RESEARCH ARTICLE

Epidemiology and clinical relevance of mutations in postpolycythemia vera and postessential thrombocythemia myelofibrosis: A study on 359 patients of the AGIMM group

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Transformation to secondary myelofibrosis (MF) occurs as part of the natural history of polycythemia vera (PPV-MF) and essential thrombocythemia (PET-MF). Although primary (PMF) and secondary MF are considered similar diseases and managed similarly, there are few studies specifically focused on the latter. The aim of this study was to characterize the mutation landscape, and describe the main clinical correlates and prognostic implications of mutations, in a series of 359 patients with PPV-MF and PET-MF. Compared with PV and ET, the *JAK2*V617F and *CALR* mutated allele burden was significantly higher in PPV-MF and/or PET-MF, indicating a role for accumulation of mutated alleles in the process of transformation to MF. However, neither the allele burden nor the type of driver mutation influenced overall survival (OS), while absence of any driver mutation (triple negativity) was associated with significant reduction of OS in PET-MF, similar to PMF. Of the five interrogated subclonal mutations (*ASXL1, EZH2, SRSF2, IDH1,* and *IDH2*), that comprise a prognostically detrimental high molecular risk (HMR) category in PMF, only *SRSF2* mutations were associated with reduced survival in PET-MF, and no additional mutation profile with prognostic relevance was highlighted. Overall, these data indicate that the molecular landscape of secondary forms of MF is different from PMF, suggesting that unknown mutational events might contribute to the progression from chronic phase disease to myelofibrosis. These findings also support more extended genotyping approaches aimed at identifying novel molecular abnormalities with prognostic relevance for patients with PPV-MF and PET-MF.

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Introduction

The chronic myeloproliferative neoplasms (MPN) polycythemia vera (PV) and essential thrombocythemia (ET) can evolve to secondary forms of myelofibrosis, known as postpolycythemia vera (PPV-MF) and postessential thrombocythemia (PET-MF) myelofibrosis, as part of their natural history [1]. Reported rates of transformation are 10–15 and 5–10%, respectively for PV and ET, at 15 years from initial diagnosis, but these figures may be inaccurate due to the retrospective nature of studies, small series reported, variability of diagnostic criteria [2]. More recently, strict criteria for diagnosing transformation to PPV-MF and PET-MF have been delineated by the International Working Group for Myeloproliferative neoplasms Research and Treatment (IWG-MRT), that should result in more homogeneous patient series and facilitate controlled studies [3]. Histopathologic findings and clinical manifestations are very similar in secondary MF and the primary form of disease (primary myelofibrosis, PMF), and also the pathophysiological mechanisms leading

Additional Supporting Information may be found in the online version of this article.

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TABLE I. Patients' Characteristics

Variables	PPV ($n = 194$)	PET ($n = 165$)	P
Males [*n* (%)]	101 (52.1%)	90 (54.5%)	0.358
Age in years; median (range)	64.4 (34–91)	63.2 (27–94)	0.238
Hemoglobin (g/L); median (range)	128 (74–18.0)	109 (50–156)	**<0.0001**
Hemoglobin <100 g/L, *n* (%)	30 (15.5%)	58 (35.1%)	**<0.0001**
Leucocytes $\times 10^9$/L; median (range)	12.3 (1.7–98.4)	7.8 (1.1–48.0)	**<0.0001**
Leucocytes >25 $\times$ 10^9/L; *n* (%)	32 (16.5%)	10 (6.1%)	**0.002**
Leucocytes >30 $\times$ 10^9/L; *n* (%)	22 (11.3%)	5 (3.0%)	**0.002**
Platelets, $\times 10^9$/L; median (range)	294 (16–1689)	375 (19–1213)	**0.017**
Platelets <100 $\times$ 10^9/L; *n* (%)	20 (10.2%)	13 (7.9%)	0.273
Circulating blasts ≥1%; *n* (%)	54 (27.8%)	49 (29.7%)	0.367
Constitutional symptoms; *n* (%)	94 (48.4%)	59 (35.7%)	**0.014**
Splenomegaly; *n* (%)	179 (92.3%)	136 (82.4%)	**0.004**
>10 cm from LCM[a]; *n* (%)	84 (43.3%)	35 (21.2%)	**<0.0001**
Cytogenetic categories; *n* (%)	*N.* evaluable = 101	N. evaluable = 82	**0.007**
Abnormal	43 (42.6%)	20 (24.4%)	
Unfavorable karyotype[b]			
High	7 (6.9%)	2 (2.4%)	0.138
Intermediate 2	4 (13.9%)	9 (11.0%)	
Intermediate 1	13 (12.9%)	5 (6.1%)	
Progression to leukemia; *n* (%)	16 (8.2%)	19 (11.5%)	0.194
Death; *n* (%)	67 (34.5%)	49 (29.7%)	0.194

[a] LCM: left costal margin.
[b] Unfavorable karyotype: high, monosomal karyotype, inv(3), i(17q), −7/7q−, 11q or 12p abnormality; intermediate2: complex nonmonosomal, two abnormalities not included in very high risk category, 5q−, +8, other autosomal trisomies except +9, and other sole abnormalities not included in other risk categories; Intermediate1: sole abnormalities of 20q−, 1q+ or any other sole translocation, and -Y or other sex chromosome abnormality; low: normal or sole abnormalities of 13q− or +9.

to deposition of fibers and other typical abnormalities of bone marrow microenvironment, including neoangiogenesis and osteosclerosis, are considered to be akin.

Few studies have specifically focused on secondary forms of myelofibrosis, and it is common practice to manage them in the same way as PMF. While this is likely appropriate in terms of therapeutic management, as supported also by the comparable efficacy of JAK2 inhibitors in subgroup analysis of patients treated with ruxolitinib [4,5] fedratinib [6], and pacritinib [7], it remains to be fully addressed whether the clinical course of secondary MF differs from PMF and which are the variables eventually influencing it. In particular as regards prognosis, there are evidences that the International Prognostic Score System (IPSS), that is routinely employed for both PMF and secondary MF, may not be performing satisfactorily in secondary forms of MF [8]. Even less is known about the relevance of the mutational background in secondary MF. It has been shown that certain somatic mutations exhibit strong prognostic relevance in PMF when considered either individually [9–11] or in combination [12,13]. Patients with PMF who have the *CALR* type 1/type 1-like mutation constitute a group with a more favorable disease compared with those who are *CALR* type 2/type 2-like, *JAK2*V617F or *MPL*W515x mutated [14–16], and patients who result negative for the 3 driver mutations mentioned above (so called "triple negative" patients) are at even greater risk of earlier death [11]. Furthermore, a category of "High Molecular Risk" (HMR) patients was defined to include those patients who harbor any mutation in *ASXL1, EZH2, SRSF2, IDH1,* and *IDH2;* HMR patients have significantly shorter overall survival and enhanced risk of transformation to acute leukemia [12,17] compared with those who lack prognostically detrimental mutations. In addition, the number of HMR mutations, and widely the number of subclonal mutations, represents a strong negative predictor of survival for patients with PMF [18,19].

The aim of current study was to describe the mutation landscape and analyze the clinical impact of mutation profile, considering both driver and selected subclonal mutations, in a well characterized multicenter cohort of patients with PPV-MF and PET-MF.

Methods

Patients. We retrospectively collected clinical and hematologic information of 359 patients with secondary MF; diagnosis of PPV-MF and PET-MF was according to the IWG-MRT criteria [3]. Patients were collected from 5 tertiary centers (Florence, Pavia, Bergamo, Torino and Varese) belonging to the Italian cooperative group AGIMM (AIRC- Gruppo Italiano Malattie Mieloproliferative). The study was performed in accordance with the Declaration of Helsinki and an informed consent was obtained; the research protocol was approved by the local Ethical committees. Clinical parameters evaluated were: leukocyte count, hemoglobin and platelet levels, peripheral blast count, presence of constitutional symptoms, palpable splenomegaly (measured as cm from the left costal margin, LCM), and patient age at diagnosis of PPV-MF and PET-MF. Specific karyotypic abnormalities and cytogenetic risk categories were defined as described for patients with PMF [20].

Mutation analysis. A peripheral blood sample was collected at the time of diagnosis; granulocytes were isolated by density gradient centrifugation and processed for DNA purification. *JAK2*V617F and *MPL*W515x mutations were detected by real-time quantitative PCR; for *MPL* mutations, also high-resolution melting analysis followed by bidirectional Sanger sequencing was employed [21]. Calreticulin (*CALR*) mutations were identified by capillary electrophoresis followed by bidirectional sequencing in case of abnormal traces, and classified as type 1/type 1-like or type 2/type 2 like, as reported [16,22]. Patients lacking mutations in driver genes, *JAK2, MPL* and *CALR,* were operationally defined as "triple negative" (TN) [11,21]. A Next Generation Sequencing (NGS) technique based on the PGM Ion Torrent platform was used to detect mutations across the entire coding region of *ASXL1*, and *EZH2*, and regions previously described as mutational hotspots for *IDH1*, *IDH2,* and *SRSF2*. A high molecular risk status (HMR) was defined by the presence of ≥1 mutated gene, as described for patients with PMF [12]. In case of mutations not previously reported in public databases, only those considered as potentially damaging by Polyphen algorithm (http://genetics.bwh.harvard.edu/pph2/) were included for analysis.

Statistical analyses. Follow-up was measured as the interval from the diagnosis of PPV-MF and PET-MF to death or last follow-up date; patients who received stem cell transplant were censored on the date of transplant procedure. The cumulative probability of overall survival (OS) and leukemia free-survival (LFS) were estimated using the Kaplan–Meier method. Differences in OS among the groups were compared by using a log-rank test in univariate analysis. All $P < 0.05$ were considered as statistically significance. Statistical analyses were performed using the IBM Statistical Package for Social Sciences (SPSS) statistics v23.

Results

This study included 359 patients with secondary MF, of which 194 had PPV-MF and 165 PET; their clinical characteristics are reported in Table I. Median age was 64 and 63 years, respectively, the proportion

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TABLE II. Mutation Profile for Driver and High Molecular Risk (HMR) Mutations[a] in Patients with PPV-MF and PET-MF

	PPV ($n = 194$)	PET ($n = 165$)	P
JAK2V617F; *n* (%)	194 (100%)	81 (49.1%)	**<0.0001**
V617F allele burden (%); mean ± SD	75.7 ± 20.3	56.2 ± 24.9	**<0.0001**
MPLW515; *n* (%)	–	16 (9.7%)	–
CALR mutated; *n* (%)	–	56 (33.9%)	–
Type 1/type 2[b]; *n* (%)		40 (71.4%)/16 (28.6%)	–
Allele burden type 1/type 2; mean ± SD		55.8 ± 7.5/51.7 ± 23.7	0.287
Triple negative; *n* (%)	–	12 (7.3%)	–
ASXL1 mutated; *n* (%)	33 (17.0%)	48 (29.1%)	**0.011**
EZH2 mutated; *n* (%)	7 (3.6%)	17 (10.3%)	**0.022**
IDH1/2 mutated; *n* (%)	11 (5.7%)	2 (1.2%)	0.055
SRSF2 mutated; *n* (%)	2 (1.0%)	7 (4.2%)	0.096
HMR patients; *n* (%)	48 (24.7%)	59 (35.7%)	**0.028**
N. HMR mutations ≥2; *n* (%)	6 (3.1%)	6 (3.6%)	0.960

[a] Genes included in the HMR category are *ASXL1, EZH2, SRSF2, IDH1, IDH2* [12].
[b] Type 1 and type 2 includes type 1-like and type 2-like mutations according to the criteria in [22].

of male patients was similar (52 and 54%). Patients with PPV-MF had higher hemoglobin levels and leukocyte count ($P < 0.0001$ for both) than those with PET-MF, while platelet counts were higher in PET-MF ($P = 0.017$). There were more anemic patients (Hb < 100 g/L) among PET-MF than PPV-MF (35.1 versus 15.5%; $P < 0.0001$); conversely, more patients with PPV-MF suffered from constitutional symptoms (48.4% versus 35.7% of PET-MF; $P = 0.014$) and had palpable splenomegaly (92.3 versus 82.4%; $P = 0.004$) and larger splenomegaly (43.3 versus 21.2%; $P < 0.0001$) than PET-MF. Information about karyotype at the time of myelofibrosis transformation was available in 52.1 and 49.7% of PPV-MF and PET-MF; there were more abnormal karyotypes among PPV-MF than PET-MF patients (42.6 and 24.4%, respectively; $P = 0.007$), while karyotype risk classes were similarly represented in the two groups (Table I).

The patients' mutation profile is reported in Table II. All PPV-MF patients harbored the *JAK2*V617F mutation compared with 49.1% of PET-MF; 9.7% and 33.9% of PET-MF were *MPL*W515x and *CALR* mutated, respectively. The mean (±SD) allele burden of *JAK2*V617F mutation was higher in PPV-MF (75.7 ± 20.3%) than PET-MF (56.2 ± 24.9%; $P < 0.0001$); furthermore, the *JAK2*V617F allele burden was significantly higher in PPV-MF and PET-MF than in a population of 369 *JAK2*V617F mutated patients with PV (44.5 ± 22.2; $P < 0.0001$) and 402 with ET (26.5 ± 13.6%; $P < 0.0001$), randomly selected from our archives. We also compared the percentage of patients with PPV-MF and PV who had more than 50 and 75% mutated alleles: the figures were 88.7% and 55.3% for PPV-MF compared with 34.0 and 10.6% for PV ($P < 0.0001$ for both). Similarly, significantly more patients with PET-MF had *JAK2*V6171F allele burden greater than 25% (91.7%) or 50% (49.1%) than those with ET (56.5 and 7.4%, respectively; $P < 0.0001$ for both).

In PET-MF, *CALR* type1/type1-like mutations were more represented than type2/type2-like (71.4 versus 28.6%; $P < 0.0001$), while the allele burden was similar for the two mutation types (55.8 ± 7.5% and 51.7 ± 23.7%, respectively; $P = 0.287$). The overall *CALR* mutated allele burden was higher in PET-MF than in ET patients (54.8 ± 13.6 versus 44.7 ± 10.7; $P < 0.0001$); specifically, it was 55.8% versus 46.3% for *CALR* type1/type1-like ($P = 0.0004$) and 51.7% versus 43.0% for *CALR* type2/type2-like mutations ($P = 0.049$), respectively in patients with PET-MF and ET. There were significantly more patients with a *CALR* allele burden greater than 50% in PET-MF than in ET (51.0 versus 20.4%; $P < 0.0001$).

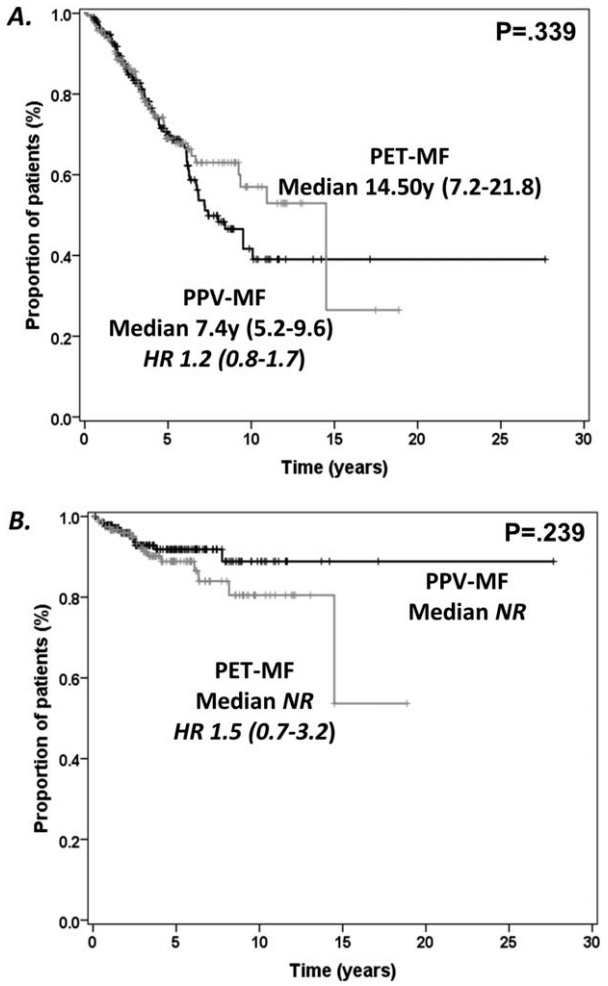

Figure 1. Overall survival (OS) and leukemia free-survival (LFS) of patients with PPV-MF and PET-MF are presented in panel *A* and *B*, respectively. In case of OS, the hazard ratio (HR, 95% CI) was calculated using PET-MF as the reference group while in case of LFS PPV-MF was used as the reference group.

A total of 48 PPV-MF patients (24.7%) and 59 PET-MF (35.8%) harbored at least one mutation in *ASXL1, EZH2, SRSF2, IDH1* and *IDH2*, and were thereby considered as HMR patients (Table II); the difference between PET-MF and PPV-MF was statistically significant ($P = 0.028$). In particular, more PET-MF patients were *ASXL1* and *EZH2* mutated compared with PPV-MF [29.1 versus 17.0% ($P = 0.011$) and 10.3 versus 3.6% ($P = 0.022$), respectively]. Six patients in each group had 2 or more mutated genes (3.1 and 3.6% for PPV-MF and PET-MF). When compared with a cohort of 483 PMF patients from our data base, there were more *EZH2* mutations in PET-MF than in PMF (10.3 versus 5.1%; $P = 0.03$) while conversely the percentage of patients with ≥2 mutations was significantly lower than in PMF (8.0%; $P = 0.003$).

With an overall median follow-up period of 3.9 years (3.9 years for PPV-MF and 3.8 years for PET-MF) 116 deaths were recorded in the whole series (32.3%), accounting for 34.5 and 29.7% of PPV-MF and PET-MF. Sixteen PPV-MF (8.2%) and 19 PET-MF (11.5%) patients transformed to acute leukemia. The median OS was 7.4 years (HR 1.2, 95% CI 0.8–2.7) for PPV-MF and 14.5 years for PET-MF, not statistically different ($P = 0.339$) [Fig. 1(A)]; median LFS was not reached in either groups [Fig. 1(B)]. The median time of transformation to PPV-MF and PET-MF was 10.1 years (range 1.1–30.7) and 11.5 years (0.9–3.6), respectively ($P = 0.101$). A longer (>10 years) duration of the chronic PV phase was associated with shorter OS

TABLE III. Analysis of the Impact of the *JAK2*V617F Allele Burden and High Molecular Risk (HMR) Mutations[b] on Survival of Patients with PPV-MF

Variable	*N* (%)[b]	Median OS (range) (years)	HR[a,c]	95% CI	*P* value
*JAK2*V617F allele burden quartile					
1–50%	24 (12.0)	NR	–	–	–
51–75%	66 (34.0)	7.18 (5.82–8.55)	3.13	0.74–13.32	0.122
76–100%	104 (54.0)	8.43 (5.06–11.80)	2.27	0.54–9.58	0.265
ASXL1 mutations	33 (17.0%)	6.12 (4.83–7.41)	1.48	0.80–2.77	0.208
EZH2 mutations	7 (3.6%)	NR	1.18	0.29–4.84	0.821
IDH1/2 mutations	11 (5.7%)	NR	1.64	0.50–5.33	0.407
SRSF2 mutations	2 (1.0%)	NR	1.24	0.17–9.00	0.832
HMR status	48 (24.7%)	6.12 (4.73–7.51)	1.64	0.93–2.89	0.084
N. HMR mutations≥2	6 (3.1%)	NR	1.75	0.42–7.31	0.442

[a] The reference group for *JAK2*V617F mutated patients was quartile 1 (1–25%).
[b] Genes included in the HMR category are *ASXL1, EZH2, SRSF2, IDH1, IDH2* [12].
[c] The reference group was patients without HMR mutations ($n = 146$).

TABLE IV. Analysis of the Impact of Driver and High Molecular Risk (HMR) Mutations[a] on Survival of Patients with PET-MF

Variable	*N* (%)[a]	Median OS (range) (years)	HR[b,c]	95% CI	*P* value
CALR type1[a]	40 (24.3)	NR	–	–	–
CALR type2[a]	16 (9.7)	NR	1.88	0.55–6.43	0.314
JAK2V617F—PET	81 (49.1)	10.9 (6.12–15.76)	2.08	0.90–4.80	0.080
MPLW515	16 (9.7)	NR	1.09	0.28–4.23	0.898
Triple negative	12 (7.3)	4.81 (2.27–7.35)	3.71	1.36–10.06	**0.010**
ASXL1 mutations	48 (29.1%)	14.50 (7.20–20.99)	1.37	0.74–2.55	0.314
EZH2 mutations	17 (10.3%)	NR	0.79	0.28–2.23	0.660
IDH1/2 mutations	2 (1.2%)	NR	0.33	0.01–101.86	0.192
SRSF2 mutations	7 (4.2%)	3.04 (2.38–3.70)	4.90	1.70–14.12	**0.001**
HMR status	59 (35.7%)	6.41 (2.50–10.88)	1.47	0.80–2.68	0.211
N. HMR mutations ≥2	6 (3.6%)	4.72 (2.11–7.34)	1.97	0.60–6.56	0.379

[a] Genes included in the HMR category are *ASXL1, EZH2, SRSF2, IDH1, IDH2* [12].
[b] The reference group for driver mutations (*JAK2*V617F, *MPL*W515x, *CALR*, and for triple negativity) was *CALR* type1/type1-like.
[c] The reference group was patients without HMR mutations ($n = 106$).

once transformed to PPV-MF (HR 2.26; 95% CI, 5.87–6.45; $P = 0.004$), while no such difference was seen for ET. Conversely, longer (>10 years) duration of chronic ET phase was associated with significantly shortened LFS after transformation to PET-MF (HR 4.42, 95% CI 1.01–20.06; $P = 0.036$). After stratifying patients according to the IPSS risk criteria, we found that 52.6% of PPV-MF and 47.3% of PET-MF were comprised in the higher risk categories (Supporting Information Table I); however, only the high risk category resulted clearly separated from the others, confirming the poor performance of IPSS in secondary MF (Supporting Information Fig. 1).

We first compared hematological and clinical characteristics of PET-MF patients who were categorized according to their driver mutation status (Supporting Information Table II). We found no significant differences among mutational groups regarding age, hemoglobin, leucocyte, and platelet count, peripheral circulating blast cells and abnormal karyotype. However, significantly more *JAK2*V617F positive patients referred constitutional symptoms ($P = 0.003$), especially in comparison with *CALR* mutated patients ($P < 0.0001$); also, large splenomegaly was found in 29.6% of *JAK2*V617F mutated compared with 12.5% of *CALR* mutated patients ($P < 0.001$). Higher IPSS risk category were more frequent among *JAK2*V617F mutated patients (59.3%) compared with the other groups (33.9% *CALR*, 37.5% *MPL*. 41.6% in TN patients). Information about thrombosis were available in 68 patients; those harboring *JAK2*V617F mutation displayed more thrombotic events than *CALR* mutated (43.3% versus 22.2% in *CALR* type1 and no case in *CALR* type2; $P = 0.023$). The interval from ET phase to myelofibrosis progression was significantly longer in *CALR* type2 (18.9 years) compared with type1 (12.2 years), *JAK2*V617F (10.6 years), *MPL* (14.4 years) and particularly TN (8.1 years) ($P = 0.015$). Finally, *CALR* type1 mutated patients had reduced rate of death in comparison with *CALR* type2, *JAK2* and TN (17.5 versus 25.0%, 32.1 and 75%, respectively; $P = 0.003$). *ASXL1* mutations were particularly associated with *CALR* type2, *MPL*W515x and TN ($P = 0.013$), while six of seven *SRSF2* mutation were associated with *JAK2*V617F mutation (Supporting Information Table II). No other meaningful correlation could be outlined.

We then evaluated the impact of driver and subclonal mutations for prognosis. Since all PPV-MF patients were *JAK2*V617F mutated, we divided patients according to quartiles of allele burden, but did not find any significant correlation with either OS (Table III) or LFS (not shown in detail). In case of PET-MF, we considered 5 mutational groups, represented by *CALR* (type1/type1-like and type2/type2-like), *JAK2*V617F, *MPL*W515x and TN patients [Table IV and Supporting Information Fig. 2(A)]. Since there was no difference between *CALR* type1 and type2 ($P = 0.29$), we used the former as reference group. We found that only triple negativity was associated with shorter survival (HR 3.71, 95% CI 1.36–10.06; $P = 0.010$) while other genotypes were irrelevant. No impact on LFS could be demonstrated. We also did not find difference in OS between PPV-MF and PET-MF patients who were *JAK2*V617F mutated [Supporting Information Fig. 2(B)]. Analysis of the impact of HMR mutations in PPV-MF failed to discover correlations of either single gene mutations, a HMR status or the number of HMR mutations with OS [Table III and Supporting Information Fig. 2(C)] and LFS (not shown). As regards PET-MF, a HMR status was not predictive of reduced OS [Supporting Information Fig. 2(D)], while we found a significant correlation of *SRSF2* mutations with shortened survival (HR 4.90, 95%CI 1.70–14.12; $P = 0.001$) [Table IV and Supporting Information Fig. 2(E)]; median survival was 14.5 years in *SRSF2* wild type compared with 4.9 years in *SRSF2* mutated patients. No impact on LFS could be demonstrated (not shown).

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Discussion

Results from this study describe for the first time the mutation landscape, and the main clinical correlates and prognostic implications of mutations, in a large series of well-characterized patients with PPV-MF and PET-MF. Current information about characteristics, disease course and prognosis in secondary myelofibrosis are quite scanty, largely due to the lack of enough powered study to address these points. Similarly, the knowledge about the molecular profile of the patients, which conversely has been well characterized in PMF patients, is poor. This also reflects the current practice of managing PMF and secondary forms of myelofibrosis as the same in terms of therapeutic approaches and prognosis stratification, although, for example, there is evidence that the IPSS score is not satisfactorily performing in this setting of patients as in those with PMF [8], for which it was specifically devised [23]. Not to mention, furthermore, that in clinical trials with newest JAK2 inhibitors or other targeted molecules, the entry criteria have been the same for the two categories of patients [24,25]; reassuringly, there was no evidence of differences in terms of hematological and/or clinical responses between PMF and secondary MF, but a potential bias when considering the impact of drugs on survival has not been clearly ruled out, although a PPV-MF and PET-MF subtype were associated with better outcome in general [26]. Therefore, it is important to specifically focus on PPV-MF and PET-MF as distinct entities to develop better performing tools to interpret disease characteristics and clinical.

There is circumstantial evidence that driver mutation profile of patients with PMF is associated with clinical characteristics of the patients and identifies different prognostic groups. Apart from PV patients, who were all *JAK2*V617F mutated as expected, the proportion of PET-MF patients with *JAK2*V617F and *CALR* mutations in this series was comparable to reports in PMF and ET; however, notably, *CALR* type1 mutation was harbored by 71% of *CALR* mutated patients compared with 29% of type2, contrasting with the roughly balanced representation of the two mutations types in ET, thereby reinforcing previous suggestions regarding a role for *CALR* type1 in myelofibrotic transformation [11,27,28]. Accordingly, we also found that the myelofibrosis-free survival was longer in *CALR* type2 patients than all other genotypes. Interestingly, in retroviral model of *CALR* mutation, mice expressing type1 mutations developed more severe myelofibrosis trait as compared with type2 [29]. On the opposite, an excess of *CALR* type2 mutations was found in Chinese patients with PMF (64% versus 32% type1) [30] suggesting possible genetic background differences. Also, we found herein that the frequency of *MPL* mutated patients in PET-MF (9.7%) was higher than reported in ET (2 to 4%) [21,31,32]. In a previous study [21], we found that *MPL* mutated ET patients had greater likelihood of transforming to PET-MF compared to other genotypes, but due to small number of events such difference did not reach the significance level; more recently, Elala et al reported shorter myelofibrosis-free survival in *MPL* mutated ET patients that remained significant in multivariate analysis, thereby corroborating our current findings [33]. On the other hand, results of this study validate and extend previous observations concerning the role of accumulation of mutated alleles of *JAK2*V617F [34–36] in the progression to PPV-MF and PET-MF, although we show here that allele burden did not impact on overall survival. We also report that *CALR* allele burden of PET-MF patients was significantly higher compared with a series of ET patients from our archives, indicating that a *CALR* allele burden greater than 50% is far more frequent in PET-MF than ET. The retrospective comparative nature of these observations imposes caution, and prospective follow-up analysis of individual cases might help to define the role of accumulating *CALR* alleles in the progression to PET-MF.

Concerning the prognostic impact of driver mutations, data in PMF patients indicated that the *CALR* type1/type1 like mutations identified patients with longer survival as compared to *JAK2*V617F, *MPL*W515x and *CALR* type2/type2 mutated [14,16,22]; in most studies, the triple negative patients experienced the shorter survival [11,16]. However, the survival advantage of *CALR* type1/type1-like versus type2/type2-like in PMF has generated some conflicting data [27,28]. In this series of PET-MF, we found that survival was largely superimposable among the varying mutational groups, with the notable exception of triple negative patients who did worse, as in PMF. Here, we did not find differences between *CALR* type1/type1-like and type2/type2-like. Therefore, in secondary MF, driver mutations do not appear to meaningfully predict survival, at variance with PMF.

Mutations in selected subclonal genes have been shown to be prognostically predictive in PMF [9,12,13,37,38]; a HMR category was devised to include patients with at least one mutation in *ASXL1, EZH2, SRSF2*, *IDH1,* and *IDH2* who suffered from shortened overall survival and more frequent transformation to leukemia. In the current series of secondary MF, we found that, although the frequency of these mutated genes was roughly comparable to PMF, they did not inform prognosis nor leukemia transformation, with the notable exception of *SRSF2* mutations in PET-MF that predicted for shorter survival. As reported in PMF, we also found that mutations in the spliceosome gene *SRSF2* are mainly associated with *JAK2*V617F mutated or triple negative patients and are infrequently combined with *CALR* mutations (no patient in this series out of 7 mutated) [15]. The percentage of patients with 2 or more HMR mutations was too low to evaluate an impact of mutation number on prognosis, as it was reported in PMF [18].

In summary, we conclude that, at variance with PMF, the mutation profile of secondary forms of myelofibrosis does not remarkably inform prognostic assessment, leaving open many questions regarding the texture of genetic changes that promote evolution from chronic phase disease to myelofibrosis and are responsible for the shortened survival associated with such transformation. For the clinical practice, these data also indicate that using current molecular target analysis for prognostication, including the HMR category, should be reserved to patients with PMF only.

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