

# Gene expression profile correlates with molecular and clinical features in patients with myelofibrosis

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## Key Points

- Gene expression-based classification of primary and secondary MF correlates with patients' clinical and molecular characteristics.
- A gene expression signature identifies patient subgroups with different overall survival.

Myelofibrosis (MF) belongs to the family of classic Philadelphia-negative myeloproliferative neoplasms (MPNs). It can be primary myelofibrosis (PMF) or secondary myelofibrosis (SMF) evolving from polycythemia vera (PV) or essential thrombocythemia (ET). Despite the differences, PMF and SMF patients are currently managed in the same way, and prediction of survival is based on the same clinical and genetic features. In the last few years, interest has grown concerning the ability of gene expression profiles (GEPs) to provide valuable prognostic information. Here, we studied the GEPs of granulocytes from 114 patients with MF, using a microarray platform to identify correlations with patient characteristics and outcomes. Cox regression analysis led to the identification of 201 survival-related transcripts characterizing patients who are at high risk for death. High-risk patients identified by this gene signature displayed an inferior overall survival and leukemia-free survival, together with clinical and molecular detrimental features included in contemporary prognostic models, such as the presence of high molecular risk mutations. The high-risk group was enriched in post-PV and post-ET MF and JAK2V617F homozygous patients, whereas pre-PMF was more frequent in the low-risk group. These results demonstrate that GEPs in MF patients correlate with their molecular and clinical features, particularly their survival, and represent the proof of concept that GEPs might provide complementary prognostic information to be applied in clinical decision making.

## Introduction

Classic Philadelphia-negative myeloproliferative neoplasms (MPNs) consist of 3 clinical entities: polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). PMF can be subdivided into a prefibrotic/early stage (pre-PMF) and an overt fibrotic stage (overt PMF), according to the degree of bone marrow (BM) fibrosis.<sup>1</sup> Moreover, PV and ET can evolve into secondary myelofibrosis (SMF) giving rise to post-PV myelofibrosis (PPV-MF) and post-ET myelofibrosis (PET-MF).<sup>2</sup> These

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Data sharing requests should be sent to Rossella Manfredini (rossella.manfredini@unimore.it).

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malignancies are grouped together according to their overlapping features. Although the absence of BM fibrosis is considered the chronic phase of these malignancies, myelofibrosis (MF) onset represents the advanced stage that might precede acute myeloid leukemia (AML) transformation, the main cause of death for these patients.<sup>3,4</sup>

MPNs originate from the mutation of hematopoietic stem cells, which generates a neoplastic clone that gives rise to the myeloproliferative phenotype. Clonal hematopoiesis in MPNs has been demonstrated by the presence of recurrent gene mutations that is shared by the 3 clinical entities.<sup>5,6</sup> Mutations in *JAK2*, calreticulin (*CALR*), and *MPL* proto-oncogene thrombopoietin receptor (*MPL*) genes are considered driver events. Although a *JAK2V617F* mutation is carried by almost all patients with PV, it occurs at a 55% frequency in PMF and a 65% frequency in ET. *CALR* indels affect 25% to 35% of PMF patients and 15% to 24% of those with ET, whereas *MPL* mutations occur in ~8% and 4% of patients with PMF and ET, respectively. Almost 5% of PMF patients and 10% of those with ET do not harbor any of the 3 driver mutations and are considered triple negative.<sup>5,7</sup> Nevertheless, many other somatic mutations and genomic aberrations have been identified that might represent driver events but, in most cases, they accompany other lesions and influence disease outcome.<sup>3</sup>

PMF and SMF patients are currently managed in the same way because they share common histopathologic features and clinical manifestations. Prediction of survival influences the therapeutic strategy used and is based on prognostic models, such as the International Prognostic Scoring System (IPSS) and Dynamic-IPSS (DIPSS), which have been designed for PMF based on clinical features. Nevertheless, because these tools are not able to accurately distinguish different risk categories in SMF,<sup>8,9</sup> the Myelofibrosis Secondary to PV and ET-Prognostic Model (MYSEC-PM)<sup>10</sup> was recently created. The need for more specific and more accurate prognostic models in PMF and SMF arises from the observation that only allogeneic stem cell transplantation (ASCT) is potentially curative and is able to prolong survival in patients; however, the incidence of mortality and severe adverse events justify this approach only in high-risk patients.<sup>11</sup> New prognostication models have been developed: the Mutation-enhanced International Prognostic Score System (MIPSS70) and the most recent MIPSS70+ version 2.0 integrate clinical information with mutation and cytogenetic data to identify high-risk PMF patients aged  $\leq 70$  years who are candidates for ASCT.<sup>12,13</sup> If cytogenetics is available, a genetically inspired prognostic scoring system (GIPSS) can be applied to predict patients' survival based on mutations and karyotype information.<sup>14</sup>

To identify disease subtypes that are characterized by a poor outcome, we studied the correlation between gene expression profiles (GEPs) of PMF and SMF patients and their clinical characteristics, with particular interest in survival. This approach has already been successfully applied in other solid and hematological malignancies, including AML<sup>15,16</sup> and myelodysplastic syndromes,<sup>17-19</sup> leading to the discovery of gene expression signatures that identify patients with inferior survival. These studies demonstrated that gene expression profiling could be a useful tool for the classification of hematological malignancies, because it might improve the identification of high-risk patients who have a poor prognosis.

Starting from these observations, in this proof-of-concept study, we analyzed the GEP of granulocytes from patients affected by PMF and SMF. To assess the correlation between GEP and survival, we applied a Cox proportional-hazards model to our data set, which led to the identification of a list of genes whose expression can divide patients into 2 subgroups with different outcomes. Overall, our results suggest that gene expression analysis may complement current methods for risk stratification in MF.

## Patients and methods

### Patients and samples

This study was conducted using granulocyte samples from 114 patients with a diagnosis of PMF or SMF recruited from 5 Italian centers. PMF was diagnosed according to 2016 World Health Organization criteria,<sup>1</sup> whereas International Working Group for Myeloproliferative neoplasms Research and Treatment criteria<sup>2</sup> were used for the diagnosis of PET-MF and PPV-MF. The study was conducted in accordance with the Declaration of Helsinki and was approved by local ethics committees. All subjects provided informed written consent.

Granulocytes were collected from peripheral blood using a density gradient. Following centrifugation, granulocytes and erythrocytes formed a cell pellet at the bottom of the tube, and granulocytes can be purified using red blood cell lysis reagent. The different units provided frozen granulocyte pellets and lysed cells.

As previously described,<sup>12,20</sup> gene mutations were detected in DNA from peripheral blood cells. Real-time quantitative polymerase chain reaction was used to identify *JAK2V617F* and *MPLW515x* mutations, whereas *CALR* mutations were detected by capillary electrophoresis, followed by bidirectional sequencing, and classified as type 1/type 1-like or type 2/type 2-like.<sup>21</sup> Next-generation sequencing was used to detect high molecular risk (HMR) mutations (ie, *ASXL1*, *EZH2*, *IDH1*, *IDH2* and *SRFSF2*).

### RNA extraction and gene expression profiling

Total cellular RNA was isolated from stored frozen granulocyte pellets using TRIzol Reagent (Invitrogen, ThermoFisher Scientific), following the manufacturer's instructions. RNA sample concentration was evaluated using a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific), and purity was assessed using 260/280 nm and 260/230 ratios. An Agilent 2100 Bioanalyzer (Agilent Technologies) was used to determine the integrity of total RNA.

Gene expression profiling was performed using HG-U219 Array Strips (Affymetrix), as previously described.<sup>22</sup> Biotin-labeled complementary RNA was synthesized starting from 100 ng of total RNA using a GeneChip HT 3' IVT PLUS Reagent Kit (Applied Biosystems), according to the standard protocol supplied, whereas microarray hybridization, staining, and scanning were performed using a GeneAtlas Hybridization, Wash, and Stain Kit (Affymetrix) and a GeneAtlas System (Affymetrix), following the manufacturer's recommendations.

Partek Genomics Suite (GS) software, version 7.0 (Partek Inc., St. Louis, MO) was used to normalize the probe level data and convert them into expression values by means of robust multiarray average. An exploratory principal component analysis was performed, and the existing batch effect due to the clinical unit of origin was removed using Partek GS. The "Remove batch effect" function in

Partek GS is based on a mixed-model analysis of variance and allows adjustment of the gene expression matrix, removing differences between batches. Principal component analysis was then used to verify the batch effect adjustment. Cox regression analysis function was run to identify probesets whose expression correlates with patient survival. Probesets with a  $P$  value  $< .005$  were selected for further analysis and were used to perform supervised hierarchical clustering with Partek GS.

### Classification model construction

The classification model was built starting from the list of probesets resulting from Cox regression analysis and risk classes defined with hierarchical clustering. The “nearest shrunken centroids” supervised learning technique, implemented in the *pamr.train* function of the R package *pamr* (v.1.56.1), was applied for the classification model generation. A standardized centroid was calculated for each risk class and then it was shrunk toward the overall centroid by 30 different thresholds. The threshold giving the lowest error rate was selected. The “nearest shrunken centroids” technique made it possible to optimize the number of probesets by excluding from the prediction rule those whose expression was not sufficiently different between the 2 groups (and thus not contributing to the classification). Briefly, the centroid is defined as the median expression of each probeset in each class divided by the standard deviation in each class. Each of the class centroids was shrunk toward the overall centroid by an amount called threshold. This shrinkage consists of moving the centroid toward 0 by threshold, setting it equal to 0 if it hits 0. If a probeset was shrunk to 0 for all classes, then it did not survive the threshold.

The resulting model was cross-validated with a  $k$ -fold cross-validation method, with  $k = 20$ , using the *pamr.cv* function in the R package *pamr*. The whole data set was split into  $k$  smaller sets and then several models were defined using  $k - 1$  of the folds. The remaining data were used for testing the model, repeating the procedure until each fold was used as the test set. The class of the test samples was predicted based on the nearest centroid.

To further optimize the number of probesets, model generation and cross-validation were repeated  $m - 1$  times (where  $m$  is the total number of probesets). As described previously,<sup>23</sup> starting from 2 probesets, each time 1 probeset was sequentially added from the top of the rank-ordered probeset list based on the hazard ratio (HR), until all of the probesets were used. The model's performance was assessed with the use of the cross-validated misclassification error calculated with the *pamr.cv* function from the *pamr* R package; the model with the optimized set of probesets that provided the lowest error was selected.

### Statistical analysis

Differences in the distribution of numerical variables were evaluated using the Mann-Whitney  $U$  test or the Kruskal-Wallis test for the comparison of 2 groups or  $>2$  groups, respectively. Categorical variables were compared using the  $\chi^2$  test or Fisher's exact test. Overall survival (OS) was calculated from the date of sample collection to the date of death or last follow-up. When calculating leukemia-free survival (LFS), the date of leukemic transformation was used in place of the date of death. OS and LFS analyses were performed with the Kaplan-Meier method, and the log-rank test was used to compare curves. Univariate and multivariate analyses were carried out using Cox proportional-hazards regression for OS.

Analyses were performed using R version 3.4.1. A  $P$  value  $< .05$  was considered statistically significant.

## Results

### Patients

A total of 114 patients (35 pre-PMF, 37 overt PMF, 26 PET-MF, and 16 PPV-MF) was studied. Table 1 shows the distribution of patients with regard to MF clinical subtype. No difference in terms of OS was detected among these groups (Table 1; supplemental Figure 1), whereas some dissimilarities were evident in terms of the clinical characteristics. Pre-PMF patients displayed increased hemoglobin (Hb) levels and were less frequently anemic; platelet count was increased in this group, and splenomegaly was more frequent.<sup>24,25</sup> Hb was also increased in PPV-MF, as were white blood cell counts. Anemia was less frequent in these patients, whereas leukocytosis was more common.<sup>20,26</sup> PET-MF patients showed increased platelet counts.<sup>20,26</sup> All PPV-MF patients harbored a JAK2V617F mutation, with the exception of 1 patient who displayed a mutation on exon 12. Patients with pre-PMF displayed an increased frequency of SRSF2 mutations and  $>2$  HMR mutations. Leukemic transformation occurred in 13 patients, whereas 49 died of causes related to the disease.

### Identification of a gene signature that correlates with OS

Cox-regression analysis identified 832 probesets (corresponding to 596 genes) that correlated with patient survival; among them, 433 genes were associated with inferior survival (supplemental Table 1). According to hierarchical clustering (Figure 1A), this list splits our data set into 2 main branches composed of 62 (left) and 52 (right) samples. As shown in Figure 1B and supplemental Table 2, the cluster on the right was characterized by significantly inferior OS ( $P = 4.38E-6$ , log-rank test). Moreover, the frequency of dead patients was significantly higher in the right branch ( $P = 3.08E-4$ , Fisher's exact test) (supplemental Table 2); therefore, we named this cluster the high-risk group, whereas the other one was termed the low-risk group.

The majority of pre-PMF samples were included in the low-risk cluster, whereas SMF was more frequent in the high-risk group (Figure 1A; supplemental Table 1). The high-risk group was also enriched in patients harboring JAK2V617F mutations; the frequency of homozygous mutations was increased in this subgroup, whereas JAK2 heterozygosity was more frequent in the low-risk group. Moreover, 26 of 52 samples (65%) in the high-risk group harbored  $\geq 1$  HMR mutation (Figure 1A; supplemental Table 2). Despite this, we identified a significant difference only in the distribution of patients with ASXL1 mutations ( $P$  value = 2.79E-4, Fisher's exact test). Considering clinical characteristics, the high-risk group displayed features of patients with predictable inferior survival. Indeed, the median age at the time of sample collection was higher in this group compared with the low-risk group. White blood cell count was increased in high-risk patients, whereas Hb levels and platelet count were decreased compared with the low-risk group. The high-risk group had greater frequencies of patients with  $>1\%$  or  $2\%$  circulating blasts, splenomegaly, and BM fibrosis grade  $\geq 2$  (supplemental Table 2). Taken together, these results demonstrated that Cox-regression analysis led to the identification of genes whose expression correlates with OS in MF patients.

**Table 1. Clinical and laboratory characteristics of patients included in our dataset, divided according to diagnosis**

Variable	Pre-PMF (n = 35)	Overt PMF (n = 37)	PET-MF (n = 26)	PPV-MF (n = 16)	P
Follow-up, median (95% CI), y	6.88 (3.39-NA)	5.54 (3.26-6.36)	4.55 (2.91-NA)	4.18 (1.72-6.79)	6.15E-01
Males	19 (54.3)	21 (56.8)	13 (50.0)	7 (43.8)	8.33E-01
<b>Age</b>					
Median (range), y	62.90 (34.6-80.9)	63.80 (31.1-84.3)	65.80 (32.1-81.0)	71.10 (42.6-85.6)	9.62E-02
>65 y	13 (37.1)	15 (40.5)	14 (53.8)	11 (68.8)	1.38E-01
<b>Hb</b>					
Median (range), g/dL	12.40 (8.0-16.6)	11.20 (5.2-15.3)	10.75 (6.5-14.6)	12.55 (9.2-15.9)	<b>2.52E-04</b>
<10 g/dL	2 (5.7)	9 (24.3)	9 (34.6)	2 (12.5)	<b>2.75E-02</b>
<b>Leukocytes</b>					
Median (range), ×10 <sup>9</sup> /L	8.70 (3.6-41.0)	10.00 (2.8-89.0)	9.58 (2.3-104.0)	14.90 (5.9-88.7)	<b>1.35E-02</b>
>25 × 10 <sup>9</sup> /L	3 (8.8)	6 (16.2)	3 (11.5)	7 (46.7)	<b>9.09E-03</b>
<b>Platelets</b>					
Median (range), ×10 <sup>9</sup> /L	410.0 (72-1299)	179.0 (22-1252)	377.5 (61-1568)	224.5 (20-1271)	<b>6.54E-03</b>
<100 × 10 <sup>9</sup> /L	2 (5.9)	9 (25.0)	2 (8.3)	1 (6.2)	6.19E-02
Circulating blasts ≥1%	5 (15.2)	8 (25.8)	8 (34.8)	3 (20.0)	3.77E-01
Circulating blasts ≥2%	5 (15.2)	4 (12.9)	6 (26.1)	2 (13.3)	5.81E-01
BM fibrosis grade ≥2	—	33 (97.1)	23 (100)	13 (92.9)	4.44E-01
Constitutional symptoms	5 (14.7)	11 (30.6)	7 (26.9)	5 (31.2)	4.08E-01
Splenomegaly	16 (45.7)	31 (86.1)	22 (84.6)	10 (71.4)	<b>6.24E-04</b>
<b>Driver mutation</b>					
JAK2 V617F	12 (34.3)	16 (43.2)	11 (42.3)	15 (93.8)	<b>8.04E-04</b>
JAK2 ex12	0	0	0	1 (6.2)	—
CALR unspecified	0	0	1 (3.8)	0	—
CALR type 1	4 (11.4)	10 (27.0)	7 (26.9)	0	1.99E-01
CALR type 2	5 (14.3)	3 (8.1)	0	0	1.31E-01
MPL	5 (14.3)	3 (8.1)	3 (11.5)	0	7.07E-01
Triple negative	9 (25.7)	5 (13.5)	4 (15.4)	0	3.69E-01
<b>ASXL1 mutation (n evaluable, total = 85)</b>	25	32	15	13	
n (%)	11 (44.0)	10 (31.2)	5 (33.3)	6 (46.2)	6.81E-01
<b>EZH2 mutation (n evaluable, total = 82)</b>	24	27	16	15	
n (%)	3 (12.5)	2 (7.4)	0	1 (6.7)	5.27E-01
<b>SRSF2 mutation (n evaluable, total = 81)</b>	23	27	16	15	
n (%)	7 (30.4)	1 (3.7)	1 (6.2)	0	<b>5.98E-03</b>
<b>IDH1/2 mutation (n evaluable, total = 81)</b>	23	27	16	15	
n (%)	2 (8.7)	1 (6.7)	0	1 (6.7)	6.36E-01
<b>HMR (n evaluable, total = 86)</b>	25	29	17	15	
n (%)	14 (56.0)	12 (41.4)	6 (35.3)	7 (46.7)	5.66E-01
≥2	8 (32.0)	2 (6.9)	0	1 (6.7)	<b>6.82E-03</b>
<b>DIPSS (n evaluable, total = 107)</b>	31	36	25	15	
Low	14 (45.2)	9 (25.0)	4 (16.0)	3 (20.0)	
Intermediate-1	10 (32.3)	13 (36.1)	13 (52.0)	5 (33.3)	
Intermediate-2	3 (9.7)	12 (33.3)	4 (16.0)	5 (33.3)	
High	4 (12.9)	2 (5.6)	4 (16.0)	2 (13.3)	3.89E-01
<b>MIPSS70 (n evaluable, total = 73)</b>	21	25	15	12	
Low	10 (47.6)	2 (8.0)	0	1 (8.3)	
Intermediate	4 (19.0)	14 (56.0)	10 (66.7)	5 (41.7)	
High	7 (33.3)	9 (36.0)	5 (33.3)	6 (50.0)	<b>1.71E-03</b>

Unless otherwise noted, data are n (%). Significant *P* values (<.05) are highlighted in bold. NA, not available. —, missing value.

**Table 1. (continued)**

Variable	Pre-PMF (n = 35)	Overt PMF (n = 37)	PET-MF (n = 26)	PPV-MF (n = 16)	P
<b>MYSEC-PM (n evaluable, total = 41)</b>			23	15	
Low	—	—	7 (30.4)	2 (13.3)	
Intermediate-1	—	—	9 (39.1)	7 (46.7)	
Intermediate-2	—	—	4 (17.4)	3 (20.0)	
High	—	—	3 (13.0)	3 (20.0)	6.70E-01
Progression to leukemia	8 (22.9)	3 (8.1)	2 (7.7)	0	6.14E-02
Death	13 (37.1)	17 (45.9)	9 (34.6)	10 (62.5)	2.78E-01

Unless otherwise noted, data are n (%). Significant *P* values (<.05) are highlighted in bold. NA, not available. —, missing value.

These genes identified 2 patient subgroups characterized by high-level differences in terms of clinical and molecular features and OS.

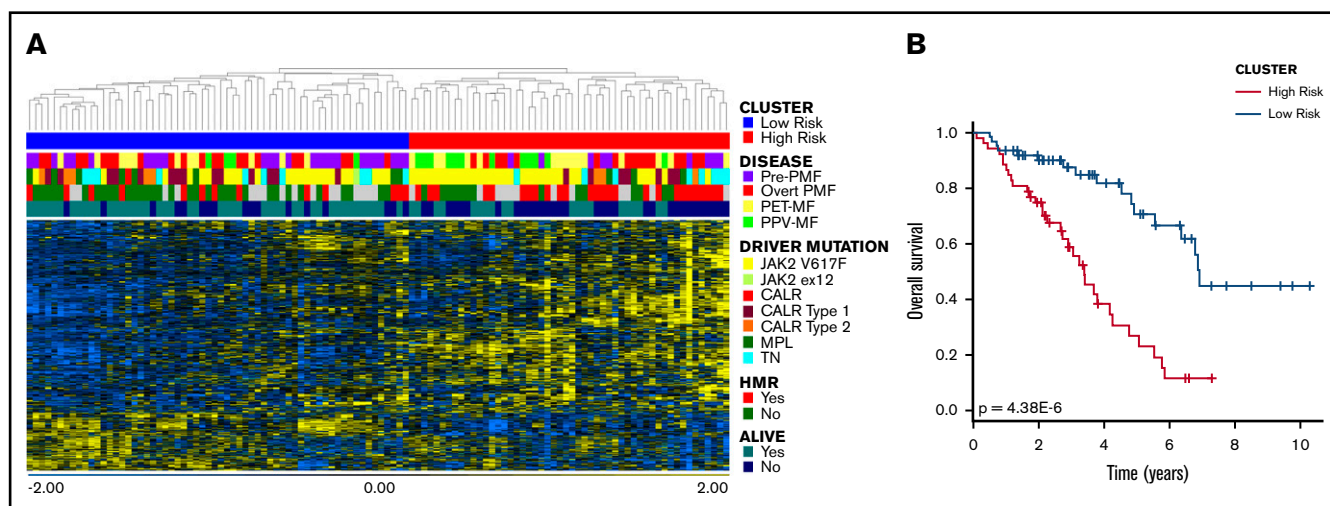
### Gene expression–based classification correlates with patients' clinical and molecular features

Starting with the list of 832 probesets derived from Cox-regression analysis, we constructed a gene expression–based classifier for the 2 risk categories defined according to hierarchical clustering. As detailed in the Patients and methods section, we applied a supervised learning technique, the “nearest shrunken centroids” method, to construct the model and cross-validated it using a 20-fold cross-validation strategy exploiting the *pamr.cv* R function to estimate the misclassification error and build a robust classifier. We obtained an optimized model using the first 351 probesets of the list, of which 273 (corresponding to 201 genes) survived the cross-validation threshold (supplemental Table 3).

According to this classification, the low-risk and high-risk groups were identified, which contained 60 and 54 patients, respectively (Table 2). Patients in the high-risk group displayed several detrimental characteristics. The high-risk class was enriched in samples derived from dead patients (Table 2) and was characterized by a significantly inferior survival compared with the low-risk

group (*P* value = 1.78E-7, log-rank test) (Figure 2A; Table 2). Moreover, the low-risk group was enriched in pre-PMF samples, whereas overt PMF samples were distributed equally between the 2 classes. On the contrary, higher percentages of PET-MF and PPV-MF were present in the high-risk group. Taking into account molecular characteristics, the high-risk group was enriched in patients harboring the JAK2V617F mutation (Table 2); in particular, homozygosity was more frequent in this group compared with the low-risk one. The frequency of patients with  $\geq 1$  HMR mutation was increased in the high-risk group; again, this group was enriched in ASXL1-mutated patients compared with the low-risk one (Table 2).

Next, we studied the distribution of clinical variables included in contemporary prognostic models and observed that high-risk classification correlated with the presence of clinical markers of inferior survival. Indeed, patient age at sample collection was higher in the high-risk group, which was also characterized by inferior Hb levels and platelet counts, superior white blood cell counts, increased incidence of splenomegaly, circulating blasts > 1% or 2%, BM fibrosis grade  $\geq 2$ , and constitutional symptoms (Table 2). Even if 9 of 13 patients who developed secondary AML clustered within the high-risk group, this difference failed to reach statistical significance (Table 2). Nevertheless, survival analysis revealed that



**Figure 1. Cox-regression analysis.** (A) Hierarchical clustering of samples according to the expression of 832 probesets that correlated with OS. The dataset was divided into 2 clusters. (B) The red cluster (on the right in the heatmap) displayed significantly inferior survival compared with the other one, as demonstrated by the result of log-rank test. Therefore, we defined the blue group as low-risk, whereas the red one was named high-risk.

**Table 2. Clinical and molecular characteristics of patients included in our dataset, classified according to our gene expression-based model**

Variable	Low-risk (n = 60)	High-risk (n = 54)	P
OS, median (95% CI), y	6.93 (5.56-NA)	3.26 (2.68-3.81)	<b>1.78E-07</b>
Males	32 (53.3)	28 (51.9)	1.00E+00
<b>Disease</b>			
Pre-PMF	25 (41.7)	10 (18.5)	
Overt PMF	20 (33.3)	17 (31.5)	
PET-MF	11 (18.3)	15 (27.8)	
PPV-MF	4 (6.7)	12 (22.2)	<b>1.17E-02</b>
<b>Age</b>			
Median (range), y	61.4 (32.1-81)	69.95 (31.1-85.6)	<b>5.73E-05</b>
>65 y	18 (30)	35 (64.8)	<b>3.15E-04</b>
<b>Hb</b>			
Median (range), g/dL	12.1 (6.5-16.6)	11.15 (5.2-14.4)	<b>1.89E-03</b>
<10 g/dL	6 (10)	16 (29.6)	<b>9.44E-03</b>
<b>Leukocytes</b>			
Median (range), $\times 10^9/L$	8.00 (2.3-38.7)	14.2 (3.15-104)	<b>3.98E-06</b>
>25 $\times 10^9/L$	1 (1.7)	18 (34)	<b>3.80E-06</b>
<b>Platelets</b>			
Median (range), $\times 10^9/L$	386 (22-1568)	226 (20-1271)	<b>6.85E-03</b>
<100 $\times 10^9/L$	4 (7)	10 (18.9)	8.62E-02
Circulating blasts $\geq 1\%$	8 (14.3)	16 (34.8)	<b>1.94E-02</b>
Circulating blasts $\geq 2\%$	5 (8.9)	12 (26.1)	<b>3.13E-02</b>
BM fibrosis grade $\geq 2$	33 (55.9)	36 (76.6)	<b>3.97E-02</b>
Constitutional symptoms	10 (16.7)	18 (34.6)	<b>4.78E-02</b>
Splenomegaly	34 (56.7)	45 (88.2)	<b>2.93E-04</b>
<b>Driver mutation</b>			
JAK2 V617F	22 (36.7)	32 (59.3)	<b>2.38E-02</b>
JAK2 ex12	1 (1.7)	0	—
CALR unspecified	1 (1.7)	0	—
CALR type 1	14 (23.3)	7 (13)	2.26E-01
CALR type 2	6 (10)	2 (3.7)	2.77E-01
MPL	8 (13.3)	3 (5.6)	2.11E-01
Triple negative	8 (13.3)	10 (18.5)	6.08E-01
<b>JAK2 V617F</b>			
Heterozygous	14 (66.7)	7 (22.6)	
Homozygous	7 (33.3)	24 (77.4)	<b>3.41E-03</b>
CALR type 1 absence	46 (76.7)	47 (87)	2.26E-01
<b>ASXL1 mutation (n evaluable, total = 85)</b>			
n (%)	46	39	
n (%)	9 (19.6)	23 (59)	<b>2.79E-04</b>
<b>EZH2 mutation (n evaluable, total = 82)</b>			
n (%)	48	34	
n (%)	3 (6.2)	3 (8.8)	6.88E-01
<b>SRSF2 mutation (n evaluable, total = 81)</b>			
n (%)	47	34	
n (%)	4 (8.5)	5 (14.7)	4.81E-01

**Table 2. (continued)**

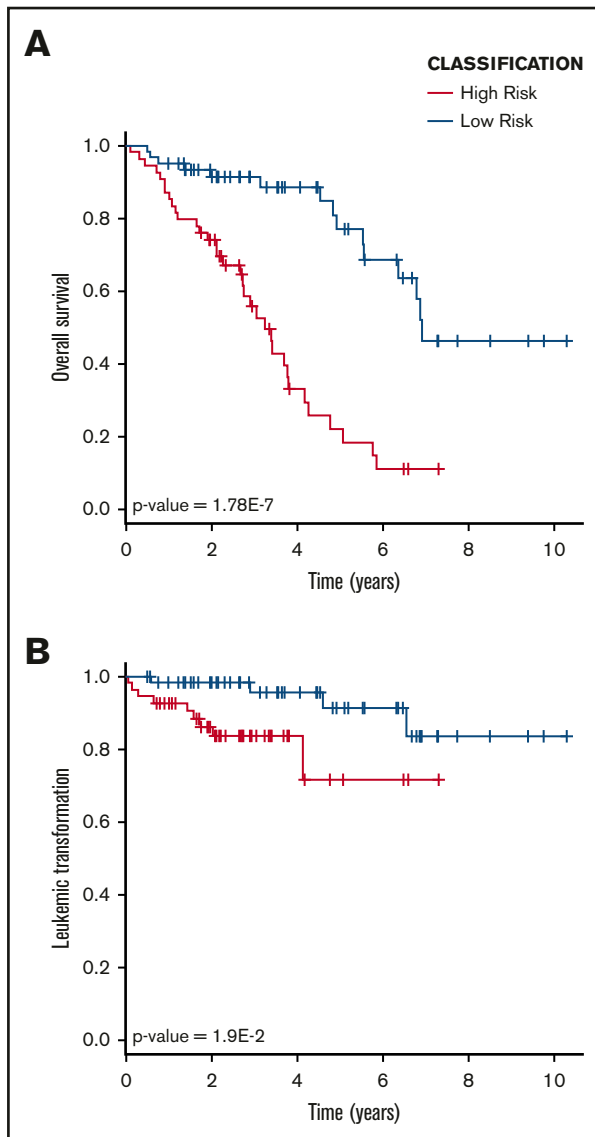
Variable	Low-risk (n = 60)	High-risk (n = 54)	P
<b>IDH1/2 mutation (n evaluable, total = 81)</b>			
n (%)	47	34	
n (%)	3 (6.4)	1 (2.9)	6.36E-01
<b>HMR (n evaluable, total = 86)</b>			
n (%)	48	38	
n (%)	14 (29.2)	25 (65.8)	<b>1.02E-03</b>
$\geq 2$	4 (8.3)	7 (18.4)	2.03E-01
<b>DIPSS (n evaluable, total = 107)</b>			
Low	57	50	
Low	23 (40.4)	7 (14)	
Intermediate-1	24 (42.1)	17 (34)	
Intermediate-2	8 (14)	16 (32)	
High	2 (3.5)	10 (20)	<b>6.00E-04</b>
<b>MIPSS70 (n evaluable, total = 73)</b>			
Low	41	32	
Low	12 (29.3)	1 (3.1)	
Intermediate	22 (53.7)	11 (34.4)	
High	7 (17.1)	20 (62.5)	<b>1.01E-04</b>
<b>MYSEC-PM (n evaluable, total = 38)</b>			
Low	14	24	
Low	5 (35.7)	4 (16.7)	
Intermediate-1	5 (35.7)	11 (45.8)	
Intermediate-2	3 (21.4)	4 (16.7)	
High	1 (7.1)	5 (20.8)	4.36E-01
Progression to leukemia	4 (6.7)	9 (16.7)	1.40E-01
Death	15 (25)	34 (63)	<b>6.01E-05</b>

Unless otherwise noted, data are n (%). Significant *P* values (<.05) are highlighted in bold.

LFS was reduced significantly in the high-risk group (*P* value = 1.9E-2, log-rank test) (Figure 2B). Collectively, these results demonstrated that belonging to the high-risk group represents a risk factor for survival (HR, 4.736; 95% confidence interval [CI], 2.5-8.9; *P* value = 1.48E-6, Wald test) and for leukemic transformation (HR, 3.976; 95% CI, 1.2-13.6; *P* value = 2.75E-2, Wald test). Of particular interest, this is also true considering samples stratified according to diagnosis. Indeed, high-risk patients displayed inferior OS when PMF and SMF samples were considered separately (supplemental Figure 2).

### Comparison with contemporary prognostic model

Next, we looked at the correlation between GEP-based and prognostic model classifications. The low-risk group was significantly enriched in patients belonging to DIPSS Low and Intermediate-1 categories (*P* value = 6.00E-4,  $\chi^2$  test), whereas the majority of patients classified as DIPSS Intermediate-2 (16/24 [66.7%]) or High (10/12 [83.3%]) risk clustered within the gene expression-defined high-risk group (Table 2). Interestingly, DIPSS Intermediate-1 and Intermediate-2 patients belonging to the high-risk group, according to our classification, showed a significantly inferior survival compared with those identified as low-risk (*P* values = 5.07E-4 and 2.53E-2, respectively, log-rank test) (Figure 3A), and Cox-regression analysis confirmed that high-risk classification represented a risk factor for inferior survival in DIPSS Intermediate-1 and Intermediate-2 groups (Figure 3B). High-risk classification retained its significance in



**Figure 2. OS and LFS.** (A) Kaplan-Meier analysis demonstrated a statistically significant difference in terms of OS between the high-risk and low-risk groups, as identified by gene expression-based classifier. (B) The high-risk group showed an inferior LFS compared with the low-risk group.

multivariate analysis when evaluated in the context of DIPSS classification ( $P$  value = 5.69E-5, Wald test) (Figure 3C), as well as when considering risk factors included in the DIPSS prognostic model ( $P$  value = 4.96E-3, Wald test) (supplemental Table 4). With regard to SMF, we found that the distinction between high-risk and low-risk patients in terms of survival reached statistical significance for MYSEC-PM lower-risk categories (Low plus Intermediate-1), whereas it approached significance for higher-risk classes (Intermediate-2 plus High) ( $P$  values = 1.39E-2 and 7.26E-2, respectively, log-rank test) (supplemental Figure 3).

More recently, outcome prediction in MF was improved by the inclusion of molecular information in prognostic models. Because of the lack of cytogenetics data for most of the samples, we decided to compare our classification using the MIPSS70 model.<sup>12</sup> Most

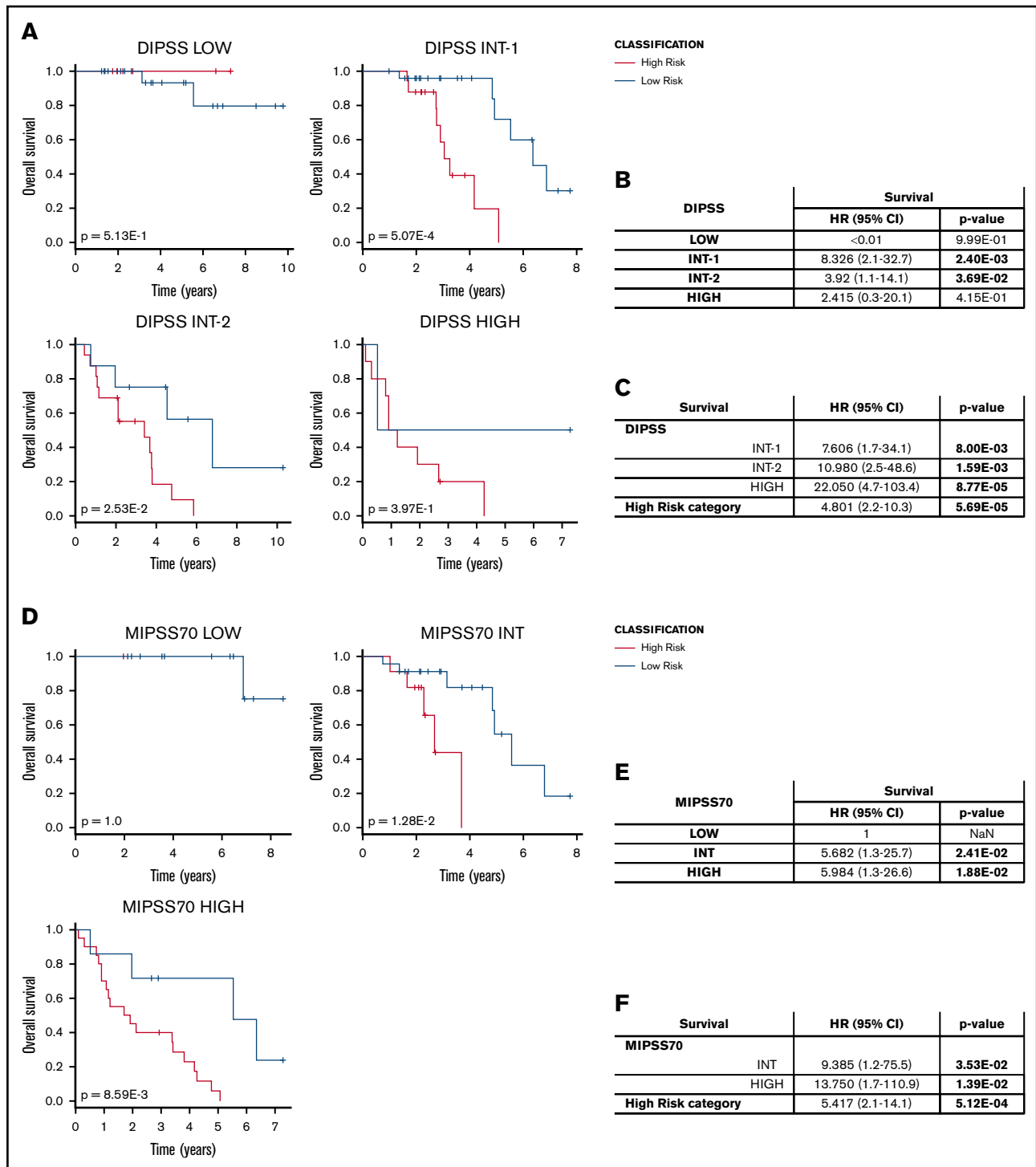
MIPSS70 High-risk patients clustered within the high-risk group, whereas the low-risk group contained higher frequencies of Low and Intermediate samples ( $P$  value = 1.01E-4,  $\chi^2$  test) (Table 2). Nevertheless, gene expression-based classification distinguished high-risk patients from low-risk ones with different survival within MIPSS70 Intermediate and High categories ( $P$  values = 1.28E-2 and 8.59E-3, respectively, log-rank test) (Figure 3D). The high-risk classification represented a risk factor for inferior survival for patients belonging to these prognostic categories (Figure 3E), also retaining its significance in multivariate analyses when considering MIPSS70 classification ( $P$  value = 5.12E-4, Wald test) (Figure 3F) or factors included in the MIPSS70 model ( $P$  value = 1.12E-4, Wald test) (supplemental Table 5).

Taken together, these results demonstrated that a gene expression-based classifier might also identify groups of patients characterized by different outcomes in the context of contemporary prognostic models.

## Discussion

MF is a complex hematologic disorder arising from the mutation of hematopoietic stem cells. Excessive proliferation of cells from the neoplastic clones gives rise to granulocyte and megakaryocyte hyperplasia, whereas the altered interaction between hematopoietic and stromal cells in the BM microenvironment leads to the development of the fibrosis that is the hallmark of the disease.<sup>27</sup> PMF originates from the acquisition of somatic driver mutations in *JAK2*, *MPL*, or *CALR* genes by hematopoietic stem/progenitor cells, even if almost 5% of patients do not harbor any of these and, therefore, are considered triple negative.<sup>7</sup> Several other mutations might be present in PMF patients; among them, those occurring in *EZH2*, *ASXL1*, *SRSF2*, and *IDH1/2* genes are termed HMR because of their negative impact on OS and LFS.<sup>28</sup>

MF can be primary or secondary to PV and ET; moreover, a prefibrotic stage and overt PMF must be distinguished within PMF, depending on the degree of BM fibrosis, because they represent distinct entities. As demonstrated by clinical features of patients included in our data set, in agreement with literature findings, PET-MF was characterized by increased platelet counts,<sup>20,26</sup> whereas pre-PMF displayed higher Hb levels and platelet counts and a lower frequency of splenomegaly.<sup>24,25</sup> Increased Hb was also evident in PPV-MF, which was also characterized by an increased leukocyte count and the presence of a *JAK2V617F* mutation in all patients.<sup>20,26</sup> Despite these differences, MF cases are currently managed in the same way.<sup>29</sup> To study whether gene expression might provide clinical and prognostic information at any time during the disease course, we included samples from diagnosis and during follow-up in our dataset and correlated GEP with patient features at those time points. Many prognostic models were developed in the last decade, allowing risk stratification in MF patients based on clinical features (ie, IPSS, DIPSS), as well as on molecular and cytogenetics characteristics (ie, DIPSS-plus, MIPSS70, MIPSS70+v2.0, GIPSS).<sup>12,13,30-32</sup> These models were developed for prognostication in PMF but are also applied in SMF cases, even if a specific model was recently developed (MYSEC-PM).<sup>10</sup> More recently, Grinfeld et al emphasized the impact of driver mutations on patient prognosis by defining a classification scheme for MPNs and related disorders based on the type of genomic alterations harbored by patients.<sup>3</sup>



**Figure 3. Comparison with DIPSS and MIPSS70 classification.** (A) Survival curves for samples stratified according to DIPSS and classified in the high-risk and low-risk groups using our gene expression–based model. *P* values were calculated using the log-rank test. (B) HR for death comparing the high-risk and low-risk groups in the context of DIPSS classification. (C) Results of multivariate analyses. (D-F) The same analysis was performed for patients classified using the MIPSS70 scoring system. (D) Survival analysis. HR for death of samples stratified according to MIPSS70 (E) and using multivariate analyses (F).

Because clinical decision making in MF is mainly influenced by survival prediction, it is fundamental to develop easy-to-use models that can identify patients who are at the highest risk of death. Gene expression analysis was recently adopted to predict the risk of

recurrence in breast cancer,<sup>33</sup> but gene expression signatures were also developed to predict survival in hematopoietic malignancies, such as AML (ie, 17-gene leukemia stem cell score [LSC17]).<sup>15</sup> Therefore, we decided to study the GEP of MF granulocytes to

evaluate its impact on disease phenotype and patient outcome. We focused on granulocytes because they represent an easily accessible myeloid cell population that belongs to the neoplastic clone, unlike peripheral blood mononuclear cells that also contain lymphoid elements.<sup>34</sup>

Using Cox-regression analysis, we identified survival-related transcripts that were used to construct a classifier based on the expression of 201 genes that could identify 2 groups of patients. High-risk patients identified by our model displayed an inferior OS and LFS compared with low-risk cases. Our results demonstrated that gene expression–based classification showed good agreement with contemporary prognostic models; indeed, the high-risk classification correlated with the presence of several detrimental features, such as advanced age, decreased Hb levels (<10 g/dL) and platelet count (<100 × 10<sup>9</sup>/L), increased white blood cell count (>25 × 10<sup>9</sup>/L), circulating blasts ≥ 1% and 2%, and the presence of constitutional symptoms and splenomegaly. Interestingly, in a recent report by Penna et al,<sup>35</sup> the absence of the same detrimental features correlated with long survival (>20 years) in patients with PMF; this condition was similar to that observed in the low-risk group identified by gene expression signature. The frequency of patients with ≥1 HMR mutation was increased in the high-risk group, and the presence of these variants correlates with MF diagnosis and a more aggressive disease with inferior OS.<sup>3</sup> Moreover, our findings underscored the impact of JAK2V617F allele burden on OS, because the high-risk group contained an increased frequency of homozygous patients, whereas heterozygous ones clustered within the low-risk group. These data are in agreement with those of Grinfeld et al, who demonstrated that patients with a JAK2V617F heterozygous disease have favorable outcomes.<sup>3</sup>

Our gene expression–based classifier was able to distinguish high-risk patients from low-risk ones within intermediate-risk classes. Indeed, in patients stratified according to DIPSS, belonging to the high-risk group represented a risk factor for inferior survival in the Intermediate-1 and Intermediate-2 categories. The high-risk group was characterized by significantly inferior OS compared with the low-risk group: 3.05 years and 3.42 years in the Intermediate-1 and Intermediate-2 classes, respectively. Likewise, within the MIPSS70 Intermediate group, high-risk patients displayed a median OS of 2.68 years, which was significantly lower compared with the low-risk group. Multivariable analysis demonstrated that gene expression–based classification might represent a risk factor for inferior survival independent from the DIPSS and MIPSS70 classifications, as well as from factors included in these models. Therefore, this suggests that gene expression analysis might provide additional information other than that included in contemporary prognostic models.

Overall, this study demonstrates the correlation between GEP and MF clinical features and provides the proof of concept that gene expression analysis should be considered to complement risk stratification in MF.

To date, treatment algorithms suggest observation accompanied by palliative drug therapy for DIPSS Intermediate-1 patients, whereas ASCT and enrollment in clinical trials must be considered for patients belonging to Intermediate-2 and High-risk categories.<sup>6</sup> Our results demonstrated that high-risk patients with an expected median survival < 5 years might also be identified within DIPSS Intermediate-1 and Intermediate-2 classes. The same is true when we consider Intermediate-risk patients based on MIPSS70 classification.

Overall, given the robustness of these analyses, we believe that these data may still be helpful to the hematological community in uncovering the possibility that GEPs might integrate contemporary prognostic models. Further studies might be able to prospectively validate these data in an independent cohort of MF cases, thus improving the identification of patients with expected inferior survival who can benefit from participation in clinical trials or ASCT.

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## Authorship

Contribution: S.R. performed GEP analyses and statistical analyses; S.C., A.G., and S.B. constructed the prediction model; R.Z., E.B., E.G., and C.C. performed granulocyte sorting; S.P., S.F., and S.M. performed total RNA purification; L.T., S. Sartini, and M.M. performed RNA quantification and quality control; S.M., L.T., and S. Sartini performed RNA quality analyses; P.G., C.M., F.G., A.P., D.P., E.R., S. Salmoiraghi, B.M., L.V., V.R., G.B., F.P., A.R., L.M., and M.C. selected and recruited patients and provided clinical data; and S.R., E.T., A.M.V., and R.M. designed the research and wrote the manuscript.

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