Genomics

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Quantitative Proteome Heterogeneity in Myeloproliferative Neoplasm Subtypes and Association with *JAK2* Mutation Status

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Abstract

Apart from well-known genetic abnormalities, several studies have reported variations in protein expression in Philadelphia-negative myeloproliferative neoplasm (MPN) patients that could contribute toward their clinical phenotype. In this context, a quantitative mass spectrometry proteomics protocol was used to identify differences in the granulocyte proteome with the goal to characterize the pathogenic role of aberrant protein expression in MPNs. LC/MS-MS (LTQ Orbitrap) coupled to iTRAQ labeling showed significant and quantitative differences in protein content among various MPN subtypes [polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF)], and according to the genetic status of *JAK2* (*JAK2V617F* presence and *JAK2V617F* allele burden). A number of differentially expressed proteins were identified, with the most frequent being members of the RAS GTPase family and oxidative stress regulatory proteins. Subsequent analysis found that calreticulin (CALR), known to be involved in calcium homeostasis and apoptotic signaling, was overexpressed in *JAK2V617F* granulocytes compared with *JAK2* wild type and independently of the *JAK2V617F* allele burden. Finally, it was demonstrated, in a Ba/F3 cell model, that increased calreticulin expression was directly linked to *JAK2V617F* and could be regulated by JAK2 kinase inhibitors.

Implications: In conclusion, these results reveal proteome alterations in MPN granulocytes depending on the phenotype and genotype of patients, highlighting new oncogenic mechanisms associated with *JAK2* mutations and overexpression of calreticulin. *Mol Cancer Res; 15(7); 852–61. ©2017 AACR.*

Introduction

Polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) are the three well-known Philadelphia-negative (Ph^-) myeloproliferative neoplasms (MPN), characterized by the clonal proliferation of hematopoietic stem cells that leads to an excess of mature blood cells (1, 2). MPNs result from molecular abnormalities in progenitor cells, such as those with the *JAK2V617F* mutation, which is the most frequent mutation in PV (>95%), *MPL* mutations (present in 5%–10% of ET and PMF), or the recently discovered *CALR* gene mutations present in 25% to 35% of ET and PMF patients who are negative for *JAK2V617F* (3, 4). These driver mutations are responsible for a constitutive activation of the JAK–STAT signaling pathway causing the enhanced proliferation of progenitor cells.

Besides genetic abnormalities, several studies have reported protein alterations in MPN patients that could be influenced by the genetic status of these patients. One "historical" example of such abnormal protein expression is the modification of serum erythropoietin (EPO) levels in PV patients. MPNs are physiopathologically characterized by EPO-independent proliferation of erythroid progenitors inducing a decrease in EPO secretion by renal cells. This alteration has been validated as a PV marker allowing, with good specificity and sensitivity, the discrimination between PV and secondary erythrocytosis, even though some contradictory results exist in some patients (5). Tefferi and colleagues (2007; ref. 6) suggested that serum EPO levels were expected to be decreased in PV regardless of any *JAK2* mutation.

Other serum proteome studies demonstrated that there were significant differences between ET and PV proteome profiles and in some serum protein levels. In PV, the Apolipoprotein A-1 concentration was correlated with the presence of *JAK2V617F* and its allele burden, unlike in ET (7). Both *JAK2V617F*-positive and *JAK2V617F*-negative ET shared 85% of their serum proteome, which differed significantly from the PV serum proteome (8). Recently, some alterations concerning cytokines have also been highlighted. Tefferi and colleagues (2011; ref. 9) found significantly elevated serum levels of 20 cytokines in PMF patients compared with controls, and increased levels of seven of these cytokines were associated with the *JAK2V617F* mutation. Pourcelot and colleagues (10) showed that cytokine variations could

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also occur in PV and ET with a significant increase in cytokine serum levels compared with normal range values.

Changes in granulocyte protein expression have also been reported in Ph^- MPNs. For instance, Hui and colleagues (11), using a Protein Pathway Array, screened 15 proteins (implicated in apoptosis and inflammatory pathways) that were differently expressed in peripheral blood neutrophils in ET compared with control patients. They also found differences in protein expression in cycle signaling pathways when comparing ET patients with and without the *JAK2V617F* mutation.

Falanga and colleagues (12) studied the involvement of neutrophils in the pathogenesis of the thrombotic predisposition observed in PV and ET patients. They showed that neutrophils in MPNs presented high levels of CD11b (a marker of neutrophil activation) and leukocyte alkaline phosphatase, suggesting the hypothesis that neutrophil degranulation raises plasma concentrations of elastase and myeloperoxidase. Arellano-Rodrigo and colleagues (13) found that, in ET patients, the higher expression of CD11b in neutrophils was associated with *JAK2V617F* presence, but not with thrombosis. Nevertheless, they found that CD11b expression was increased in monocytes from thrombotic patients along with monocyte tissue factor, which may be involved in blood coagulation. More recently, Gallardo and colleagues (14) found 61 upregulated proteins in PV compared with ET patients (among them HSP70, LTA4H, and SERPINB1) using 2D-DIGE and mass spectrometry analysis. Moreover, using an HSP70 inhibitor in an *ex vivo* model, they showed that this protein has a potential role in the JAK–STAT signaling pathway in the erythroid lineage.

Taken together, these data highlight differences in the proteomes of MPNs that could play a critical role in the clinical characteristics of patients, but whose link with the genetic status of patients remains unclear. In this context, we performed a quantitative proteomic study of granulocytes from Ph^- MPN patients, harboring different *JAK2V617F* allele burdens, using an iTRAQ labeling method so as to compile an inventory of quantitative differences in the proteomes of the MPN subtypes (according to WHO classification; refs. 1, 2) and/or the genetic status of patients.

Materials and Methods

Patients

All patients agreed to participate in the experimental procedure and signed the informed consent. A first set of patients from the Institute for Medical Research of the University of Belgrade (Belgrade, Serbia) was selected so as to allow a quantitative analysis of their granulocyte proteome. We selected 6 PV patients with high *JAK2V617F* allele burden (88%–93%), mean 92%, 6 PMF patients with *JAK2V617F* allele burden ranging from 38% to 94%, mean 63%, and 5 ET patients ranging from 35% to 50%, mean 40.5%. The group of *JAK2(−)* patients included 4 ET (2 *CALR(+)* and 2 *CALR(−)*) and 3 PMF (*JAK2(−)/CALR(+)*). These four groups were representative of four levels of *JAK2V617F* allele burden: high, medium, low, and null. A further set of 32 patients belonging to the Grenoble University Hospital-CHUGA France was selected for a study of calreticulin expression in erythrocytes: 12 *JAK2V617F(+)* including 7 PV, 4 ET, and 1 PMF; 4 *JAK2(−)/CALR(−)*; 6 *CALR(+)* ET; and 10 healthy donors (control set).

Granulocyte preparation

Thirty milliliters of peripheral blood was collected in 10% sodium citrate tubes. Lymphocytes were separated by adding 15 mL of lymphocyte separation medium (PAA Laboratories GmbH). After centrifugation (400 × *g*, 20 minutes, 20°C), the pellet, composed of erythrocytes and granulocytes, was submitted to erythrocyte lysis with lysing solution (0.15 mol/L NH_4Cl, 0.1 mmol/L Na_2EDTA, 12 mmol/L $NaHCO_3$). Protein extracts were then prepared from fresh granulocytes. The high quality of purified granulocytes was confirmed by cytospin preparations and Wright–Giemsa staining. Viability was measured with Trypan blue (BioWhittaker).

Protein preparation

Granulocytes were washed twice with PBS and resuspended in 10 mL cold PBS with 2 mmol/L EDTA and 1 mmol/L Na-orthovanadate and centrifuged for 10 minutes at 1,000 × *g*. The cell pellet was dried by inversion over a paper towel, vortexed, and placed on ice. Then, granulocytes were lysed with cold lysis buffer (50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1% NP-40, 2 mmol/L EDTA, 50 mmol/L NaF) with protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L ε-aminocaproic acid, 2 μg/mL aprotinin, 50 μg/mL leupeptin, 1 μmol/L pepstatin A) and phosphatase inhibitors (1 mmol/L Na-orthovanadate) for 30 minutes at 4°C. Cell suspensions were centrifuged at 10,000 × *g* for 15 minutes at 4°C. The supernatant was transferred to cold Eppendorfs, mixed, and aliquoted into precooled small Eppendorfs and frozen at −70°C.

iTRAQ labeling

The same amount of protein was taken from each patient of each MPN subtype (PV, ET, PMF, and *JAK2(−) MPN*) and pooled to obtain four grouped samples, containing 250 μg of protein each, representing the four subgroups (PV, ET, PMF, and *JAK2(−)* MPN). Proteins from each group were precipitated with acetone and digested according to the Applied Biosystems digestion protocol for iTRAQ marking. Protein pellets were suspended in 500 mmol/L triethylammonium bicarbonate buffer 0.1% SDS pH 8.5. Cysteines were reduced with TCEP (tris(2-carboxyethyl)phosphineand), alkylated with MMTS (methyl methanethiosulfonate), and proteins were digested by trypsin overnight. Then, pooled samples were divided in two parts of 100 μg each and labeled in 2 hours with a specific iTRAQ label (113 and 117 for the group *JAK2(−) MPN*; 115 and 119 for PV; 116 and 121 for ET, and 114 and 118 for PMF). Then, samples were combined and desalted in Sep-Pak C18 Cartridges (Waters). Prefractionation of peptides prior to LC/MS-MS was done using a 3100 off-gel fractionator (24 wells). The resulting fractions were desalted using C18 micro spincolumns (Harvard Apparatus).

Protein identification

For the LC/MS-MS analysis, about 200 ng of each fraction was injected. Peptides were separated by nano LC/MS-MS (Ultimate 3000, Dionex) coupled to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). The LTQ-Orbitrap operated with Xcalibur software. Survey MS spectra were acquired on the Orbitrap in the 300 to 2,000 m/z range with 60,000 as the resolution. The five most intense ions per survey were selected for collision-induced dissociation fragmentation to be analyzed in the linear trap (LTQ). A dynamic exclusion period of 60 seconds was used to prevent repetitive selection of the same peptide.

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For protein analysis, we used Mascot Daemon software (version 2.3.0, Matrix Science). The following parameters were set for creation of the peak lists: parent ions in the mass range 400 to 4,500, no grouping of MS/MS scans, and a threshold at 1,000. A peak list was created for each sample, and individual Mascot searches were performed. Protein identification was performed by comparing data with *Homo sapiens* entries in the Uniprot protein database (http://www.uniprot.org/). Protein hits were automatically validated if they satisfied one of the following criteria: identification with at least one top ranking peptide with a Mascot score >39 ($P < 0.001$), or at least two top ranking peptides each with a Mascot score >22 ($P < 0.05$). When several proteins matched exactly the same set of peptides, only one member of the protein group was reported in the final list. To evaluate the false-positive rate in these experiments, all the initial database searches were performed using the "decoy" option of Mascot.

Protein quantification

For peptide quantification, the intensity of the different ions (m/z: 114, 115, 116, 118, 119, and 121) was divided by the intensity of the reporter ion (m/z: 117 – JAK2(−) MPN) for each measured compound. All ratios were normalized against the median intensity of reporters. Only proteins quantified with at least two peptide ratios for each condition were validated. Ratios were transformed into natural logarithms and plotted against the number of peptides subjected to MS/MS analysis. Student *t* tests were performed for ratio comparison corresponding to each replicate (i.e., 116/117 vs. 121/117). Only proteins without a significant difference between the replicates were retained for comparison between subgroups of patients. To reinforce statistical analysis and to avoid technical bias, we calculated medians and SDs of ratios (i.e., 115/117 and 119/117 for PV). Thereby, we obtained a range of ratios from 0.76 to 1.24 corresponding to 2× SD of the ratio value. Therefore, we defined as high significant difference between two peptides when the ratio of the two values was under 0.76 or over 1.24 and the Student *t* test under 0.01, and low significant difference when the ratio was between 0.76 and 1.24 but the statistical *P* was under 0.05

Erythrocyte preparation

Fresh red blood cells (RBC) from peripheral blood were separated from polynuclear and mononuclear cells by Ficoll gradient centrifugation. Cells were lysed by hypotonic shock with distilled water in the presence of protease inhibitors (cOmplete, EDTA-free, Roche). After centrifugation at 36,000 × *g* for 10 minutes, the protein extract of the supernatant was incubated with FluidMAG-Q (4112; Chemicell GmbH) for hemoglobin depletion as described by Villanueva and colleagues (15).

Cell culture

Ba/F3 EpoR, Ba/F3 EpoR JAK2 wild-type (WT), and Ba/F3 EpoR JAK2V617F were provided by Dr. I. Plo, Gustave Roussy (Villejuif, France). Cells were cultured in DMEM culture media with 1% penicillin/streptomycin, 1% glutamine, 10% FBS, and 1 IU EPO as described previously (16). Cell culture under deprivation (or stress) conditions was the same as for controls but in the absence of EPO and FBS. Third, cell culture with JAK2 inhibitors was performed under deprivation conditions in the presence of ruxolitinib (INCB018424; Novartis) and AZD1480 (Selleckchem). Cell lines were washed in PBS and lysed for Western blotting.

Western blotting

Western blotting was performed using a standard protocol. Nitrocellulose membranes were incubated with primary antibodies overnight at 4°C and then 90 minutes at room temperature with secondary antibodies. β-Actin was used as a loading control. For protein quantification, we used Image Lab Software version 4.1 (2012; Bio-Rad Laboratories).

Statistical analysis

Comparison of means was performed by Student *t* test using GraphPad Prism software with a 95% confidence interval. Functional classification was performed using Reactome software (http://www.reactome.org/).

Results

Granulocyte proteome displays variation, including CALR expression, between different Ph$^-$ MPN patients

Thanks to LC/MS-MS analysis, we could identify 1,048 proteins from Ph$^-$ MPN granulocytes. To quantify the differences in expression in these proteins, we calculated medians and SDs of intensity ratios (i.e., 115/117 and 119/117 for PV, see Materials and Methods). This analysis showed that MPN granulocytes display significant quantitative variation of various proteins among MPN subtypes (ET, PV, and PMF) but also according to the presence (or absence) of *JAK2V617F* mutation. Table 1 shows the number of proteins differently expressed between these different subgroups. Proteins expressed with highly significant differences between groups are presented as a comparative histogram in Fig. 1. The list of all differently expressed proteins is accessible in the Supplementary Tables S1 to S6.

When comparing *JAK2(+)* patients (PV, ET, and PMF) versus the *JAK2(−)* (ET and PMF patients), we could identify several proteins that were differently expressed between *JAK2*-mutated and nonmutated patients. We highlighted that there were four proteins commonly overexpressed in PV and ET *JAK2(+)*

Table 1. Number of proteins expressed differently: pairwise comparison between *JAK2(+)* PV, *JAK2(+)* ET, *JAK2(+)* PMF, and *JAK2(−)* MPN

	PV vs. *JAK2(−)*	ET vs. *JAK2(−)*	PMF vs. *JAK2(−)*	PV vs. PMF	ET vs. PMF	PV vs. ET
Proteins $P < 0.01$	54	75	77	62	67	39
Proteins $P < 0.05$	22	22	69	15	34	7
Total	76	97	146	79	101	46
Ratio	1.01 ± 0.17	1.01 ± 0.19	1.02 ± 0.12	1.00 ± 0.19	0.99 ± 0.21	1.06 ± 0.35
Median	0.94	0.94	0.96	0.97	0.94	0.93
Range	0.74–1.56	0.36–1.60	0.63–1.55	0.64–1.69	0.54–2.23	0.66–2.85
Ratio > 1	30	40	62	34	42	21
Ratio < 1	46	57	84	43	59	25

NOTE: This table shows the number of proteins with differences in expression levels between the different subgroups: *P* value, ratio, median, and range of each comparison.

Abbreviations: ET, *JAK2(+)* ET; *JAK2(−)*, *JAK2(−)* MPNs; PMF, *JAK2(+)* PMF; PV, *JAK2(+)* PV.

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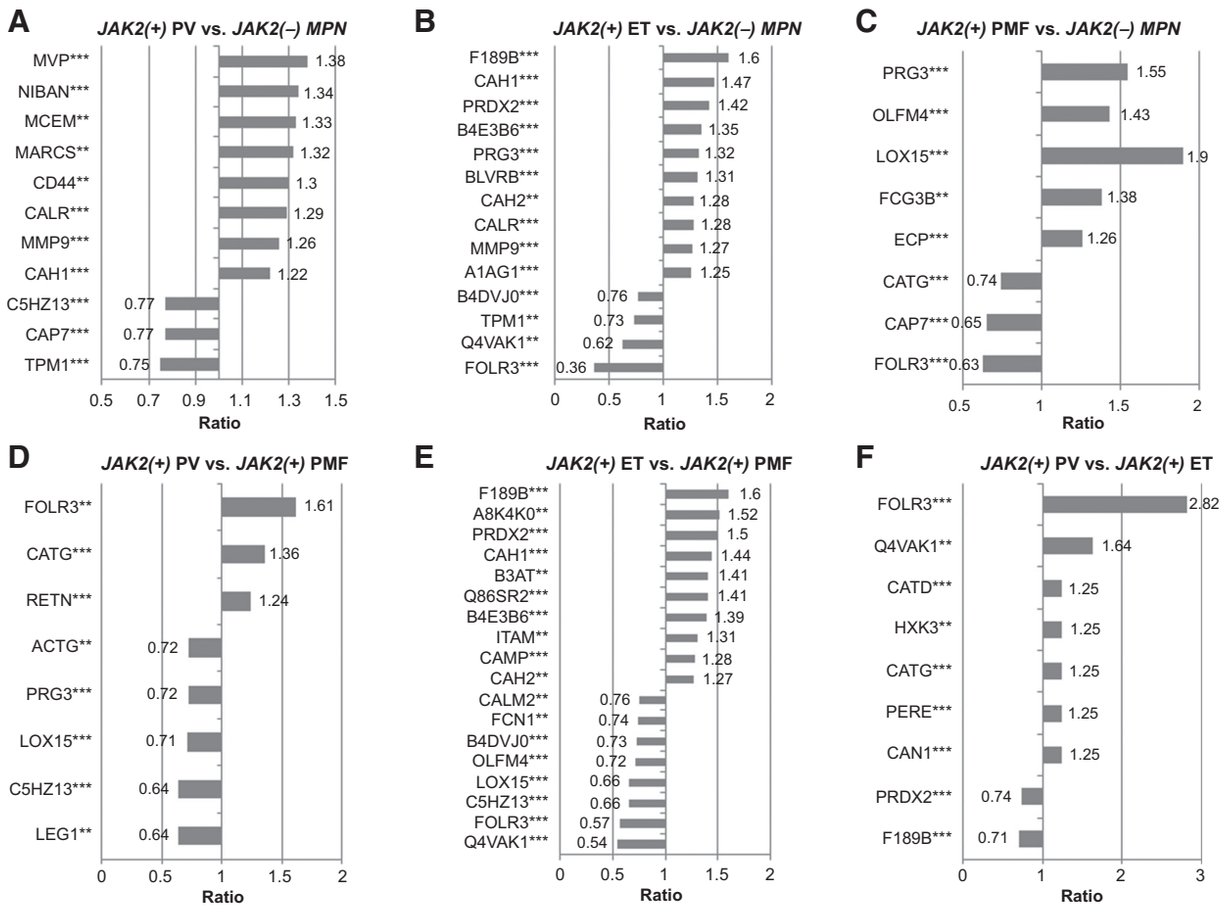

Figure 1.

Proteins with large significant differences in expression between the different subgroups. This figure shows the protein expression ratios for the comparisons between the different subgroups. **A,** *JAK2(+)* PV versus *JAK2(−) MPN*. **B,** *JAK2(+)* ET versus *JAK2(−) MPN*. **C,** *JAK2(+)* PMF versus *JAK2(−) MPN*. **D,** PV versus PMF. **E,** ET versus PMF. **F,** PV versus ET. *P* values: **, $P < 0.01$; ***, $P < 0.001$.

(vs. *JAK2(−)*), two in both mutated ET and PMF, and one in both PV and PMF patients versus *JAK2(−)*. However, we failed to find any protein commonly overexpressed among all subtypes of *JAK2(+)* MPNs.

Interestingly, among these proteins, we found that calreticulin was overexpressed in all *JAK2(+)* Ph^- MPNs compared with *JAK2(−) MPN*s: ET versus *JAK2(−)* MPN (ratio 1.28, $P < 0.001$); PV versus *JAK2(−)* MPN (ratio 1.29, $P < 0.001$); and PMF versus *JAK2(−)* MPN (ratio 1.22, $P < 0.001$). We also highlighted that the surface protein CD11b (ITAM) was significantly overexpressed in *JAK2V617F* ET granulocytes compared with nonmutated ones (overexpressed principally in *JAK2V617F* PV and ET granulocytes and underexpressed in mutated *JAK2* PMF granulocytes). In addition, we noticed that some of the differently expressed proteins appeared in more than one comparison, meaning that their expression was altered in at least two subgroups of patients (Table 2).

Impact of JAK2V617F allele burden in granulocyte protein expression

The fact that we could identify several proteins that were selectively over or underexpressed between patients carrying mutated and nonmutated *JAK2* may suggest a possible influence of *JAK2V617F* allele burden on protein expression. With this goal, we classified patients into four subgroups according to their *JAK2V617F* allele burden, high, medium, low, and null (see Materials and Methods) and then looked at significant quantitative differences between them. First, we compared patients with very high (>88%) allele burden who could be considered primarily as homozygous with patients harboring less than 50% allele burden (considered primarily as heterozygous). We found that the number of differently expressed proteins (97) was higher for patients carrying low allele burdens than for those with high allele burden (76). At low *JAK2V617F* allele burdens, 40 proteins were overexpressed and 57 were significantly underexpressed compared with high allele burden. Patients with high allele burden over- and underexpressed 30 and 46 proteins, respectively, compared with those with low allele burden.

As some proteins seemed to be influenced by *JAK2V617F* allele burden, we looked for proteins whose ratio (compared with patients without the mutation) was correlated with higher or lower allele burdens. Table 3 shows that the expression of some proteins increased in parallel with the *JAK2V617F* allele burden.

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Table 2. Proteins most often expressed differently between the various subgroups of MPN

	PV vs. *JAK2(−)*	ET vs. *JAK2(−)*	PMF vs. *JAK2(−)*	PV vs. PMF	ET vs. PMF	PV vs. ET
B4DVJ0		↓			↓	
C5HZ13	↑			↓	↓	
CAH1	↑	↑			↑	
CAH2		↑			↑	
CALR	↑	↑				
CAP7	↓		↓			
CATG			↓	↑		↑
F189B		↑			↑	↓
FOLR3		↓	↓	↑	↓	↑
LOX15			↑	↓	↓	
MMP9	↑	↑				
OLFM4			↑		↓	
PRDX2		↑			↑	↓
PRG3		↑	↑	↓		
Q4VAK1		↓			↓	↑
TPM1	↓	↓				

NOTE: This table shows the proteins that were most frequently expressed differently between pairs of MPN subtypes and whether they were overexpressed (↑) or underexpressed (↓).
Abbreviations: ET, *JAK2(+)* ET; *JAK2(−), JAK2(−)* MPNs; PMF, *JAK2(+)* PMF; PV, *JAK2(+)* PV.

This is the case for mast cell–expressed membrane protein 1, the major vault protein, Niban or resisitin. It is noticeable that the folate receptor gamma was strongly underexpressed when the allele burden was low and, although remaining largely underexpressed, its expression increased with higher burdens of *JAK2V617F*. On the contrary, even though Flavin reductase and carbonic anhydrase 1 are overexpressed in *JAK2V617F* granulocytes, their overexpression decreased when the *JAK2V617F* allele burden rose. Likewise, Peroxiredoxin 2, which was highly expressed in low-*JAK2V617F* allele burden granulocytes (ratio = 1.42), had a huge drop in expression when the allele burden increased (ratio = 0.94).

We noted in particular that calreticulin levels were significantly increased in all *JAK2(+)* MPN granulocytes (compared with *JAK2(−)*), but independently of the *JAK2V617F* allele burden, suggesting that only the presence of the *JAK2V617F* mutation but not the amount of this mutated allele influences calreticulin expression.

Pathway alterations in Ph-MPN granulocytes

To further explore functional alterations that could be linked to changes found in the proteome, we analyzed the pathways that could be most impacted by modifications in the pattern of protein expression using Reactome software.

Among all the modified pathways (see Supplementary Tables S7–S12), we remarked a significant deregulation of the RAS GTPase pathways in *JAK2V617F* MPN granulocytes compared with *JAK2(−)* granulocytes and also in the pathways involved in reactive oxygen species (ROS) detoxification.

RAS GTPase deregulation in Ph-MPN granulocytes. We found that some members of the RAS GTPase family were over or underexpressed in Ph-MPN granulocytes (See Supplementary Tables S1–S6). Although some differences were found between MPN subtypes, the number of differently expressed proteins depended on the presence (or not) of *JAK2* mutation. Ten members of the RAS GTPase family were differently expressed in *JAK2(+)* and

Table 3. Impact of *JAK2V617F* allele burden in protein expression

Protein group	Description	JAK2 <50%	JAK2 38%–94%	JAK2 >88%
A8K9E4_HUMAN	cDNA FLJ76459, highly similar to *Homo sapiens* matrix metallopeptidase 8 (neutrophil collagenase; MMP8)	1.13	1.10	1.24
ANXA1_HUMAN	Annexin A1	1.09	1.05	
B4DV10_HUMAN	cDNA FLJ59142, highly similar to Epididymal secretory protein E1	0.79		0.84
BLVRB_HUMAN	Flavin reductase	1.31		1.18
BPI_HUMAN	Bactericidal permeability–increasing protein	0.81	0.94	0.84
C5HZ13_HUMAN	Galectin-10	0.78	1.21	0.77
CAH1_HUMAN	Carbonic anhydrase 1	1.47		1.22
CALR_HUMAN	Calreticulin	1.28	1.22	1.29
CAP7_HUMAN	Azurocidin	0.86	0.65	0.77
CATG_HUMAN	Cathepsin G	0.81	0.74	
FLNA_HUMAN	Filamin-A	0.94	0.94	0.94
FOLR3_HUMAN	Folate receptor gamma	0.36	0.63	
HPT_HUMAN	Haptoglobin	1.14		1.17
MCEM1_HUMAN	Mast cell-expressed membrane protein 1		1.19	1.33
MMP9_HUMAN	Matrix metalloproteinase-9	1.27	1.14	1.26
MVP_HUMAN	Major vault protein		1.22	1.38
NIBAN_HUMAN	Protein Niban		1.15	1.34
PRDX2_HUMAN	Peroxiredoxin-2	1.42	0.94	
RETN_HUMAN	Resistin	1.07		1.22

NOTE: This table shows protein expression ratios for the comparisons between low, medium, and high *JAK2V617F* allele burdens and *JAK2 WT*.

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Table 4. RAS GTPase proteins differently expressed in granulocytes, from patients with different diagnoses

Protein description	Comparison	Ratio	*P*
GDI2 protein	PMF vs. *JAK2(−) MPN*	0.94	0.0063
	PV vs. *JAK2(−) MPN*	0.92	0.0172
GTP-binding nuclear protein Ran	ET vs. *JAK2(−) MPN*	0.92	0.0002
Rab GDP dissociation inhibitor alpha	ET vs. PMF	1.09	0.0192
Ras-related C3 botulinum toxin substrate 2, (RAC2)	PMF vs. *JAK2(−) MPN*	0.85	0.0386
	ET vs. *JAK2(−) MPN*	0.74	0.0147
Ras-related protein Rab-11B	PV vs. *JAK2(−) MPN*	0.88	0.0006
Ras-related protein Rab-31	PMF vs. *JAK2(−) MPN*	0.93	0.0043
Ras-related protein Rab-35	PMF vs. *JAK2(−) MPN*	0.95	0.0041
Rho GDP-dissociation inhibitor 1	PMF vs. *JAK2(−) MPN*	0.95	0.0017
Rho GDP-dissociation inhibitor 2	PMF vs. *JAK2(−) MPN*	0.93	0.0007
	ET vs. *JAK2(−) MPN*	0.93	0.0049
	PV vs. *JAK2(−) MPN*	0.86	0.000003
	PV vs. PMF	0.94	0.0180
Rho-related GTP-binding protein RhoG	PMF vs. *JAK2(−) MPN*	0.88	0.00009
	ET vs. *JAK2(−) MPN*	0.85	0.0080
Transforming protein RhoA	PV vs. PMF	0.95	0.0059
	PV vs. *JAK2(−) MPN*	0.94	0.0016

NOTE: This table shows RAS GTPase proteins that are differentially expressed between the different subgroups with the ratio and the *P* value for the comparison. Abbreviations: ET, *JAK2(+)* ET; PMF, *JAK2(+)* PMF; PV, *JAK2(+)* PV.

JAK2(−) MPNs. Interestingly, all of these proteins, including Rho GDP-dissociation inhibitors 1 and 2, GDI protein2, Rab-11B, Rab-35, Rab-31, and the nuclear GTP-binding protein Ran, were underexpressed in *JAK2V617F* MPNs (compared with *JAK2(−)* MPNs; all of them ratio < 0.95, $P < 0.01$; Table 4).

Moreover, the RAS GTPase-activating–like protein IQGAP1, a scaffold protein linked to the RAS GTPases that is significantly overexpressed in erythrocytes (17), was also slightly overexpressed in *JAK2(+)* granulocytes of PMF patients compared with *JAK2(+)* ET and PV (ratio 1.05 and 1.08, respectively, $P < 0.01$). However, in the comparison between *JAK2(+)* PV versus *JAK2(−)* MPNs, we found a weak but significant decrease (ratio 0.96, $P < 0.01$) unlike what we had previously observed in erythrocytes. Indeed, we also noted that the IQGAP1-regulatory protein calmodulin was significantly underexpressed in ET versus PMF patients (ratio 0.76, $P < 0.01$) and in PV versus PMF patients (ratio 0.80, $P < 0.01$). Calmodulin is thus overexpressed in PMF patients compared with PV and ET patients.

Oxidative stress in granulocytes. Neutrophils are known to produce large amounts of free radicals, including ROS, which can cause oxidative stress leading to multiple deleterious effects. Cells have several mechanisms to counteract oxidative stress; however, many of the proteins involved in countering ROS (scavengers) were found to be aberrantly expressed in our proteomic analyses (Table 5). We observed that the *JAK2* mutation seemed to have more impact in ROS scavengers than the subtype of MPN. In particular, catalase was underexpressed in the presence of the *JAK2* mutation, as were glutathione S-transferase P, GAPDH, L-lactate dehydrogenase, and myeloperoxidase. In contrast, Flavin reductase was overexpressed in mutated MPNs.

Regarding protein expression in terms of patient diagnosis, we found that Flavin reductase, glutathione S-transferase omega-1, and myeloperoxidase were overexpressed in PV and ET compared with PMF patients. On the contrary, glutaredoxin was underexpressed in PV and ET compared with PMF. Eosinophil reductase is underexpressed in ET patients compared with PV and PMF ones, whereas peroxiredoxin-2 is overexpressed in ET (compared with PV and PMF).

Calreticulin expression

As our results highlighted significant overexpression of calreticulin in *JAK2V617F* PV, ET, and PMF compared with *JAK2(−)* MPN granulocytes, we attempted to further study the levels of this protein in other MPN cell types. First, we asked whether calreticulin was expressed in erythrocytes and if so, whether its levels were also influenced by the *JAK2V617F* mutation. Interestingly, we found that calreticulin was expressed both in the erythrocytes of MPN patients (Fig. 2A) and in normal erythrocytes from healthy donors (controls). However, we found no difference in calreticulin expression between *JAK2(+)*, *JAK2(−)*, *CALR(+)*, and control RBC lysates.

Taking into account the driver role of the *CALR* mutation in MPNs and our data about its *JAK2V617F*-dependent expression granulocytes, we studied its expression and regulation in a murine cell model composed of Ba/F3 cells expressing the EPO receptor (EpoR), Ba/F3 EpoR *JAK2 WT*, and Ba/F3 EpoR *JAK2V617F* cells cultured under different conditions. At steady state, under normal conditions of culture, Ba/F3 cells expressed significant levels of calreticulin that did not seem to be modulated by the expression of *JAK2 WT* or *JAK2V617F*. However, when cells were deprived of serum and growth factors, we observed a very significant increase in calreticulin expression in *JAK2V617F* cells, in comparison with normal conditions. In contrast, calreticulin levels in *JAK2 WT* or EpoR cells, in the same deprivation conditions, were not altered. Moreover, in the presence of JAK2 inhibitors, this increase in calreticulin expression was annihilated (Fig. 2B).

Discussion

The aim of our work was to provide an inventory of granulocyte proteins that are aberrantly expressed in MPN subtypes according to their *JAK2V617F* status. We chose an iTRAQ labeling method to quantify the relative amount of each identified protein between pairs of subgroups. Because of this choice, we cannot consider that the proteins we identified constitute the total proteome of granulocytes. Nevertheless, we were able to identify 1,048 proteins and consider that they give a representative picture of the total granulocyte proteome.

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Table 5. Oxidative stress proteins differently expressed in granulocytes

Description	Comparison	Ratio	*P*
Catalase	PV vs. ET	0.93	7.55E−04
	PMF vs. *JAK2(−) MPN*	0.94	1.55E−06
	ET vs. *JAK2(−) MPN*	0.97	1.63E−01
	PV vs. *JAK2(−) MPN*	0.90	1.91E−08
Eosinophil peroxidase	ET vs. PMF	0.88	7.90E−04
	PMF vs. *JAK2(−) MPN*	0.95	2.17E−02
	PV vs. ET	1.25	1.59E−05
Flavin reductase	PMF vs. *JAK2(−) MPN*	1.09	1.97E−02
	ET vs. *JAK2(−) MPN*	1.31	2.68E−06
	PV vs. *JAK2(−) MPN*	1.18	1.49E−04
	PV vs. PMF	1.09	8.27E−03
	ET vs. PMF	1.21	1.28E−05
Glutaredoxin	PMF vs. *JAK2(−) MPN*	1.08	1.24E−02
	PV vs. PMF	0.91	9.49E−03
	ET vs. PMF	0.89	5.82E−03
Glutathione reductase, mitochondrial	PMF vs. *JAK2(−) MPN*	1.04	2.42E−02
Glutathione S-transferase omega-1	PMF vs. *JAK2(−) MPN*	0.93	4.37E−05
	PV vs. *JAK2(−) MPN*	1.09	2.37E−03
	PV vs. PMF	1.17	2.99E−05
	ET vs. PMF	1.10	1.02E−02
Glutathione S-transferase P	PMF vs. *JAK2(−) MPN*	0.91	1.04E−07
	ET vs. *JAK2(−) MPN*	0.94	9.37E−04
	PV vs. PMF	1.00	1.35E−02
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	PMF vs. *JAK2(−) MPN*	0.98	2.74E−03
	PV vs. *JAK2(−) MPN*	0.94	4.80E−07
	ET vs. *JAK2(−) MPN*	0.94	6.15E−07
L-lactate dehydrogenase	PMF vs. *JAK2(−) MPN*	0.85	5.52E−03
	PV vs. *JAK2(−) MPN*	0.90	4.38E−02
Malate dehydrogenase, cytoplasmic	PMF vs. *JAK2(−) MPN*	0.95	4.48E−02
Myeloperoxidase	PMF vs. *JAK2(−) MPN*	0.80	2.53E−15
	ET vs. *JAK2(−) MPN*	0.96	8.32E−03
	PV vs. *JAK2(−) MPN*	0.94	1.69E−03
	PV vs. PMF	1.21	1.69E−09
	ET vs. PMF	1.22	1.82E−09
NAD(P)H dehydrogenase, quinone 2	ET vs. *JAK2(−) MPN*	1.23	1.84E−02
Peroxiredoxin-2	PMF vs. *JAK2(−) MPN*	0.94	9.70E−03
	ET vs. *JAK2(−) MPN*	1.42	7.53E−08
	ET vs. PMF	1.50	3.38E−09
	PV vs. ET	0.74	2.24E−05
Protein disulfide-isomerase	ET vs. *JAK2(−) MPN*	0.99	2.11E−01
Ribosyldihydronicotinamide dehydrogenase	ET vs. *JAK2(−) MPN*	1.23	1.84E−02
Sulfhydryl oxidase 1	ET vs. *JAK2(−) MPN*	1.17	4.96E−03
Superoxide dismutase [Cu-Zn]	ET vs. PMF	1.09	8.73E−03
	PMF vs. *JAK2(−) MPN*	0.97	1.97E−02
Thioredoxin	PMF vs. *JAK2(−) MPN*	0.97	8.32E−03
Thioredoxin-dependent peroxide reductase, mitochondrial	PMF vs. *JAK2(−) MPN*	0.92	2.18E−02

NOTE: RAS GTPase proteins that are differently expressed between different subgroups of MPN; with the ratio and *P* value of the comparison.
Abbreviations: ET, *JAK2(+)* ET; PMF, *JAK2(+)* PMF; PV, *JAK2(+)* PV.

The first message from our data is that the proportion of all identified proteins that were differently expressed in the various MPNs or according to *JAK2V617F* mutation is quite low. Overall, we showed that a small part of the proteome varied between the different subtypes of MPNs. These variations ranged from 4.4% (PV vs. ET) to 9.6% (ET vs. PMF) of the total number of proteins identified by our methods. The amounts of a significant number of proteins were also different between mutated *JAK2* MPN granulocytes compared with nonmutated ones (76 in PV, 97 in ET, and 146 in PMF), but these proteins account for a relatively small proportion of the proteome, from 7.3% (*JAK2(+)* PV vs. *JAK2* WT MPNs) to 13.9% (*JAK2(+)* PMF vs. *JAK2* WT MPNs). This suggests that probably, a relatively low number of pathways play a critical role in MPN physiopathology and that alterations in the expression of a limited number of proteins are sufficient to produce phenotypic and clinical variations between MPNs. A knowledge of which proteins are concerned could be particularly useful for the development of new therapeutic strategies.

We also showed and confirmed that *JAK2V617F* status (presence and allele burden) could impact the protein content of MPN cells and, in particular, the granulocyte proteome. We found variations in the proteomes between *JAK2(+)* and *JAK2(−)*

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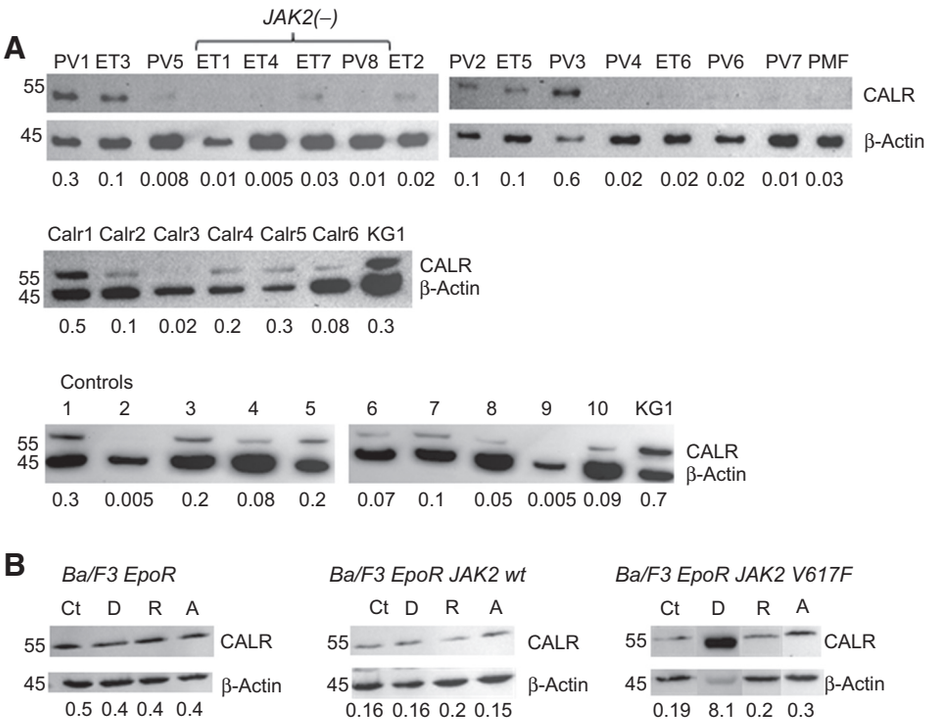

Figure 2.
Calreticulin expression in MPN and Ba/F3 cell lines. **A,** MPN erythrocytes. Patients: *JAK2(+)*: PV1-PV7, ET2, ET3, ET5, ET6, and PMF. *JAK2(−)*: ET1, ET4, ET7, and PV8. Calreticulin(+): Calr1-Calr6 and control patients. **B,** Ba/F3 cell lines. Cells were cultured under different conditions (Ct, control; D, deprivation of FBS and EPO; R, ruxolitinib 500 nmol/L; A, AZD1480 500 nmol/L); after 6 hours of culture, cells were lysed, run on SDS-PAGE gels followed by Western blotting, and incubated with suitable antibodies. β-Actin was the loading control and KG1 lysates the positive controls. Molecular weights of target proteins: calreticulin, 48 kDa; β-actin, 42 kDa. Calreticulin/β-actin ratios of the signal intensity are noted under the images.

patients, and for some proteins, we identified a correlation, either positive or negative, between allele burden and protein levels. In particular, we found that 10 proteins belonging to the RAS-GTPase family, including RAC2, RAS related RAB proteins, Rho GTPases, and IQGAP1, a protein whose level can vary considerably in erythrocytes, were significantly impacted by the presence of the *JAK2* mutation and all in the same way: *JAK2V617F* patients all displayed underexpression of these proteins compared with *JAK2(−)* ones. Altogether, these relative variations of proteins suggested that *JAK2V617F* mutation could alter RAS GTPase signaling in MPN granulocytes. Rho GTPases are principally involved in actin cytoskeleton remodeling, cell mobility, cell-cycle progression, and gene expression (18). Modifications in their relative amount could explain why leukocyte levels above 15×10^9/L have been associated with an increased risk of major thrombosis or myocardial infarction in PV (19) and in ET patients (20, 21). Neutrophil activation involves ROS production, proteolytic enzymes, such as elastase and cathepsin G, and the increased expression of CD11b on their cell surface. All of these elements can alter hemostasis and induce a prothrombotic condition (22). Interestingly, we showed that CD11b was significantly overexpressed in *JAK2V617F* ET granulocytes compared with nonmutated ones (overexpressed principally in *JAK2V617F* PV and ET granulocytes and underexpressed in PMF). These data are in line with those of Arellano-Rodrigo and colleagues (13) who linked CD11b to *JAK2V617F* ET neutrophils but not to thrombotic risk.

Furthermore, we showed that the level of expression of some proteins could be directly impacted by the allele burden of *JAK2V617F*. For instance, A8K9E4 (neutrophil collagenase) expression seemed to be positively dependent on the *JAK2V617F* allele burden. Conversely, the azurocidin (CAP7), found in the antibacterial granules of granulocytes, which is cytotoxic to Gram-negative bacteria, and tropomyosin alpha-1 chain (TPM1), which plays a central role in the stabilization of cytoskeleton actin filaments, appeared highly overexpressed in nonmutated granulocytes. Taken together, our data suggest another influence of *JAK2V617F* on granulocyte functions.

Interestingly, calreticulin was highly overexpressed in *JAK2V617F/CALR(−)* granulocytes compared with *JAK2(−)* ones. The relatively higher amounts observed in *JAK2(+)* patients suggest that calreticulin levels could be impacted by the *JAK2V617F* mutation in granulocytes. This hypothesis was reinforced by our results obtained in the Ba/F3 cell model. In this model, we demonstrated that calreticulin overexpression was not only dependent on *JAK2V617F* load but could also be regulated by JAK2 inhibitors that restored normal levels of calreticulin. Calreticulin has already been implicated in the development and progression of pancreatic cancer through the MEK/ERK signaling pathway, independently of p53 (23). In addition, calreticulin overexpression has been associated with increased sensitivity to drug-induced apoptosis in calreticulin-inducible HeLa cells (24), as well as in human MCF-7 breast cancer cells (25). Thus, our data suggest that, in addition to the well documented recruitment of the STAT pathway, *JAK2V617F* could be implicated on the deregulation of WT calreticulin. Hence, calreticulin could itself play a role in MPN pathogenesis due to its overexpression, besides the impact of the truncated form that results from *CALR* gene insertions or deletions.

Our data highlighted that different pathways could be implied on the deregulation of calcium homeostasis in MPNs. Calreticulin is known to be a regulator of this calcium homeostasis. Thus, its aberrant expression along with that of S100 calcium-binding proteins, also observed in MPNs (Supplementary Tables S1 to S6), could imply possible alterations in calcium levels in MPN

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granulocytes, influencing their functions. S100 alterations have already been described in other cancers, such as breast, melanoma, head and neck, or colorectal tumors (26), and the expression of S100A8 in leukemic cells was identified as a marker of poor prognosis in AML patients (27). Finally, calmodulin, which was overexpressed in PMF granulocytes compared with PV and ET ones, is known to modify IQGAP1 function via Ca^{2+} regulation (28, 29), thus explaining the differences in IQGAP1 levels between subgroups. In addition, changes in Ca^{2+} levels could influence the IQGAP1 functional state reinforcing the functional alterations induced by the quantitative variation of Rho GTPases.

Our proteomic analysis revealed also significant differences in the expression of many proteins involved in ROS detoxification pathways between MPN subtypes or according to the presence of *JAK2V617F*. Although we were unable to delineate an overall pattern, our results confirm that several of the effectors of ROS homeostasis are underexpressed in MPNs. Transcriptomics has previously highlighted deregulation of gene transcripts implicated in oxidative stress in MPNs (see Cokic and colleagues; ref. 30). Similarly, Hurtado-Nedelec and colleagues (31) described an increased production of ROS in MPN neutrophils from *JAK2V617F* patients compared with nonmutated ones, together with increased phosphorylation of p47phox and ERK1/2. Moreover, Marty and colleagues (32) reported that increased ROS production in PV and PMF $CD34^+$ cells and in Ba/F3-EpoR-*JAK2V617F* cell cultures led to an increase in DNA oxidative damage (8-oxo-guanines and DNA double-strand breaks). Among ROS scavengers, we found that catalase was underexpressed in *JAK2V617F* MPNs compared with *JAK2(−)*. Unfortunately, we had no control granulocytes from healthy donors for comparison, but low catalase levels in *JAK2V617F* MPNs could suggest that this mutation leads to a decrease in catalase expression contributing to the oncogenic role of *JAK2* mutations. Indeed, Marty and colleagues (32) reported decreased catalase expression in mouse models and showed that PI3K/Akt activation via *JAK2V617F* negatively regulated the Forkhead box class O transcription factor, leading to reduced expression of antioxidant enzymes, such as catalase in bone marrow cells of knock-in mice. Moreover, we found that the protein RAC2 was underexpressed in *JAK2V617F* ET and PMF patients compared with nonmutated ones. RAC2 is directly linked to the NADPH complex and has thereby been implicated in ROS production. In chronic myeloid leukemia chronic phase cells and primitive leukemia stem cells, it alters the mitochondrial membrane potential and electron flow producing high levels of ROS (33). Taken together, our data confirm that in MPNs, at the protein level, regulators of ROS homeostasis are aberrantly expressed, leading to alterations in both ROS production and ROS scavenging.

In conclusion, our data highlighted quantitative differences in the granulocyte proteome between *JAK2V617F* PV, ET, and PMF and nonmutated MPNs as well as between MPNs harboring different *JAK2V617F* allele burdens. These data demonstrated that MPN granulocyte proteome variations on pathways implicated in ROS detoxification and RAS signaling could be associated with the oncogenic role of *JAK2V617F* in MPNs. Moreover, we showed that calreticulin was overexpressed in *CALR(WT)*/*JAK2V617F* MPN granulocytes, highlighting a potential unknown oncogenic role of *JAK2V617F* mutation through calreticulin overexpression, besides those ones due to calreticulin-mutated forms.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: V.P. Čokić, P. Mossuz
Development of methodology: P. Mossuz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V.P. Čokić, J. Mondet
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Socoro-Yuste
Writing, review, and/or revision of the manuscript: N. Socoro-Yuste, P. Mossuz
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V.P. Čokić, I. Plo
Study supervision: P. Mossuz

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References

1. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood 2002;100:2292–302.
2. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood 2009;114:937–51.
3. Klampfl T, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. N Engl J Med 2013;369:2379–90.
4. Nangalia J, Massie CE, Baxter EJ, Nice FL, Gundem G, Wedge DC, et al. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N Engl J Med 2013;369:2391–405.
5. Mossuz P, Girodon F, Donnard M, Latger-Cannard V, Dobo I, Boiret N, et al. Diagnostic value of serum erythropoietin level in patients with absolute erythrocytosis. Haematologica 2004;89:1194–8.
6. Tefferi A, Pardanani A. Evaluation of "increased" hemoglobin in the JAK2 mutations era: a diagnostic algorithm based on genetic tests. Mayo Clin Proc 2007;82:599–604.
7. Mossuz P, Bouamrani A, Brugiere S, Arlotto M, Hermouet S, Lippert E, et al. Apolipoprotein A1: a new serum marker correlated to JAK2 V617F proportion at diagnosis in patients with polycythemia vera. Proteomics Clin Appl 2007;1:1605–12.
8. Mossuz P, Arlotto M, Hermouet S, Bouamrani A, Lippert E, Girodon F, et al. Proteomic study of the impact of the JAK2-V617F mutation on the phenotype of essential thrombocythemia. Exp Hematol 2008;36:1642–7.

9. Tefferi A, Vaidya R, Caramazza D, Finke C, Lasho T, Pardanani A. Circulating interleukin (IL)-8, IL-2R, IL-12, and IL-15 levels are independently prognostic in primary myelofibrosis: a comprehensive cytokine profiling study. J Clin Oncol 2011;29:1356–63.
10. Pourcelot E, Trocme C, Mondet J, Bailly S, Toussaint B, Mossuz P. Cytokine profiles in polycythemia vera and essential thrombocythemia patients: clinical implications. Exp Hematol 2014;42:360–8.
11. Hui W, Ye F, Zhang W, Liu C, Cui M, Li W, et al. Aberrant expression of signaling proteins in essential thrombocythemia. Ann Hematol 2013;92:1229–38.
12. Falanga A, Marchetti M, Barbui T, Smith CW. Pathogenesis of thrombosis in essential thrombocythemia and polycythemia vera: the role of neutrophils. Semin Hematol 2005;42:239–47.
13. Arellano-Rodrigo E, Alvarez-Larran A, Reverter JC, Villamor N, Colomer D, Cervantes F. Increased platelet and leukocyte activation as contributing mechanisms for thrombosis in essential thrombocythemia and correlation with the JAK2 mutational status. Haematologica 2006;91:169–75.
14. Gallardo M, Barrio S, Fernandez M, Paradela A, Arenas A, Toldos O, et al. Proteomic analysis reveals heat shock protein 70 has a key role in polycythemia Vera. Mol Cancer 2013;12:142.
15. Villanueva J, Lawlor K, Toledo-Crow R, Tempst P. Automated serum peptide profiling. Nat Protoc 2006;1:880–91.
16. Plo I, Nakatake M, Malivert L, de Villartay JP, Giraudier S, Villeval JL, et al. JAK2 stimulates homologous recombination and genetic instability: potential implication in the heterogeneity of myeloproliferative disorders. Blood 2008;112:1402–12.
17. Socoro-Yuste N, Dagher MC, Gonzalez De Peredo A, Mondet J, Zaccaria A, Roux Dalvai F, et al. Ph- myeloproliferative neoplasm red blood cells display deregulation of IQGAP1-Rho GTPase signaling depending on CALR/JAK2 status. Biochim Biophys Acta 2016;1863:2758–65.
18. Wennerberg K, Rossman KL, Der CJ. The Ras superfamily at a glance. J Cell Sci 2005;118(Pt 5):843–6.
19. Landolfi R, Di Gennaro L, Barbui T, De Stefano V, Finazzi G, Marfisi R, et al. Leukocytosis as a major thrombotic risk factor in patients with polycythemia vera. Blood 2007;109:2446–52.
20. Carobbio A, Finazzi G, Guerini V, Spinelli O, Delaini F, Marchioli R, et al. Leukocytosis is a risk factor for thrombosis in essential thrombocythemia: interaction with treatment, standard risk factors, and Jak2 mutation status. Blood 2007;109:2310–3.
21. Carobbio A, Antonioli E, Guglielmelli P, Vannucchi AM, Delaini F, Guerini V, et al. Leukocytosis and risk stratification assessment in essential thrombocythemia. J Clin Oncol 2008;26:2732–6.
22. Falanga A, Marchetti M. Thrombotic disease in the myeloproliferative neoplasms. Hematology Am Soc Hematol Educ Program 2012;2012: 571–81.
23. Sheng W, Chen C, Dong M, Zhou J, Liu Q, Dong Q, et al. Overexpression of calreticulin contributes to the development and progression of pancreatic cancer. J Cell Physiol 2014;229:887–97.
24. Nakamura K, Bossy-Wetzel E, Burns K, Fadel MP, Lozyk M, Goping IS, et al. Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis. J Cell Biol 2000;150:731–40.
25. Prathyuman S, Sellappa S, Joseph S, Keyan KS. Enhanced calreticulin expression triggers apoptosis in the MCF-7 cell line. Asian Pac J Cancer Prev 2010;11:1133–6.
26. Bresnick AR, Weber DJ, Zimmer DB. S100 proteins in cancer. Nat Rev Cancer 2015;15:96–109.
27. Nicolas E, Ramus C, Berthier S, Arlotto M, Bouamrani A, Lefebvre C, et al. Expression of S100A8 in leukemic cells predicts poor survival in de novo AML patients. Leukemia 2011;25:57–65.
28. Briggs MW, Sacks DB. IQGAP1 as signal integrator: Ca2+, calmodulin, Cdc42 and the cytoskeleton. FEBS Lett 2003;542:7–11.
29. Brown MD, Sacks DB. IQGAP1 in cellular signaling: bridging the GAP. Trends Cell Biol 2006;16:242–9.
30. Cokic VP, Mossuz P, Han J, Socoro N, Beleslin-Cokic BB, Mitrovic O, et al. Microarray and proteomic analyses of myeloproliferative neoplasms with a highlight on the mTOR signaling pathway. PLoS One 2015;10:e0135463.
31. Hurtado-Nedelec M, Csillag-Grange MJ, Boussetta T, Belambri SA, Fay M, Cassinat B, et al. Increased reactive oxygen species production and p47phox phosphorylation in neutrophils from myeloproliferative disorders patients with JAK2 (V617F) mutation. Haematologica 2013;98:1517–24.
32. Marty C, Lacout C, Droin N, Le Couedic JP, Ribrag V, Solary E, et al. A role for reactive oxygen species in JAK2 V617F myeloproliferative neoplasm progression. Leukemia 2013;27:2187–95.
33. Nieborowska-Skorska M, Kopinski PK, Ray R, Hoser G, Ngaba D, Flis S, et al. Rac2-MRC-cIII-generated ROS cause genomic instability in chronic myeloid leukemia stem cells and primitive progenitors. Blood 2012; 119:4253–63.

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Quantitative Proteome Heterogeneity in Myeloproliferative Neoplasm Subtypes and Association with *JAK2* Mutation Status

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