups (*JAK2*-mutant, *CALR*-mutant, *MPL*-mutant, and triple-negative patients) were compared by the log-rank test. Multivariate analysis of OS was carried out by Cox regression. Prognostic scores were analyzed as both a fixed and a time-dependent covariate. The Akaike information criterion (AIC) was applied to compare quality of models.[20] This criterion provides a measure of the relative goodness of fit of a statistical model and a means for comparison among models, a lower AIC value indicating a better tradeoff between fit and complexity.

All *P* values were considered statistically significant when <.05 (2-tailed). Statistical analyses were performed using Stata 12.1 (StataCorp LP) software.

Results

Presenting hematologic and clinical features of PMF patients according to *JAK2*, *CALR*, and *MPL* mutation status

Of the 617 patients studied, 399 (64.7%) carried *JAK2* (V617F), 140 (22.7%) a *CALR* exon 9 indel, 25 (4.0%) an *MPL* (W515) mutation, and 53 (8.6%) had nonmutated *JAK2*, *MPL*, and *CALR* (ie, triple-negative subjects). Clinical phenotypes at diagnosis are reported in Table 1. *CALR*-mutant patients were significantly younger, had lower leukocyte count, higher platelet count, and lower IPSS risk. On the opposite, triple-negative patients were older, had lower hemoglobin level, lower platelet count, and higher IPSS risk (*P* values in Table 1).

Different types of *CALR* exon 9 mutations and their frequency

Within 140 *CALR*-mutant patients, 101 (72%) had the 52-bp deletion (L367fs*46, type 1 mutation), 22 (16%) had the 5-bp insertion (K385fs*47, type 2 mutation), and 17 (12%) carried other less frequent indels. The frequency of type 1 mutation in patients with PMF was significantly higher than that previously reported by us in patients with ET[16] (72% vs 46%, $P < .001$).

Risk of development of anemia, thrombocytopenia, marked leukocytosis, and large splenomegaly during the clinical course according to *JAK2*, *CALR*, and *MPL* mutation status

We estimated the time to development of anemia (here defined as a hemoglobin level <10 g/dL), thrombocytopenia (platelet [PLT] count $<100 \times 10^9$/L), and marked leukocytosis (white blood cell [WBC] count $>25 \times 10^9$/L) using a competing risk approach.

As shown in Figure 1A, *CALR*-mutant patients had a lower cumulative incidence of anemia compared with *JAK2*-mutant ($P < .001$), *MPL*-mutant ($P = .004$), and triple-negative patients ($P < .001$). On the opposite, triple-negative patients were more likely to develop anemia compared with either *CALR*-mutant ($P < .001$) or *JAK2*-mutant subjects ($P = .013$).

The cumulative incidence of thrombocytopenia was significantly lower in *CALR*-mutant patients compared with the remaining ones ($P = .001$), whereas no significant difference was observed between triple-negative and *JAK2*-mutant ($P = .292$) or *MPL*-mutant patients ($P = .627$) (Figure 1B).

The cumulative incidence of marked leukocytosis was significantly lower in *CALR*-mutant patients compared with *JAK2*-mutant ($P = .004$) or triple-negative patients ($P < .001$). These latter showed a particularly high risk at 3 years (>30%, Figure 1C).

Large splenomegaly was defined as a spleen tip extending >10 cm from left costal margin, as previously reported.[21] Time to large splenomegaly could be estimated only in patients from the Pavia Center for the Study of Myelofibrosis. *CALR*-mutant patients had a significantly longer large-splenomegaly-free survival compared with the remaining patients with PMF ($P < .001$, data not shown).

Cumulative risk of thrombosis according to *JAK2*, *CALR*, and *MPL* mutation status

Thrombotic complications occurred in 76 of 617 patients (12%) with PMF. The 10-year cumulative incidence of thrombosis was 18.3% (95% confidence interval [CI], 13.3-24.1) in patients carrying *JAK2* (V617F), 13.6% (95% CI, 6.9-22.7) in those with *CALR* mutation, 17.9% (95% CI, 4.1-39.6) in those with *MPL* mutation, and 16.2% (95% CI, 6.3-30.1) in triple-negative subjects (Figure 2). Patients carrying a *CALR* mutation had a lower risk of thrombosis than those carrying *JAK2* (V617F) ($P = .021$), whereas no significant difference was observed by comparing the other genotypic subgroups.

The above difference in risk of thrombosis remained statistically significant after adjusting for age. In fact, when comparing *JAK2*-mutant and *CALR*-mutant patients, the subdistribution hazard ratio (SHR) was 2.19 (95% CI, 1.15-4.18, $P = .017$), indicating that the risk of thrombosis was about twofold in the former. When taking into account the type of *CALR* mutation, both patients with type 1 and those with type 2 mutation had a lower risk of thrombosis compared with patients carrying *JAK2* (V617F) ($P = .042$ and $P = .021$, respectively).

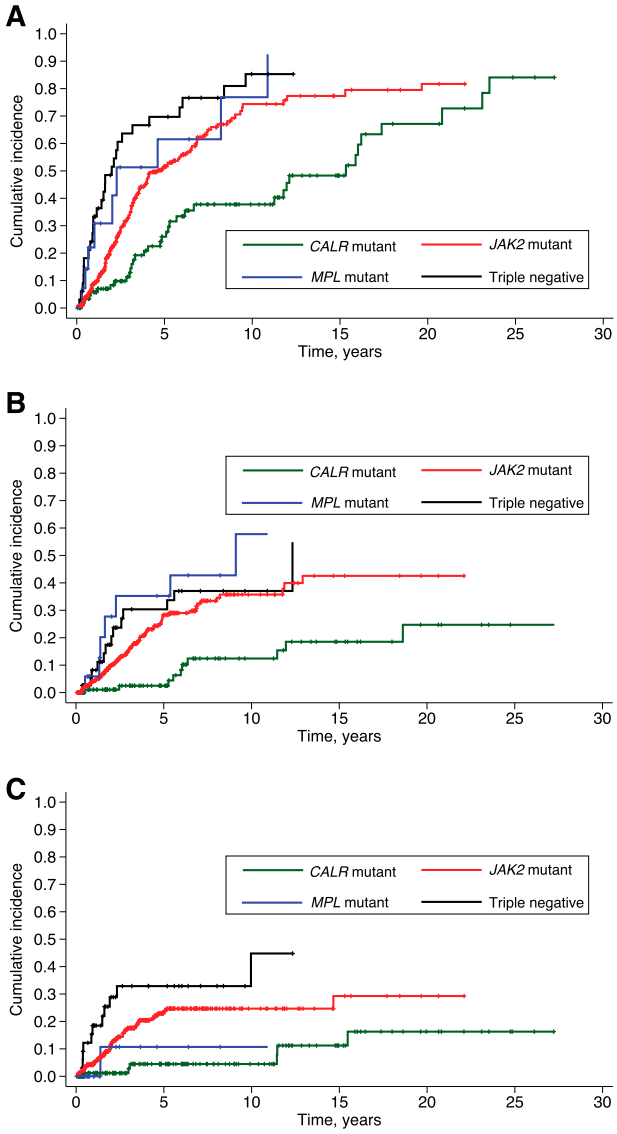


Figure 1. Cumulative incidence of anemia, thrombocytopenia, and marked leukocytosis in PMF patients stratified according to their driver mutation. The thresholds for hemoglobin level and WBC count are those of the IPSS,[3] whereas that for PLT count is the lower limit of normal range. Cumulative incidences were estimated with a competing risk approach, considering death for any cause as a competing event. Vertical tick marks indicate right-censored patients. (A) Cumulative incidence of anemia (hemoglobin <10 g/dL). *CALR*-mutant patients had a lower incidence of anemia compared with the remaining patients (maximum *P* value equal to .004). (B) Cumulative incidence of thrombocytopenia (PLT count $<100 \times 10^9$/L). The cumulative incidence of thrombocytopenia was significantly lower in *CALR*-mutant patients compared with the remaining ones ($P < .001$ in all comparisons). (C) Cumulative incidence of marked leukocytosis (WBC count $>25 \times 10^9$/L). The cumulative incidence of marked leukocytosis was significantly lower in *CALR*-mutant patients compared with *JAK2*-mutant ($P = .004$) or triple-negative patients ($P < .001$).

Progression to blast phase according to *JAK2*, *CALR*, and *MPL* mutation status

Seventy-four of 617 patients (12%) progressed to blast phase (leukemic transformation), including 43 of 399 subjects (11%) with *JAK2* mutation, 17 of 140 (12%) with *CALR* mutation, 3 of 25 (12%) with *MPL* mutation, and 11 of 53 triple-negative subjects (21%).

The 10-year cumulative incidence of leukemic transformation was 19.4% (95% CI, 13.9-25.6) in *JAK2*-mutant, 9.4% (95% CI, 4.3-16.8) in *CALR*-mutant, 16.9% (95% CI, 4.1-37.1) in *MPL*-mutant, and 34.4% (95% CI, 16.8-52.8) in triple-negative subjects (Figure 3).

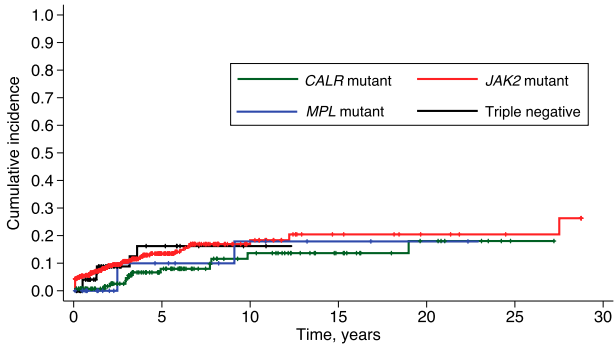


Figure 2. Cumulative incidence of thrombosis in PMF patients stratified according to their driver mutation. Vertical tick marks indicate right-censored patients. *JAK2*-mutant patients had a higher incidence of thrombosis than those with *CALR* mutation ($P = .021$). This difference remained statistically significant after adjusting for age (SHR, 2.19; 95% CI, 1.15-4.18; $P = .017$), the estimated risk of thrombosis being about 2-fold in *JAK2*-mutant compared with *CALR*-mutant patients.

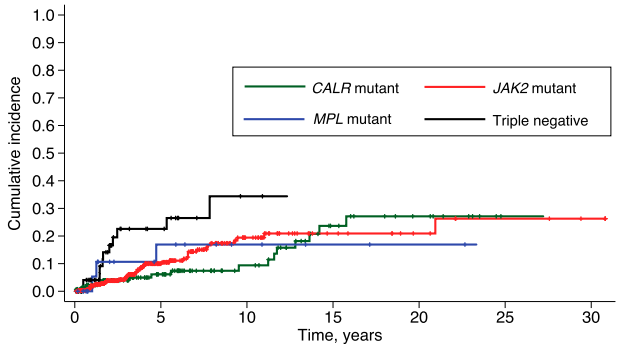


Figure 3. Cumulative incidence of leukemic transformation in PMF patients stratified according to their driver mutation. Vertical tick marks indicate right-censored patients. Triple-negative patients had higher incidence of leukemic transformation compared with both *CALR*-mutant and *JAK2*-mutant patients (maximum *P* value equal to .043).

These latter patients showed a higher incidence of leukemic transformation compared with both *CALR*-mutant ($P = .016$) and *JAK2*-mutated patients ($P = .043$). After adjusting for age, the risk of leukemic transformation remained higher in triple-negative patients compared with *JAK2*-mutant patients ($P = .04$), whereas the significance of the difference between triple-negative and *CALR*-mutant patients was borderline ($P = .052$).

OS according to *JAK2*, *CALR*, and *MPL* mutation status

The median follow-up of the study population was 3.5 years (range, 0.1-30.8 years). Death occurred in 176 patients (28.5%), including 115 of 399 patients with *JAK2* (V617F) (29%), 27 of 140 with a *CALR* exon 9 indel (19%), 10 of 25 (40%) with an *MPL* (W515) mutation, and 24 of 53 triple-negative patients (45%).

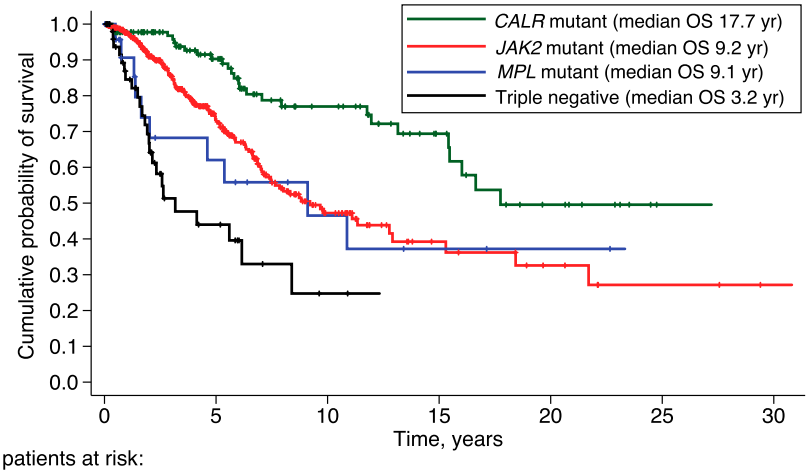
Median OS was 17.7 years in *CALR*-mutant, 9.2 years in *JAK2*-mutant, 9.1 years in *MPL*-mutant, and 3.2 years in triple-negative patients, as shown in Figure 4. In univariate analysis, *CALR*-mutant patients had a better OS than *JAK2*-mutant (hazard ratio [HR] 2.3, $P < .001$), *MPL*-mutant (HR 2.6, $P = .009$), and triple-negative patients (HR 6.2, $P < .001$). In a multivariate analysis corrected for age, *CALR*-mutant patients maintained a better OS compared with either *JAK2*-mutant ($P = .019$) or triple-negative patients ($P < .001$).

When considering the type of *CALR* mutation, patients carrying a type 1 *CALR* mutation had a better OS compared with patients carrying *JAK2* (V617F) ($P < .001$). No difference in OS was observed between patients with type 1 and those with type 2 *CALR* mutation ($P = .235$), and between patients with type 2 *CALR* mutation and those with *JAK2* (V617F) ($P = .311$). These results were confirmed after adjusting for time-dependent DIPSS, with a better OS of patients with type 1 *CALR* mutation compared with those with *JAK2* (V617F) (HR 2.01, $P = .04$), and no difference between patients with type 1 and those with type 2 *CALR* mutation, as well as between patients with type 2 *CALR* mutation and those with *JAK2* (V617F).

Relative contribution of *JAK2*, *CALR*, and *MPL* mutation status to OS as predicted by IPSS or DIPSS

The impact of the driver mutations on OS was independent of IPSS at diagnosis and of time-dependent DIPSS (maximum *P* value equal to .033).

To further examine the impact of driver mutations on the clinical course of the disease, we subdivided PMF patients into 2 subgroups according to their IPSS risk: the "lower-risk" subgroup included



No. of patients at risk:

CALR mutant	140	72	37	19	9	1
JAK2 mutant	396	135	39	13	7	3
MPL mutant	25	10	5	3	2	0
Triple negative	53	11	2	0	0	0

Figure 4. Kaplan-Meier analysis of survival of PMF patients stratified according to their driver mutation. Vertical tick marks indicate right-censored patients. In univariate analysis, *CALR*-mutant patients had a better OS than *JAK2*-mutant (HR 2.3, $P < .001$), *MPL*-mutant (HR 2.6, $P = .009$), and triple-negative patients (HR 6.2, $P < .001$). Three *JAK2*-mutant patients had short follow-up and were not included in the analysis.

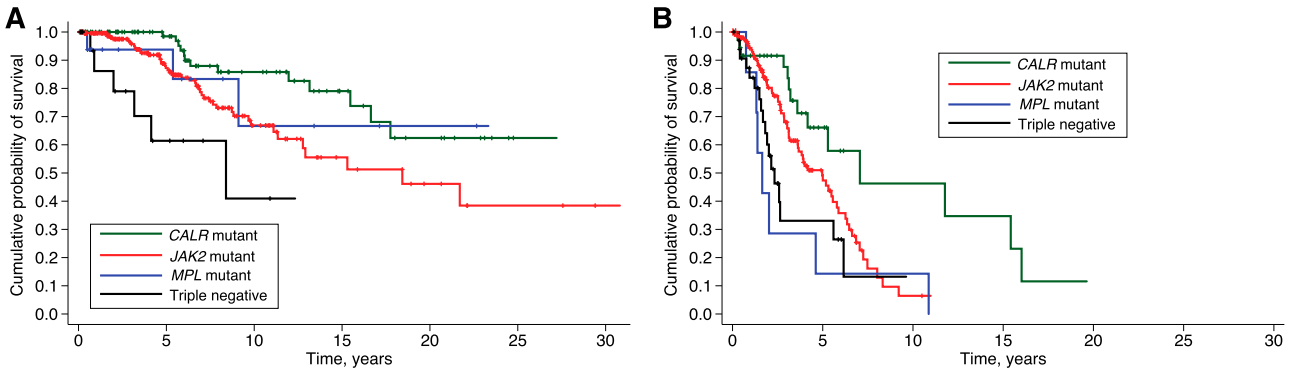


Figure 5. Kaplan-Meier analysis of survival of PMF patients stratified according to their driver mutation and subdivided according to their IPSS risk. Vertical tick marks indicate right-censored patients. (A) "Lower" IPSS risk subgroup, including patients with low or intermediate-1 IPSS risk: *CALR*-mutant patients had longer survival compared with either *JAK2* (V617F)-mutant ($P = .011$) or triple-negative patients ($P < .001$). (B) "Higher risk" subgroup, including patients with intermediate-2 or high IPSS risk: *CALR*-mutant patients had longer survival compared with the remaining genetic subgroups (maximum *P* value equal to .023).

patients with low or intermediate-1 IPSS risk, whereas the "higher-risk" subgroup included patients with intermediate-2 or high IPSS risk. Within "lower-risk" subjects (Figure 5A), *CALR*-mutant patients had a better OS compared with either *JAK2* (V617F)-mutant ($P = .011$) or triple-negative patients ($P < .001$). Within "higher-risk" subjects (Figure 5B), *CALR*-mutant patients had a better OS compared with all the remaining genetic subgroups ($P = .023$ compared with *JAK2*-mutant, $P = .003$ compared with *MPL*-mutant, and $P = .001$ compared with triple-negative patients).

Evidence that accounting for *JAK2*, *CALR*, and *MPL* mutation status improves the risk stratification provided by IPSS

We performed a multivariate Cox proportional hazard regression considering type of mutation (*CALR*, *JAK2*, *MPL*, or none of the previous mutations) and each single variable included in the IPSS score at diagnosis (age >65 years, hemoglobin <10 g/dL, WBC count $>25 \times 10^9$/L, peripheral blood blasts ≥1%, and presence of constitutional symptoms). As shown in Table 2, all of these variables retained a significant independent prognostic effect on OS.

Considering the independent significance of driver mutations, we developed a prognostic model that includes *JAK2*, *CALR*, and *MPL* mutation status in addition to the IPSS variables. All factors of Table 2 were therefore included in the new prognostic model, here defined as clinical-molecular prognostic model. We assigned each factor an integer weight according to the corresponding HR in the multivariable-Cox regression (Table 2): weight 1 for presence of constitutional symptoms, peripheral blood blasts ≥1%, hemoglobin <10 g/dL, and presence of *JAK2* mutation; weight 2 for *MPL* mutation or nonmutated *JAK2*, *CALR*, and *MPL*, WBC count $>25 \times 10^9$/L, and age >65 years.

To assess the prognostic impact of the resulting score, we included the score as a continuous covariate in a Cox survival regression model. The HR was 1.83 (95% CI, 1.69-1.99, $P < .001$), that is, there was a 1.83-fold increase in hazard for a 1-point increase in the sum of weights. To simplify the implementation of the score, we recoded it into 5 broader categories of adequate numerosity by pooling consecutive score values. The resulting risk categories were very low (score = 0), low (score = 1), intermediate (score 2 or 3), high (score 4 or 5), and very high (score ≥6). Of the 617 PMF patients, the clinical-molecular risk was very low in 71 patients, low in 150 patients, intermediate in 202 patients, high in 141 patients, and very high in 53 patients. Kaplan-Meier survival curves corresponding to the 5 score categories were significantly different by log rank-test (Figure 6, $P < .001$).

We then analyzed the categorical clinical-molecular score as a covariate in a Cox survival regression model. Compared with the

Table 2. Multivariate Cox proportional hazard regression analysis of driver mutations (*JAK2*, *CALR*, and *MPL*) and IPSS variables evaluated as risk factors for survival in patients with PMF

Covariates	HR	95% CI	*P*
Driver mutation (*JAK2*, *CALR*, and *MPL* mutation status)			
CALR exon 9 indel*	1		
JAK2 (V617F)	1.9	1.2-3.0	.004
MPL exon 10 mutation	2.7	1.3-5.6	.009
Nonmutated *JAK2*, *CALR*, and *MPL*	2.6	1.4-4.6	.002
IPSS variables			
Age >65 y	3.5	2.5-4.9	<.001
WBC count $>25 \times 10^9$/L	3.4	2.2-5.2	<.001
Hemoglobin <10 g/dL	2.0	1.4-2.9	<.001
Peripheral blood blasts ≥1%	2.0	1.4-2.8	<.001
Presence of constitutional symptoms	1.9	1.4-2.6	<.001

*Reference category.

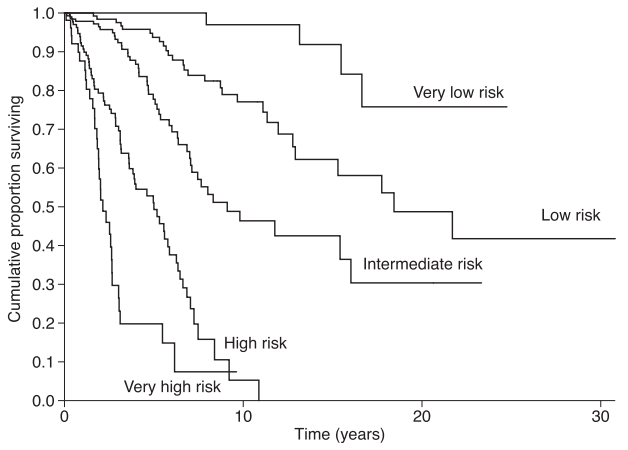


Figure 6. Kaplan-Meier analysis of survival of PMF patients stratified according to the risk categories defined by a clinical-molecular prognostic model. This model includes the variables reported in Table 2, that is, IPSS variables plus *CALR*, *JAK2*, and *MPL* mutation status. We assigned each factor an integer weight according to the corresponding HR in the multivariable-Cox regression of Table 2. Scores were then recoded into the 5 risk categories shown in this figure: details are reported in the last section of "Results." Based on the Akaike information criterion, which compares quality of models, the clinical-molecular model provided a better stratification than the IPSS. This analysis serves as a proof of concept that accounting for driver mutations improves the risk stratification provided by IPSS.

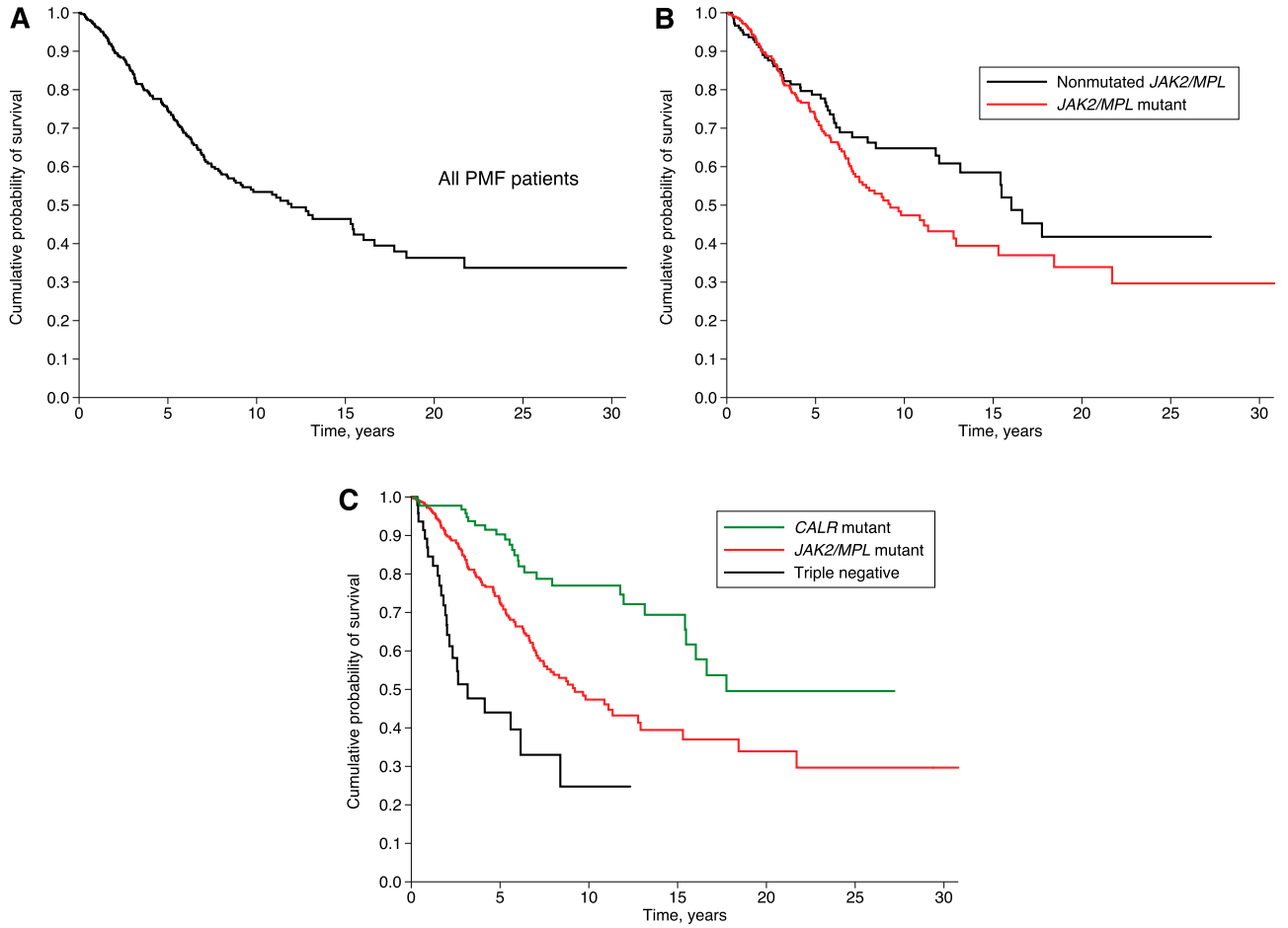


Figure 7. Kaplan-Meier analysis of survival of PMF patients stratified according to their genotype, as it was known in different time periods. (A) OS of the whole population of PMF patients: the genetic basis of MPNs was unknown before 2005, and therefore no genotypic subgroup could be defined. (B) PMF patients stratified according to *JAK2* or *MPL* mutation status: these mutant genes where identified in 2005 and 2006, respectively. (C) PMF patients stratified according to *JAK2, CALR*, or *MPL* mutation status: somatic mutations of calreticulin were identified in 2013.

very-low-risk category, the estimated HR were 4.2 (95% CI, 1.4-12, $P = .007$) for the low risk, 10.2 (95% CI, 3.6-28.6, $P < .001$) for the intermediate risk, 37.5 (95% CI, 13.3-105.8, $P < .001$) for the high risk, and 88.6 (95% CI, 30.3-259, $P < .001$) for the very-high-risk category.

Finally, we used the Akaike information criterion as a measure of the relative quality of the clinical-molecular prognostic model compared with the IPSS. The former had a lower AIC value (1744.5 vs 1764.3), indicating a better quality for the given set of data.

Discussion

The findings of this study provide a proof of concept that a genetic classification of PMF is not only feasible but also highly relevant to clinical decision-making as regards diagnostic approach and prognostication. In addition, they indicate that PMF genotypes should now be considered also in designing clinical trials on the use of novel drugs for treatment of PMF.

The diversity of PMF subtypes was not fully appreciated as long as the only known mutant genes associated with this condition were *JAK2* and *MPL*, as illustrated in Figure 7. The identification of somatic mutations of calreticulin[6,7] has substantially modified our knowledge of PMF. In fact, this identification has split PMF patients with nonmutated *JAK2* and *MPL* (about one-third of all patients with PMF) into 2 distinct subtypes (Figure 7): (1) *CALR*-mutant PMF, a condition with an indolent clinical course and (2) PMF with nonmutated *JAK2*, *CALR*, and *MPL*, a very aggressive myeloid neoplasm. This advance clearly illustrates the clinical relevance of defining the molecular basis of hematologic malignancies.

CALR-mutant ET and *CALR*-mutant PMF have a relatively indolent clinical course compared with the respective *JAK2*-mutant disorders.[6,16,22-25] On the contrary, PMF with nonmutated *JAK2*, *CALR*, or *MPL* has a poor prognosis with a particularly high risk of leukemic transformation, as shown by a study of the Mayo Clinic[23] and by the present work. It should be noted, however, that there are major differences in median OS between the same genotypic entities as estimated in the Mayo Clinic vs the present study: 2.5 vs 3.2 years in triple-negative, 4.1 vs 9.1 years in *MPL*-mutant, 4.3 vs 9.2 years in *JAK2*-mutant, and 8.2 vs 17.7 years in *CALR*-mutant patients. At least part of the difference is likely due to the fact that, in the Mayo Clinic study, OS was estimated from the date of diagnosis or first referral, whereas in this study it was always estimated from the date of diagnosis. Analysis from first referral may clearly lead to underestimation of survival.

Ongoing investigations are trying to identify the molecular basis of PMF with nonmutated *JAK2*, *CALR*, or *MPL*, and to better define the distinctive features of these patients. In terms of clinical features, triple-negative PMF is similar to the myelodysplastic syndrome

associated with bone marrow fibrosis that we described previously.[26] This latter condition is characterized by increased bone marrow cellularity, multilineage dysplasia, severe cytopenia involving high transfusion requirement, unfavorable cytogenetics, and poor survival.[26] Compared with PMF patients, those with the myelodysplastic syndrome associated with bone marrow fibrosis have more profound cytopenia and lower circulating CD34-positive cell count.[5,27] In addition, they do not carry *JAK2* (V617F), which is instead found in two-thirds of PMF patients. Studies are now needed to specifically compare triple-negative PMF with the myelodysplastic syndrome associated with bone marrow fibrosis, but a nonnegligible overlap between the 2 conditions is predictable.[27]

The remarkable differences in clinical course and outcomes observed among the diverse genetic subtypes suggest that, in spite of similar clinical features (bone marrow fibrosis, abnormal stem cell trafficking, and myeloid metaplasia), the disease biology varies considerably according to the different genetic lesions. Recent observations indicate that the JAK-STAT (Janus kinase–signal transducer and activator of transcription) pathway is activated in all MPNs regardless of founding driver mutations.[28] However, the diverse mutations likely involve additional abnormalities in other metabolic pathways; for instance, mutant calreticulin might have peculiar effects on megakaryocyte biology.[29]

As previously observed in ET,[16,22] *JAK2* (V617F) appears to be highly thrombophilic also in patients with PMF, indicating that this mutation likely causes thrombosis through multiple mechanisms, including activation of platelets and granulocytes.[30,31] On the contrary, despite the fact that calreticulin mutations involve high platelet counts also in PMF, the risk of thrombosis of these patients is relatively low, at least compared with that of *JAK2*-mutant patients. We did not find major differences between *CALR*-mutant patients with the 52-bp deletion (type 1 mutation) and those with the 5-bp insertion (type 2 mutation). The only relevant observation was the higher frequency of *CALR* type 1 mutation in PMF compared with ET, which may suggest a particularly active role of the 52-bp deletion in causing bone marrow fibrosis.

So far, risk stratification in PMF has been essentially based on demographic, clinical, and hematologic parameters.[3,4] The findings of this study clearly indicate that the genetic basis of disease and, more specifically, the driver mutation responsible for clinical phenotype is an independent predictor of clinical course and outcomes, and that accounting for that can improve the risk stratification provided by IPSS. Facing a patient with PMF, his or her genetic lesion must now be taken into account carefully because this has an impact on clinical decision-making. As illustrated in Figures 4-5, the prognosis of a triple-negative patient is markedly worse than that of a *CALR*-mutant patient: in these cases, therapeutic decisions may span from a watchful waiting strategy to allogeneic stem cell transplantation.

The different genetic subtypes of PMF should also be considered in designing clinical trials on the use of novel drugs. As illustrated in Figure 7, at least 3 genetic subgroups should now be taken into account in the interpretation of results: *CALR*-mutant, *JAK2/MPL* mutant, and triple-negative patients. It should also be noted that future trials might involve treatments conceived for specific genetic subgroups.

As shown recently by us and by others,[9,32] the clinical course of MPNs is profoundly influenced not only by the founding driver mutations but also by subclonal events. The occurrence of subclonal driver mutations might be closely related to the early initiating genetic lesions, as recently observed in myelodysplastic syndromes.[33] This latter observation generated the hypothesis of genetic "predestination," in which early driver mutations dictate future trajectories of subclonal evolution with distinct clinical outcomes.[33] The current challenge is to develop a prognostic model that accounts for both clinical and molecular parameters, including relevant subclonal mutations,[24] and that in perspective may be useful also for predicting response to different treatments.[34] The model reported in Figure 6 was developed with the only objective of showing, as a proof of concept, that genetic data can significantly improve prognostication of PMF. We are not proposing it as clinical tool for several reasons, including the fact that to this purpose it should be validated in an independent patient cohort. As an international collaborative effort, we are currently analyzing clinical, hematologic, and molecular data of a large population of PMF patients with the aim of developing a clinically useful prognostic tool.

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Authorship

Contribution: M.C., G.B., F.C., C.P., E.R., and A.M.V. designed research; D.P., D.C., M.P., and C. Milanesi performed molecular investigations; P.G., A.M.-T., I.C., L.P., G.R., E.S., M.B., C.C., C. Mannarelli, E.B., C.A., V.R., F.C., G.B., and A.M.V. collected molecular, histologic, and clinical data; C.P. and V.F. performed statistical analyses; and M.C. and E.R. wrote and finalized the manuscript.

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A complete list of the members of the Associazione Italiana per la Ricerca sul Cancro Gruppo Italiano Malattie Mieloproliferative Investigators appears in "Appendix."

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Appendix

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Bergamo Unit: Alessandro Rambaldi (coordinator), Ariel Amaru, Tiziano Barbui, Clara Belotti, Chiara Boroni, Maria Luisa Ferrari, Guido Finazzi, Maria Chiara Finazzi, Josée Golay, Giuseppe Gritti, and Silvia Salmoiraghi.

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