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ORIGINAL ARTICLE

Genomic characterization in triple-negative primary myelofibrosis and other myeloid neoplasms with bone marrow fibrosis

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Abstract

Triple-negative primary myelofibrosis (TN-PMF) and other myeloid neoplasms with associated bone marrow fibrosis such as the myelodysplastic syndromes (MDS-F) or the myelodysplastic/myeloproliferative neoplasms (MDS/MPN-F) are rare entities, often difficult to distinguish from each other. Thirty-four patients previously diagnosed with TN-PMF ($n = 14$), MDS-F ($n = 18$), or MDS/MPN-F ($n = 2$) were included in the present study. After central revision of the bone marrow histology, diagnoses according to the 2016-WHO classification were TN-PMF ($n = 6$), MDS-F ($n = 19$), and MDS/MPN-F ($n = 9$), with TN-PMF genotype representing only 4% of a cohort of 141 molecularly annotated PMF. Genomic classification according to next-generation sequencing and cytogenetic study was performed in 28 cases. Median number of mutations was 4 (range 1–7) in cases with *TP53* disruption/aneuploidy or with chromatin-spliceosome mutations versus 1 mutation (range 0–2) in other molecular subgroups ($p < 0.0001$). The number of mutations and the molecular classification were better than PMF and MDS conventional scoring systems to predict survival and progression to acute leukemia. In conclusion, TN-PMF is an uncommon entity when the 2016 WHO criteria are strictly applied. Genomic classification may help in the prognostic assessment of patients with myeloid neoplasms with bone marrow fibrosis.

Keywords Primary myelofibrosis · Myelodysplastic syndromes · Diagnosis · Prognosis · Genomic classification

Introduction

Primary myelofibrosis (PMF) is the less frequent of the classic myeloproliferative neoplasms (MPN) and the one with the worst prognosis, with an estimated median survival around 6 years in contemporary series [1–4]. The majority of PMF patients have mutations in *JAK2*V617F, *CALR*, or *MPL* genes. However, up to 10% of them are negative for these mutations, with these triple-negative PMF (TN-PMF) patients showing a more unfavorable prognosis [2–4]. In such cases, the absence of a driver mutation can often complicate their distinction from other myeloid neoplasms with associated bone marrow fibrosis.

Myelodysplastic syndrome with associated fibrosis (MDS-F) represents 10–20% of MDS and, despite not being recognized as a separate entity, it is associated with an unfavorable outcome [5–7]. In addition, myelodysplastic/myeloproliferative neoplasms (MDS/MPN) are rare entities sharing clinical and morphological features with MDS and MPN and including chronic myelomonocytic leukemia

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(CMML), atypical BCR-ABL1-negative chronic myeloid leukemia (aCML), MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T), and MDS/MPN unclassified (MDS/MPN-U) [1]. In all these entities, if bone marrow fibrosis is present, differential diagnosis with TN-PMF is challenging.

It has recently been shown that a significant proportion of patients with MDS and MPN have mutations in a large variety of genes associated with myeloid neoplasms [8, 9]. In addition, the presence of mutations in certain genes and the number of mutated genes has been associated with a more unfavorable prognosis in both MDS and PMF [8–13].

In the present study, we have characterized by next-generation sequencing (NGS) techniques a cohort of patients homogeneously diagnosed with TN-PMF, MDS-F, or MDS/MPN-F according to the 2016 WHO criteria. The aim of the study was to evaluate if mutations detected by NGS can help in establishing a definite diagnosis among these entities or refining the prognostic assessment of the patients.

Patients and methods

A search for TN-PMF and other chronic myeloid neoplasms with bone marrow fibrosis was performed in two Spanish institutions. Cases with CMML, aCML, MDS/MPN-RS-T, and MDS with excess blasts type-2 were excluded. Initial data, including the main clinical, hematological, and cytogenetic features, as well as the therapies administered during follow-up, transformation to acute leukemia, and the causes of death were collected.

Bone marrow biopsies were performed at diagnosis in all cases. Hematoxylin/eosin and reticulin stained slides and CD34 immunostaining when available of formalin-fixed or Bouin's solution fixed, paraffin embedded trephine biopsies were centrally reviewed by two expert hematopathologists. The review was performed blindly without knowledge of age, clinical symptoms, original diagnosis, or gene mutational status. Histological features assessed were cellularity (measured as percentage), presence of lymphoid aggregates (yes or no), granulocytic precursors (increased, normal or decreased, left shifted), erythrocytic precursors (increased, normal or decreased, left shifted, megaloblastoid changes), myeloid/erythroid ratio (increased, normal or decreased), presence of sinusoidal hyperplasia (yes or no), presence of intrasinusoidal hematopoiesis (yes or no), and grade of reticulin fibrosis according to the European Consensus Grading system (ranging from MF-0 to MF-3) [14]. An extensive quantification and description of the morphology and distribution of the megakaryocytes was performed. Presence of dense and loose clusters of megakaryocytes, paratrabecular localization, megakaryocyte size (small and/or giant), and nuclear features, including bulbous morphology, hypolobulation with disperse nuclei, hyperlobulation (stag horn-like), and hyperchromatism, were also evaluated. Taking into consideration the above-mentioned features, all cases were finally classified according to the current WHO criteria [1].

Twenty-seven cases with available samples for NGS study were analyzed. Targeted NGS was performed using the Sophia Genetics Myeloid Tumor Solution Panel including the following genes: *ABL1*, *ASXL1*, *BRAF*, *CALR*, *CBL*, *CEBPA*, *CSF3R*, *CSNK1A1*, *DNMT3A*, *ETV6*, *EZH2*, *FLT3*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *KIT*, *KMT2A*, *KRAS*, *MPL*, *NPM1*, *NRAS*, *PTPN11*, *RUNX1*, *SETBP1*, *SF3B1*, *SRSF2*, *TET2*, *TP53*, *U2AF1*, *U2AF2*, *WT1*, and *ZRSR2*. Libraries were sequenced 2×300 bp on an Illumina MiSeq. Sequencing data were analyzed using the commercial software Sophia DDM v4.4 (Sophia Genetics). Only variants categorized as highly or potentially pathogenic by Sophia software were considered. The minimum variant allele frequency (VAF) considered was 2%. Synonymous, intronic, and polymorphic variants were discarded. Additionally, selected variants were reviewed in COSMIC and ClinVar databases to select clinically relevant mutations.

Genomic classification combining cytogenetics and NGS studies was performed as reported by Grinfeld et al. For such purpose, patients were hierarchically allocated into eight molecular subgroups as follows: group 1: *TP53* disruption or aneuploidy (*TP53* mutation, Chr17pLOH or Chr5-/Chr5q-), group 2: ≥ 1 chromatin or spliceosome mutation (*EZH2*, *IDH1*, *IDH2*, *ASXL1*, *PHF6*, *CUX1*, *ZRSR2*, *SRSF2*, *U2AF1*, *KRAS*, *NRAS*, *GNAS*, *CBL*, Chr7/7qLOH, Chr4q/LOH, *RUNX1*, *STAG2*, and *BCOR*), group 3: *CALR* mutation, group 4: *MPL* mutation, group 5: homozygous *JAK2* mutation, group 6: heterozygous *JAK2* mutation, group 7: myeloid neoplasm with other driver mutation, and group 8: myeloid neoplasm with no known driver mutation [9].

The clinical and biological data at diagnosis, the cytogenetic findings, and the results from NGS analysis were included in the statistical analysis. Comparisons among diagnostic groups were performed after central review of bone marrow histology. The prognostic group was calculated according to the International Prognostic Scoring Systems (IPSS) designed for PMF [15] and MDS [16], respectively. The probabilities of survival and progression to acute leukemia were estimated by the Kaplan Meier method using the log rank test for comparisons. Informed consent for the scientific use of the patients' clinico-hematological data and biological samples was obtained, and the study was approved by the Ethics Committee of the Hospital Clínic of Barcelona.

Table 1 Main clinical and hematological characteristics at diagnosis in 34 patients with myeloid neoplasms with bone marrow fibrosis

	TN-PMF $N = 6$	MDS-F $N = 19$	MDS/MPN-U-F $N = 9$	*P* value
Age, year*	61 (48–78)	64 (35–80)	65 (42–79)	ns
Male sex, *n* (%)	3 (50)	12 (63)	8 (89)	ns
Palpable spleen, *n* (%)	3/6 (50)	6/18 (33)	8/9 (89)	0.02
WBC count, $\times 10^9$/L*	10.1 (3.8–19)	3 (0.3–11.4)	15.4 (5.1–69)	< 0.001
Hemoglobin, g/L*	88 (78–144)	88 (69–140)	110 (50–140)	ns
Platelets, $\times 10^9$/L*	241 (26–600)	85 (4–465)	116 (39–357)	0.02
Circulating blasts $\geq$ 1%, *n* (%)	1/6	10/19	5/9	ns
Abnormal cytogenetics, *n* (%)**	1/4 (25)	8/14 (57)	4/6 (67)	ns

Diagnosis allocation after central review of bone marrow histology. *P* values correspond to ANOVA test

TN-PMF triple-negative primary myelofibrosis, *MDS-F* myelodysplastic syndrome with fibrosis, *MDS/MPN-U-F* myelodysplastic/myeloproliferative neoplasm unclassifiable with fibrosis. Diagnosis allocation after central review of bone marrow histology

[a] Median (range)

[b] Cytogenetics available in 4, 14, and 6 cases of TN-PMT, MDS-F, and MDS/MPN-U-F, respectively. *P* values correspond to ANOVA test

Table 2 Bone marrow histology in 34 patients with myeloid neoplasms with associated fibrosis

	TN-PMF $N = 6$	MDS-F $N = 19$	MDS/MPN-U-F $N = 9$	*P* value
Cellularity, %*	80 (50–95)	65 (20–100)	95 (90–100)	0.001
Lymphoid clusters	83%	32%	22%	0.039
Increased granulopoiesis	83%	33%	100%	0.002
Increased M/E	80%	11%	67%	0.002
Increased MK	83%	74%	56%	ns
MK clustering				
Loose	83%	42%	33%	0.1
Dense	67%	16%	11%	0.02
MK abnormalities				
Small size	33%	84%	67%	0.06
Giant size	33%	5%	0%	0.06
Hyperlobulated nuclei	50%	0%	0%	< 0.0001
Bulbous nuclei	67%	21%	0%	0.01
Hypolobulated nuclei	33%	74%	44%	0.1
Grade of fibrosis**				ns
1	0%	16%	22%	
2	67%	58%	67%	
3	33%	26%	11%	
CD34+ cells, %*	2.5 (0–17)	5 (0–15)	10 (0–15)	ns

Diagnosis allocation was performed after central review of bone marrow histology.

TN-PMF triple-negative primary myelofibrosis, *MDS-F* myelodysplastic syndrome with fibrosis, *MDS/MPN-U-F* myelodysplastic/myeloproliferative neoplasm unclassifiable with fibrosis. *M/E* myeloid/erythroid ratio. *MK* megakaryocytes

[a] Median (range)

[b] *P* values correspond to ANOVA or chi-square test for continuous or categorical variables, respectively

Table 3 Molecular classification in 28 patients with myeloid neoplasms with bone marrow fibrosis

	TN-PMF $N=5$	MDS-F $N=15$	MDS/MPN-U-F $N=8$	Total $N=28$
MN with *TP53* disruption or aneuploidy	0	2 (13)	0	2 (7)
MN with chromatin or spliceosome mutation	2 (40)	9 (60)	6 (75)	17 (61)
MN with other driver mutation	2 (40)	3 (20)	1 (12.5)	6 (21)
Myeloproliferation with no known driver mutation	1 (20)	1 (7)	1 (12.5)	3 (11)

*TN*Diagnosis allocation was performed after central review of bone marrow histology

TN-PMF triple-negative primary myelofibrosis. *MDS-F* myelodysplastic syndrome with fibrosis. *MDS/MPN-U-F* myelodysplastic/ myeloproliferative neoplasm unclassifiable with fibrosis, *MN* myeloid neoplasm

Results

Patient's characteristics and bone marrow histology

From a total cohort of 141 patients with molecularly annotated PMF, 16 (11%) triple-negative cases (TN-PMF) were selected for the present study. Two of them were excluded because of inadequate material for histological assessment. Twenty additional patients with the diagnosis of MDS-F ($n=18$) or myelodysplastic/ myeloproliferative neoplasm unclassifiable with fibrosis (MDS/MPN-U-F, $n=2$) were also added for comparison. Therefore, the final number of patients included in the study was 34.

After central review of the bone marrow histology, 5 out 14 TN-PMF cases remained in the same diagnostic category, whereas 3 and 6 cases were reclassified as MDS-F and MDS/MPN-U-F, respectively. Alternatively, MDS-F was confirmed in 16 out of 18 (89%) cases, whereas the remaining two were reclassified as TN-PMF and MDS/MPN-U-F, respectively. The two cases originally diagnosed as MDS/MPN-U-F patients were confirmed after histological revision. After reclassification according to the histological review, only 6 patients remained in the TN-PMF category, accounting for 4% of the total PMF cohort. Two of these patients showed bone marrow fibrosis in the absence of abnormal megakaryocytes; carried mutations in *KMT2A* and *ETV6*, respectively; and had a favorable therapeutic outcome, with resolution of transfusion-dependent anemia.

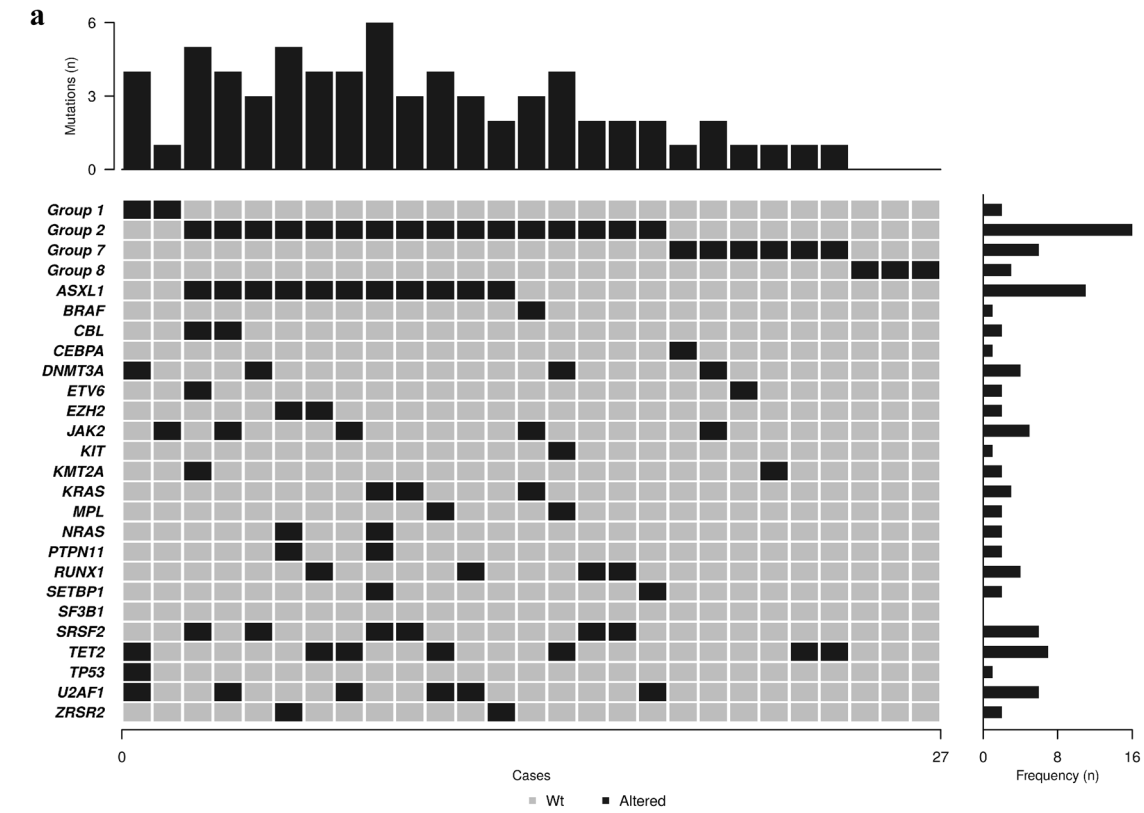
The main clinical and hematological characteristics at diagnosis, after central revision of bone marrow histology according to the WHO 2016 diagnostic criteria, are shown in Table 1. Patients with TN-PMF showed higher platelet counts, whereas splenomegaly and leukocytosis were more prominent in MDS/MPN-U-F. Cytogenetic abnormalities were detected in 13 (54%) out of 24 assessable patients, being categorized as follows: low risk ($n=14$), intermediate risk ($n=5$), and high risk ($n=5$). There were no significant differences among the diagnostic groups regarding cytogenetic risk.

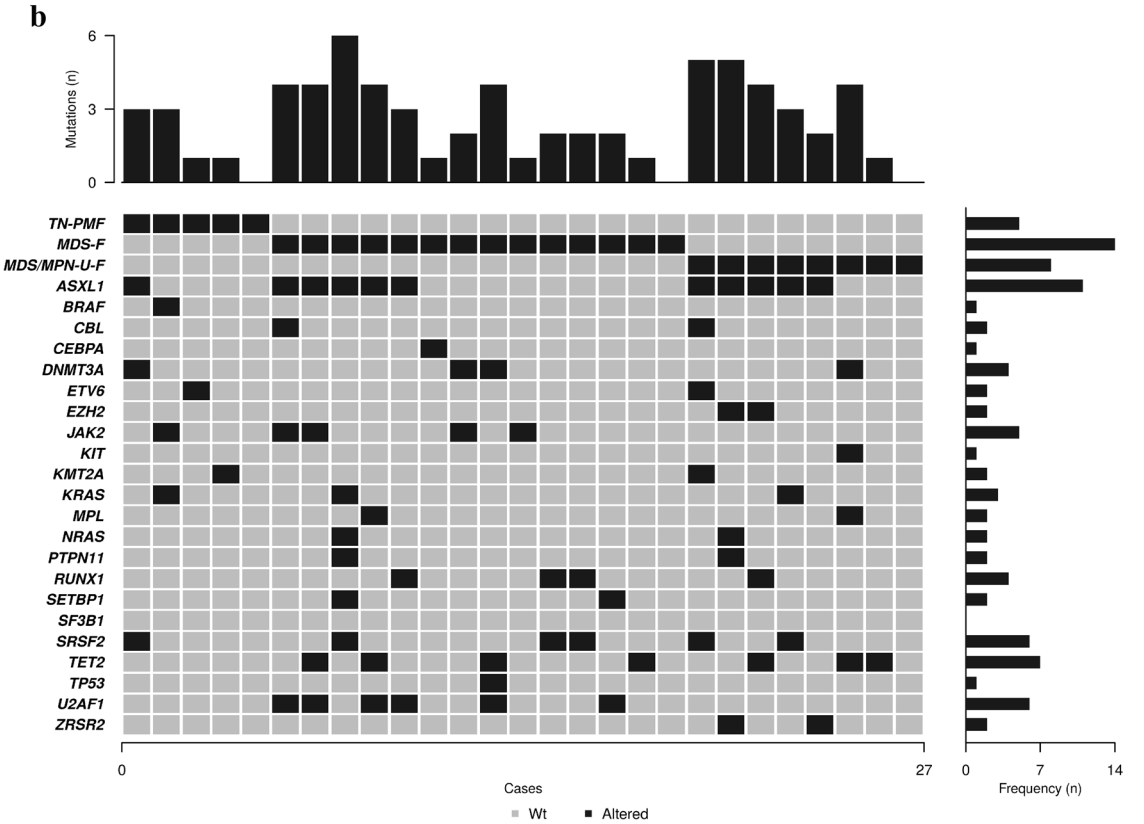
The main characteristics of the patients' bone marrow histology are shown in Table 2. Diagnosis allocation was performed after central review of bone marrow histology, with TN-PMF and MDS/MPN-U-F sharing increased cellularity due to prominent granulocytic proliferation resulting in an increased myeloid/erythroid ratio in comparison with MDS-F. In contrast, MDS/MPN-U-F and MDS-F shared some megakaryocytic abnormalities such as small size, cloud-shaped, or hypolobulated nuclei, while giant hyperlobulated elements and dense clustering were less frequent than in TN-PMF.

Molecular characterization by NGS

NGS studies were performed in samples obtained at diagnosis ($n=23$) or during follow-up ($n=4$). Mutations were detected in 24 out of 27 (93%) patients, with 13 (48%) patients presenting ≥ 3 mutations. Median number of mutations was 3 (range 1–7). The most frequently mutated genes were *ASXL1* ($n=11$; 41%), *TET2* ($n=7$; 26%), *SRSF2* ($n=6$; 22%), *U2AF1* ($n=6$; 22%), *JAK2* ($n=5$; 18.5%), *DNMT3A* ($n=4$; 15%), *RUNX1* ($n=4$; 15%), *KRAS* ($n=3$; 11%), *CBL*, *ETV6*, *EZH2*, *KMT2A*, *MPL*, *NRAS*, *PTPN11*, *SETBP1*, *ZRSR2* ($n=2$ each; 7%), and *TP53* ($n=1$; 4%). The mutated genes are shown in the supplementary table. Based on NGS and/or cytogenetic studies, genomic classification according to Grinfeld et al. [15] could be performed in 28 cases (Table 3). One case without available sample for NGS could

Fig. 1 Distribution of the mutations detected by NGS in 27 patients with myeloid neoplasm with associated bone marrow fibrosis according to genomic classification (**a**) or histological classification (**b**). Group 1: *TP53* disruption or aneuploidy (*TP53* mutation, Chr17pLOH or Chr5-/Chr5q-). Group 2: ≥ 1 chromatin or spliceosome mutation (*EZH2*, *IDH1*, *IDH2*, *ASXL1*, *PHF6*, *CUX1*, *ZRSR2*, *SRSF2*, *U2AF1*, *KRAS*, *NRAS*, *GNAS*, *CBL*, Chr7/7qLOH, Chr4q/LOH, *RUNX1*, *STAG2*, and *BCOR*). Group 7: Myeloid neoplasm with other driver mutation. Group 8: myeloid neoplasm with no known driver mutation. TN-PMF: triple-negative primary myelofibrosis. MDS-F: myelodysplastic syndrome with fibrosis. MDS/MPN-F: myelodysplastic/myeloproliferative neoplasm unclassifiable with fibrosis. Each column represents an individual case. Diagnosis allocation was performed after central review of bone marrow histology


a
Mutations (n)
6
3
0
Group 1
Group 2
Group 7
Group 8
ASXL1
BRAF
CBL
CEBPA
DNMT3A
ETV6
EZH2
JAK2
KIT
KMT2A
KRAS
MPL
NRAS
PTPN11
RUNX1
SETBP1
SF3B1
SRSF2
TET2
TP53
U2AF1
ZRSR2
0
27
Cases
0
8
16
Frequency (n)
Wt
Altered


b
Mutations (n)
6
3
0
TN-PMF
MDS-F
MDS/MPN-U-F
ASXL1
BRAF
CBL
CEBPA
DNMT3A
ETV6
EZH2
JAK2
KIT
KMT2A
KRAS
MPL
NRAS
PTPN11
RUNX1
SETBP1
SF3B1
SRSF2
TET2
TP53
U2AF1
ZRSR2
0
27
Cases
0
7
14
Frequency (n)
Wt
Altered

be classified within group 2 according to cytogenetics. Median number of mutations was 4 (range 1–7) in cases with *TP53* disruption/aneuploidy or with chromatin-spliceosome mutations versus 1 (range 0–2) in other molecular subgroups ($p < 0.0001$). The distribution of mutations according to the genomic classification is shown in Fig. 1a.

Mutations in *JAK2* were detected in five patients, with three of them corresponding to non-canonical variants (R867G, F694S, and R1063H). Two patients carried *MPL* mutations located at 505 and 591 aminoacid position, respectively (supplemental table). All *U2AF1* mutations were detected in patients with MDS-F ($p = 0.028$) and corresponded to the *U2AF*1Q157P and R156H variants in 5 and 1 cases, respectively. Mutations in *ZRSR2* ($p = 0.08$) and *EZH2* ($p = 0.08$) were restricted to patients with MDS/MPN-U-F. The distribution of mutations in individual cases according to the histological diagnosis after central review is shown in Fig. 1b. Three out of six MDS-F cases with mutated *U2AF1* showed a recurrent combination of mutations characterized by *U2AF*1Q157P/*ASXL1*G646Wfs*12 plus a driver mutation at low allele burden (*JAK2*V617F and *MPL*505 in 2 and 1 cases, respectively). The median number of mutations was 1 (range 0–4) in TN-PMF, 2 (range: 0–7) in MDS-F, and 4 (range: 0–7) in MDS/MPN-U-F, but the differences were not statistically significant. There were no significant differences in the frequency of the different genomic subgroups according to histological diagnosis.

Survival and disease progression

Twenty-four patients had died, resulting in a median survival of 1.7 years (95%CI 1.1–2.3) for the overall group of patients. Median survival was 2.6 years in TN-PMF, 1.5 years in MDS-F, and 1.6 years in MDS/MPN-F ($p = 0.3$). Of the different variables included in the currently used prognostic scoring systems for PMF and MDS, leukocyte count $> 25 \times 10^9$/L ($p = 0.001$), blood blasts $\geq 1\%$ (0.02%), blood blasts $> 5\%$ ($p = 0.001$) and high-risk cytogenetics ($p = 0.01$) were associated with shorter survival, whereas other variables such as age, presence of constitutional symptoms, hemoglobin level < 10 g/dL, platelet count $< 100 \times 10^9$/L, or bone marrow blast percentage were not.

PMF-IPSS could be applied in all patients regardless of their original diagnosis, whereas MDS-IPSS could be calculated in only 18 out of 34 (54%) patients due to the lack of information regarding cytogenetics or bone marrow blast quantification. When applied to the whole cohort of patients, MDS-IPSS discriminated better for survival than the IPSS-PMF. Nevertheless, the MDS-IPSS was only useful to identify a low-risk group, with a median survival of 8.3 years, but failed to show survival differences between the intermediate-risk groups. Risk stratification and survival according to the two scoring systems are shown in Table 4.

According to the molecular classification, median survival was 1.6 years in patients with *TP53* disruption/aneuploidy or with chromatin-spliceosome mutations and not reached in those cases classified in other subgroups ($p = 0.01$, Fig. 2a). Of note, the number of mutations detected by NGS was significantly associated with survival. Median survival was 1.3 years in patients with three or more mutations as compared with 8.2 years in those with two or less mutations ($p = 0.009$, Fig. 2b).

Progression to acute myeloid leukemia was observed in nine patients, eight of them within 3 years after diagnosis. There was one case of late progression to acute leukemia after 7 years of follow-up. Three-year probability of acute leukemia was 20% in TN-PMF, 37% in MS-F, and 50% in MDS/MPN-U-F ($p = 0.4$). All progressions were observed in the *TP53* disruption/aneuploidy or with chromatin-spliceosome mutation groups (3-year probability of 52% vs. 0% in other genomic subgroups, $p < 0.026$, Fig. 3a). Time to acute leukemia was significantly shorter in patients with ≥ 3 mutations detected by NGS than in those with two or less mutations (probability at 3 years: 60% versus 7%, respectively; $p = 0.01$, Fig. 3b).

Discussion

In the present work, a comprehensive review of all cases of TN-PMF and other myeloid neoplasms with bone marrow fibrosis from two academic hospitals has been performed. The main findings were the low frequency of TN-PMF when the current WHO criteria are strictly applied and the major contribution of NGS in defining prognosis and risk of leukemic transformation in these infrequent entities. Due to the

Table 4 Risk stratification and survival according to the prognostic scoring systems for PMF and MDS in 34 patients with myeloid neoplasms with bone marrow fibrosis

	Number of patients, *n* (%)	Median Survival, years	*P* value
IPSS-PMF risk group:	34 (100)		0.4
Low	4 (12)	0.7	
Intermediate-1	6 (18)	2.2	
Intermediate-2	13 (38)	2.3	
High	11 (32)	1.7	
IPSS-MDS risk group:	18 (53)		0.02
Low	5 (28)	8.3	
Intermediate-1	9 (50)	1.5	
Intermediate-2	4 (22)	1.2	
High	–	–	

PMF primary myelofibrosis, *MDS* myelodysplastic syndrome, *IPSS* international prognostic scoring system

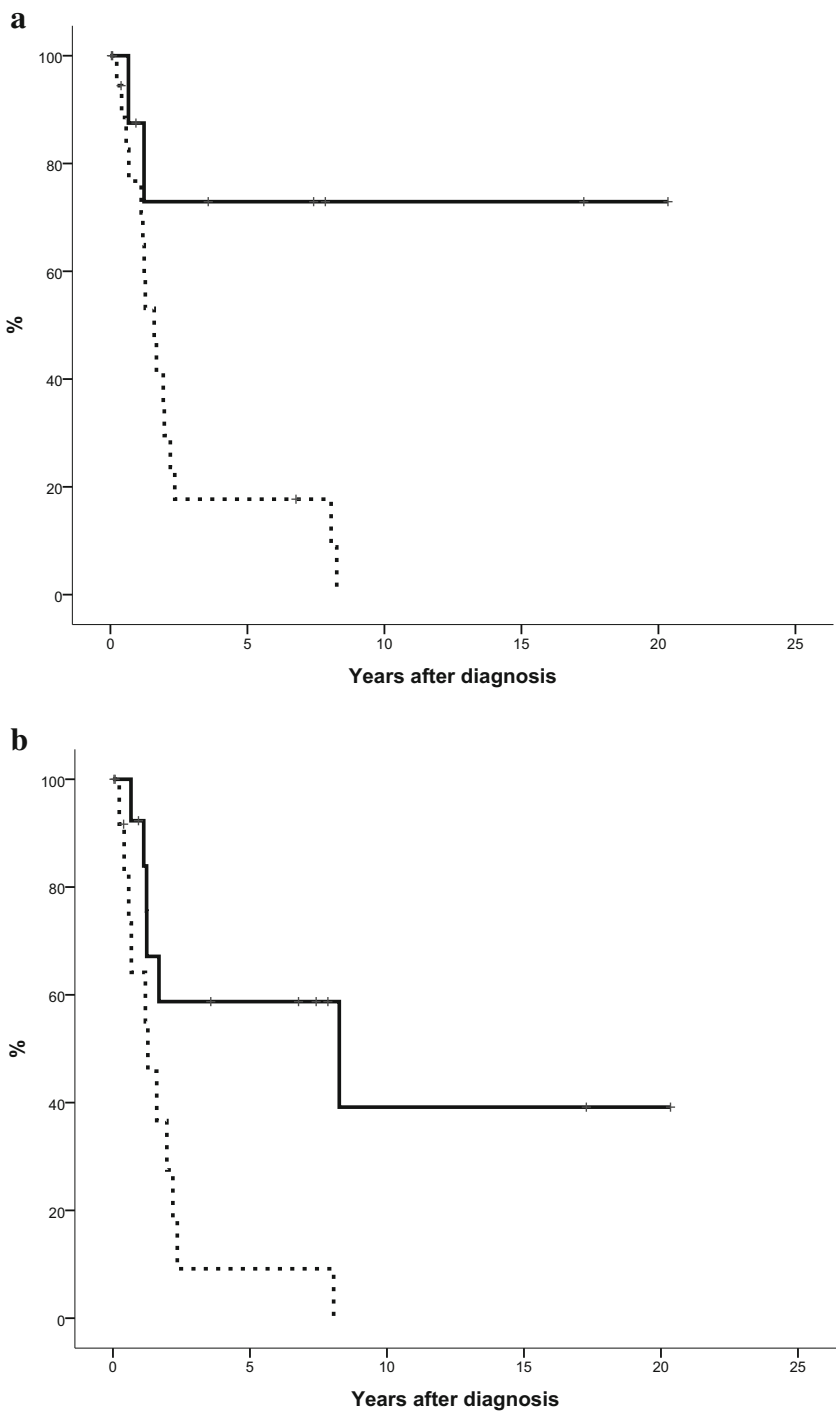


Fig. 2 **a** Probability of survival according to the genomic classification in patients with myeloid neoplasms with bone marrow fibrosis. The dotted line corresponds to patients with *TP53* disruption/aneuploidy or with chromatin-spliceosome mutations and the solid line to patients with other driver mutation or not known driver mutation ($p = 0.01$). **b** Probability of survival according to the number of mutations in patients with myeloid neoplasms with bone marrow fibrosis. The dotted line corresponds to patients with ≥ 3 mutations and the solid line to patients with ≤ 2 mutations ($p = 0.009$).

reclassification of TN-PMF into MDS-F or MDS/MPN-U-F categories after histological review, TN-PMF represented only 4% of all patients with PMF, a figure significantly lower than the 10% reported in most contemporary series [4, 9, 17].

TN-MFP-TN, MDS-F, and MDS/MPN-U-F are associated with a poor prognosis, with allogeneic hematopoietic stem cell transplantation (allo-HSCT) being usually indicated in suitable patients [18, 19]. For that reason, an accurate prognostic evaluation is critical for treatment decision-making. Due to its simplicity, we could retrospectively calculate the PMF-IPSS in all patients from the present series [15]. However, its ability to discriminate subgroups with different survival was limited in this particularly poor-prognosis cohort of patients. On the contrary, the IPSS designed for myelodysplastic syndromes [16] was able to identify a low-risk group of patients, with a median survival of 8.3 years. However, the absence of material for cytogenetics and the difficulty in quantifying bone marrow blasts due to bone marrow fibrosis limited the

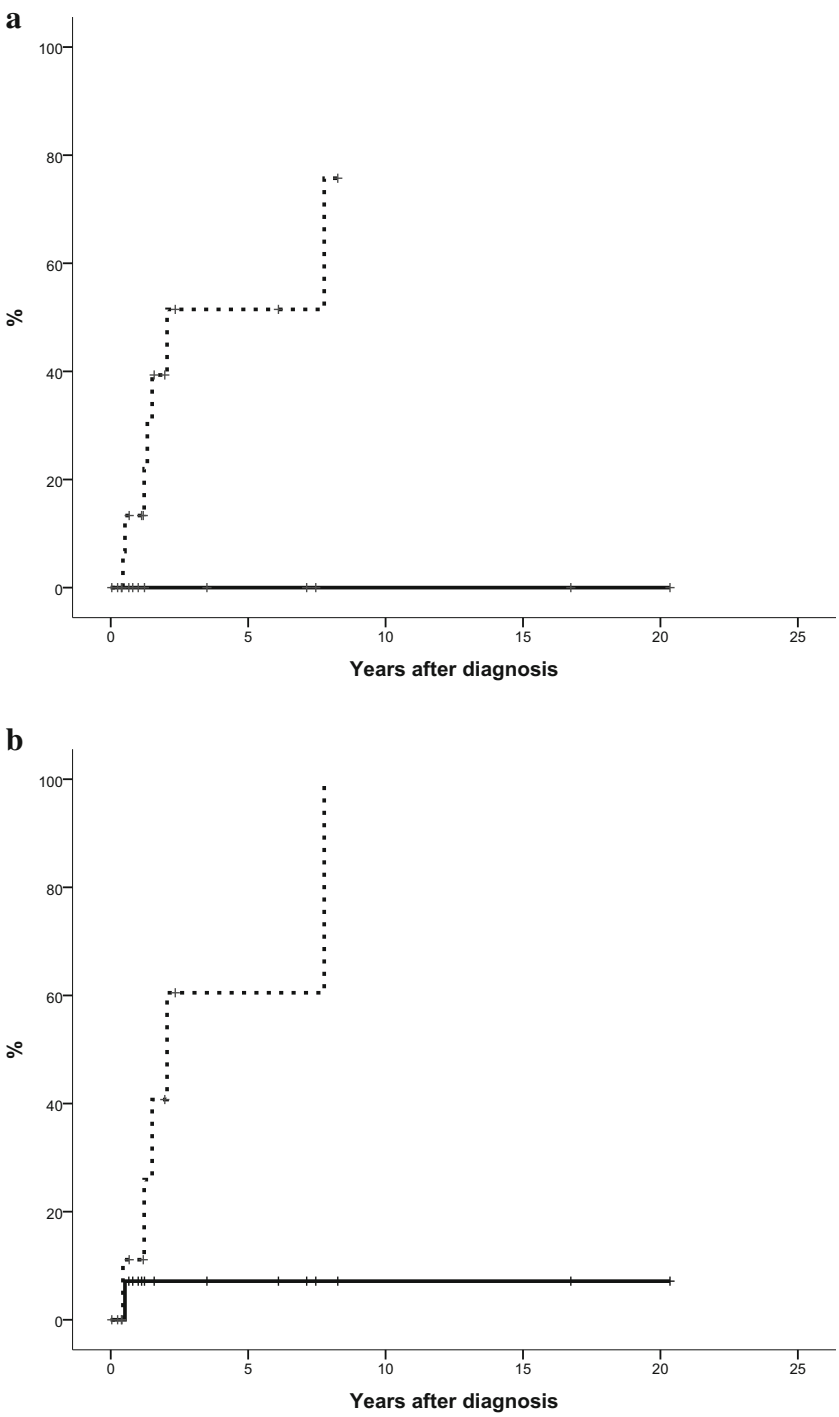


Fig. 3 **a** Probability of progression to acute leukemia according to genomic classification in patients with myeloid neoplasms with bone marrow fibrosis. The dotted line corresponds to patients with *TP53* disruption/aneuploidy or with chromatin-spliceosome mutations and the solid line to patients with other driver mutation or not known driver mutation ($p = 0.026$). **b** Probability of progression to acute leukemia according to the number of mutations in patients with myeloid neoplasms with bone marrow fibrosis. The dotted line corresponds to patients with ≥ 3 mutations and the solid line to patients with ≤ 2 mutations ($p = 0.01$)

applicability of the MDS-IPSS in a significant proportion of patients. In this sense, NGS proved to be a useful tool to estimate prognosis in these rare entities. First, it can be used in all patients as DNA can be readily obtained from peripheral blood. Second, the number of mutations detected by NGS allowed the identification of two groups of patients with different outcome in terms of survival and leukemic risk.

Molecular classification according to an algorithm recently reported by Grinfeld et al. proved to be useful in predicting survival and leukemic transformation, with these authors questioning the current WHO classification based on hematological variables and histology [9]. This is especially true for TN-PMF, in which disease heterogeneity and overlapping with other myeloid neoplasms with bone marrow fibrosis make it difficult an accurate histological diagnosis, as illustrated by the high rate of histological reclassification in a substantial proportion of our patients. In addition, we and others [17] have shown the prognostic contribution of mutations in genomic subgroups

defined by the presence of disruption/aneuploidy or with chromatin-spliceosome mutations, with these results further supporting the utility of such genomic classification in this subset of patients. For the abovementioned reasons, NGS techniques could play an important role in the selection of therapy in patients with TN-PMF and other myeloid neoplasms with bone marrow fibrosis [20]. Thus, patients belonging to low-risk genomic subgroups have a good outcome and could be treated with a conservative approach, whereas those allocated to the high-risk genomic subgroups may be candidates to bone marrow transplantation or experimental therapies.

It must be remarked that exclusion of cases with MDS/MPN-RS-T and MDS with excess blasts type-2, as well as the fact that cohesin complex genes were not included in our NGS panel, might explain some differences in the frequency of mutated genes when compared with a recent report of 63 cases with MDS-F [21]. In addition, it is especially remarkable that mutations in *U2AF1* were restricted to patients with MDS-F and all of them carried variants located at the 157 position. This finding might appear in contradiction with previous reports in which the *U2AF*1Q157P mutation was more frequent in PMF than in MDS, where the S34 variants accounted for 70% of cases [22, 23]. However, the Q157P variant has been associated with bone marrow fibrosis in MDS, which would explain our findings in a cohort restricted to MDS-F [24]. Moreover, we could identify a recurrent combination of mutations in *U2AF*1Q157P/*ASXL1*G646Wfs*12 plus a driver mutation at low allele burden in three out of six cases with *U2AF1*-mutated MDS-F. To the best of our knowledge, this is the first study reporting the recurrent association of *ASXL1*G646Wfs*12 with both *U2AF*1Q157P and *JAK2*V617F or *MPL*505.

The main limitation of the present study is the low number of cases, which has probably precluded the identification of additional subgroups within the TN-PMF category. The histological findings, with two out of six patients showing bone marrow fibrosis without abnormal megakaryocytes, and the results obtained in the molecular studies reinforce the current perception that TN-PMF is a biologically heterogeneous entity. However, it should be noted that this work includes all patients from two referral institutions with large experience on the diagnosis and management of patients with MPNs, thus allowing an accurate evaluation of the real frequency of TN-PMF.

In conclusion, TN-PMF is an uncommon entity when 2016 WHO criteria are rigorously applied. Besides, NGS-based mutational analysis may be a useful tool for prognostic stratification in patients with TN-PMF or other myeloid neoplasms with bone marrow fibrosis.

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Author contributions AAL designed the study, collected the data, performed the statistical analysis, analyzed and interpreted the results, and wrote the paper. MLG and DC performed the molecular studies, interpreted the results, and wrote the paper. DM and MR reviewed the bone marrow biopsies, interpreted the results, and wrote the paper. JGC, IM, and MT collected the data and approved the final version. JCHB, JE, and FC collected the data, interpreted the results, and wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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