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Characterization of Philadelphia-negative Chronic Myeloproliferative Neoplasms: identification of novel biomarkers by Next Generation Sequencing and study of interactions between hematopoietic stem cell and the inflammatory cell micro-environment

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ABSTRACT

Myeloproliferative Neoplasms (MPN) are a biologically and clinically heterogeneous group of hematological malignancies, consisting in clonal disorders of the hematopoietic stem/progenitor cell (HSC/PC). Molecular alterations and inflammatory microenvironment represent the two main etiopathogenic factors of MPN.

The overall aim of this study was the molecular characterization of patients diagnosed with Ph-negative Chronic MPN and the study of interactions between HSC/PC and the inflammatory cell micro-environment.

We investigated young (< 40 years at diagnosis) patients with ET and early-PMF, and patients with a low (0,1-3%) *JAK2*^{V617F} allele burden (AB), demonstrating that the determination of *JAK2*^{V617F} AB is relevant both at diagnosis and during follow up. Indeed, it allows to prove the presence of a clonal hematopoiesis and, also, to predict clinical outcome. Of note, an AB $\geq$0,8% always corresponds to an overt MPN phenotype. In this context, coordinating a network of 19 Italian laboratories, we identified the ipsogen *JAK2* MutaQuant kit as the most sensitive and efficient assay for the quantification of samples with different mutation loads (in particular those with AB ≤1%). With regard to the role of inflammatory microenvironment in the pathogenesis of MF, IL-1β and TIMP-1 seemed to confer a survival advantage to MF-derived HSPCs, enhancing their proliferation and *in vitro* migration, as well as their clonogenic ability. Finally, in this study we tested three different gene panels for mutations detection, obtaining promising results in terms of coverage analysis (more than 95% of target regions with depth greater than 500X) and identifying gene variants with very low mutation load (<1%) in all patients.

In conclusion, this study set the basis for the standardization of molecular techniques for the determination of *JAK2*^{V617F} AB, and for the validation of a robust NGS approach to be translated into a diagnostic setting. Moreover, IL-1β and TIMP-1 emerged as novel promoting factors of the *in vitro* maintenance of MF-derived HSPC, which may be exploited as potential targets of therapy.

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INTRODUCTION

Myeloproliferative Neoplasms

The first formal classification of chronic myeloid neoplasms is credited to William Dameshek, who in 1951 described the concept of "myeloproliferative disorders (MPD)" by grouping together Chronic Myelogenous Leukemia (CML), Polycythemia Vera (PV), Essential Thrombocythemia (ET), and Primary Myelofibrosis (PMF). In 2001, the World Health Organization (WHO) classification of myeloid neoplasms included the Dameshek-defined MPDs under the broader category of chronic myeloproliferative diseases (CMPDs). Finally, in the revised 2008 WHO classification system, the word “disease” was replaced by “neoplasm” (MPNs). MPNs represent a biologically and clinically heterogeneous group of hematological malignancies. To date, we refer to PV, ET and MF [both primary (PMF) and secondary (post-PV/ET-MF)] with the term “classical” MPNs, which differ from CML for the absence of the transcript *BCR-ABL* (Philadelphia chromosome)[1-6].

The estimated incidence of PMF ranges from 0.8–2.1 per 100.000/year with a comparable overall incidence between men and women; PV has an incidence ranged from 0.4–2.8 per 100.000/year with a prevalence higher in male than in female (male-to-female ratio, 1.8:1), whereas ET is more prevalent in women than men (male-to-female ratio, 1:2) with an incidence ranged from 0.4–3.4 per 100.000/year. Most patients are diagnosed with MPNs after age 60; however, the diseases can occur at any age. Median age of diagnosis of PMF, ET and PV is 65, 56 and 61 years, respectively[7-12].

Classical MPNs are clonal disorders of the hematopoietic stem/progenitor cell (HSC/PC) characterized by abnormal proliferation of one or more myeloid lineages, because of hypersensitivity or independence from normal cytokine regulation. This proliferation results in increased numbers of mature erythrocytes (for PV), megakaryocytes (MK)/platelets (for ET), and MK/granulocytes (for PMF) in the peripheral blood. MPNs are associated with a normal maturation but increased numbers of mature blood cells, except in PMF, in which abnormalities in MK differentiation might be responsible for the bone marrow (BM) fibrosis. The BM fibrosis is the hallmark of this disease and is associated with extramedullary hematopoiesis with resultant enlargement of liver and spleen, dysregulated hematopoiesis, and overexpression of inflammatory cytokines accounting for many of the symptoms. They all carry an increased risk of vascular thrombosis and a potential to evolve into life-threatening

marrow dysfunction resembling myelodysplastic syndrome (MDS) or acute myeloblastic leukemia (AML)[13].

The etiopathogenesis of MPNs is multifactorial, and genetic mutations are among crucial factors to be studied. Indeed, their phenotypic diversity is attributed to differences in the specific genetic rearrangements or mutation(s) that underlie the clonal myeloproliferation[2,14-15]. The molecular pathogenesis of classical MPNs has been enigmatic until 2005, when William Vainchenker et al. described a Janus kinase 2 mutation (*JAK2*^{V617F}) in MPN. This discovery was followed by a series of additional descriptions of mutations that directly or indirectly activate JAK-STAT signaling: *JAK2* exon 12, myeloproliferative leukemia virus oncogene (*MPL*) and calreticulin (*CALR*) mutations. The discovery of these, mostly mutually exclusive, "driver" mutations has contributed to revisions of the WHO diagnostic criteria and risk stratification in MPN. However, the panorama of mutations in MPN has become more complex as a result of the emergence of several other mutations, which are usually expressed in a subclone of hematopoietic compartment and affect genes involved in DNA regulation and transcription, as well as some oncogenes; these mutations, which are present both in MDS and in some case of acute leukemia, suggest a hierarchy of mutational events that should be considered for a more complete definition of disease mechanism and, more importantly, in view of a personalized therapeutic approach[16-29]. MPNs are not thought to be inherited; however, some evidence suggests a genetic predisposition to developing MPN in general. The JAK2 46/1 haplotype has been shown to predispose patients to developing *JAK2*^{V617F}-positive MPN, and an association has been reported between a single nucleotide polymorphism in the *TERT* gene and risk of developing an MPN[30].

In addition to molecular abnormalities, the inflammatory microenvironment has emerged in the last few years as a key-player in MPNs pathogenesis[31-32]. Up-regulated production of proinflammatory cytokines by HSPCs and surrounding stromal cells generates a microenvironment that selects for the malignant clone[33-38]. This correlates with more severe marrow fibrosis[39,40], worsening systemic symptoms[41] and decreased survival[42]. However, the key players linking inflammation and cancer in MF are still to be defined.

Current drug therapy in PV, ET, or MF is neither curative nor capable of prolonging life or preventing disease progression[43,44]. Accordingly, treatment indications in PV and ET are, primarily, directed at prevention of thrombohemorrhagic complications and, secondarily,

towards alleviation of symptoms, and in PMF mostly at alleviation of symptoms with the possibility of cure limited to patients undergoing allogeneic stem cell transplant (ASCT)[45]. This is, perhaps, because of the clonal complexity of these disorders and the increasing molecular evidence of the need for *JAK2*^{V617F} mutation to cooperate with other genetic aberrations in the initiation and progression of the disease. This clonal complexity needs to be elucidated further in order to recognize clinically relevant candidate therapeutic targets[46].

Ruxolitinib is a JAK1/2 inhibitor that represents the first and only targeted therapy in MF, in the context of an otherwise orphan disease. Ruxolitinib suppresses clonal myeloproliferation and also the release of pro-inflammatory cytokines. It is effective in reducing splenomegaly and constitutional symptoms, but is burdened by on-target haematological toxicity and by increased susceptibility to potentially severe infections. In Phase 3 studies, most patients discontinued therapy due to adverse events or disease progression. Optimal use of the drug is of utmost concern in younger patients suitable for allogeneic stem cell transplantation, in which a delay of transplant in favour of targeted therapy may be detrimental[47,48].

The perspectives of chronic inflammation as the driver of mutagenesis in MPNs intend to be a new target for therapy. Indeed, early intervention with interferon-alpha2 and potent anti-inflammatory agents (e.g. JAK1-2 inhibitors, histone deacetylase inhibitors, DNA-hypomethylators and statins) may disrupt the self-perpetuating chronic inflammation state and accordingly eliminate a potential trigger of clonal evolution and disease progression[49].

Molecular pathogenesis of MPNs

The molecular landscape of the chronic MPN is much more complex than was initially assumed on the basis of a relatively large number of studies. Indeed, it encompassed defined *driver* or *phenotypic* mutations (i.e., mutations that are sufficient *per se* to determine the clinic phenotype of these disease), such as mutations in *JAK2, CALR* and *MPL*, which constitute major diagnostic criteria in the 2016 revision of the WHO classification of myeloid neoplasms; and additional mutations, which are usually acquired in variably sized sub-clones of hematopoietic progenitors and which can occur most commonly concurrently with one of the phenotypic mutations.

Overall, most mutant genes in MPN fall in three functional classes: JAK/STAT signaling (mutations in *JAK2*, *CALR*, *MPL*, *CBL*, *LNK*, and rare mutations in *SOCS*), epigenetic gene regulation (*TET2*, *EZH2*, *ASXL1*, *DNMT3A*, *IDH1*) and spliceosome factors (*SF3B1*, *U2AF1* and *SRSF2*)[27]. Patients with MPN who transform to acute myeloid leukemia (AML) often acquire additional mutations, such as *TP53*, *NPM1*, *FLT3*, *RUNX-1* and several others[50,51].

JAK2 mutations

Frequency and distribution in MPN

Although by 1951, William Dameshek had highlighted the common pathogenic and clinical characteristics of PV, ET and PMF, the molecular pathogenesis of these disease remains unknown until 2005. In that year, four different groups described a mutation (c.1849 G>T) in exon 14 of the *Janus kinase 2* (*JAK2*) gene resulting in a valine to phenylalanine amino acid substitution at codon 617 (V617F) of the corresponding protein.

The *JAK2* gene maps to 9p24 and codes for a member of the tyrosine kinase proteins family. With JAK2, three other members of this family (JAK1, JAK3 and TYK2) form functional complexes with the intracellular domain of cytokine receptors of hematopoietic cells to whom are associated, thus allowing to transduce the specific ligand signal. Indeed, binding of the cytokine to the receptor causes receptor dimerization or modifies the three-dimensional structure with the juxtaposition and transactivation of the receptor-associated JAK molecules. Once activated, these phosphorylate tyrosine residues of the receptor, creating “anchor sites” for adapter or effector proteins containing Src-homology-2 (SH2) domains, including one or

more proteins of the seven-membered STAT family (STAT-1, -2, -3, -4, -5a, -5b, and 6). Thus, the JAK/STAT pathway, through the four JAK members and seven STAT members, is the main system of intracellular signaling. The type of STAT dimers that are recruited by activated receptors, as well as the kinetics and the extent of STAT activation, and the possible parallel activation of other signaling pathway (e.g. the mitogen-activated protein kinase (MAPK) pathway and phosphatydilinositol-3'-kinase (PI3K) pathway) ensure transduction specificity for individual cytokines. JAK1, JAK2 and TYK2 proteins are expressed ubiquitously, while JAK3 is exclusive to hematopoietic cells. JAK2 has an important role in maintaining stem cell viability and function through transmission of the thrombopoietin (TPO) receptor signal, and is also crucial for the various stage of myeloid differentiation through binding to erythropoietin (EPO), TPO, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrofage colony-stimulating factor (GM-CSF), interleukine-3 (IL-3) and interleukine-5 (IL-5) receptors (Figure 1).

The *JAK2*^{V617F} mutation represents the most common molecular feature of the three main types of MPN, being found in 95% of PV, and between 50 and 60% of ET and PMF. Therefore, *JAK2*^{V617F} mutation can be used as a unique molecular marker for distinguishing PV, ET, and MF from reactive hematopoietic disorders. It leads to a "gain of function" of the JAK2 protein, hence, the constitutive tyrosine kinase activation of molecular signaling pathway, providing hematopoietic precursors with their proliferative characteristic.

Figure 2A shows the structure of JAK2 protein. Starting from the N terminus, JAK protein contains four main domains: the FERM domain, through which JAK interacts with the intracellular part of the cytokine receptor, the SH2 domain, through which it interacts with effector proteins, a pseudo-kinase domain (JH2), and a JAK homology 1 carboxy-terminal domain (JH1), with tyrosine-kinase activity. The pseudo-kinase domain, which is most likely inactive, plays a crucial role in regulating JAK2 protein function, as it interacts with JH1 inhibiting its catalytic activity. The majority of *JAK2* mutations described in MPN (Table 1), including V617F, falls within the JH2 domain, activating the enzymatic function of JAK2 by decreasing the capacity of this domain to interact with the tyrosine-kinase domain, resulting in a dysregulation of self-inhibitory capacity[52] (Figure 2B) and in the cytokine-independent activation of the various membrane receptors in the corresponding hematopoietic MPN clone. This concept was further strengthened by the subsequent identification of various other

mutations (amino acid substitutions, deletions and duplications, predominantly affect exon 12 of the gene), described in PV patients (but not in ET and PMF) V617F- wild type, as well as in congenital forms of MPN. The most frequent of these, present in about 40% of *JAK2*^{V617F} - negative patients with PV, causes an in-frame deletion of six nucleotides between codons 542 and 543 (N542_E543del)[53]. Finally, mutations causing exon 14 splicing variants are much rare and lead to the production of a truncated JAK2 protein[54].

The *JAK2*^{V617F} mutation is also infrequently present (3–5 %) in refractory anemia with ringed sideroblasts associated with thrombocytosis (RARS-T), CMML, and other atypical chronic myeloid disorders. Additionally, this mutation is occasionally detected even in patients with primary AML, MDS and CML[55,56].

The *JAK2*^{V617F} mutation can be present in a heterozygous state or can progress to homozygosity most frequently by a mitotic recombination event resulting in uniparental disomy (UPD). Given the acquired growth advantage with respect to the rest of MPN clone, the homozygous cells quickly constitute a large portion of the bone marrow neoplastic clone, bringing the allele burden (AB), i.e. the number of mutant alleles, over the 50% threshold. Homozygous *JAK2*^{V617F} mutation is present in about 30% of PV and PMF patients, and in only 2-4% of ET patients. Interestingly, homozygosity leads to greater activation of JAK/STAT pathway by increasing the amount of intracellular mutated JAK2 protein, contributing to the different phenotypes (clinical features) of the three forms of MPN[57].

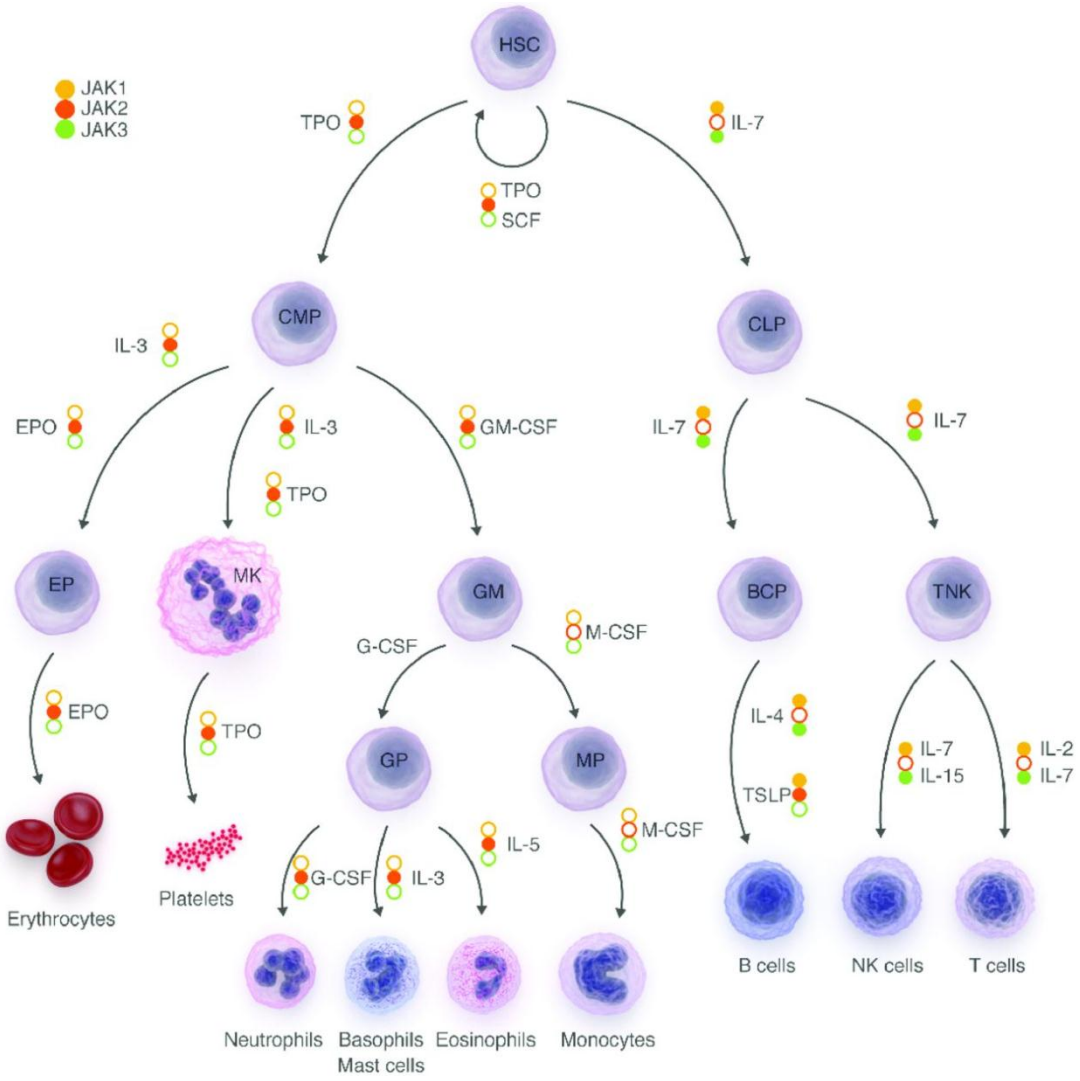

Figure 1. *JAK2* plays an important role in maintaining stem cell viability and function and myeloid differentiation (*Springuel L., Haematologica. 2015 Oct;100(10):1240-53*)

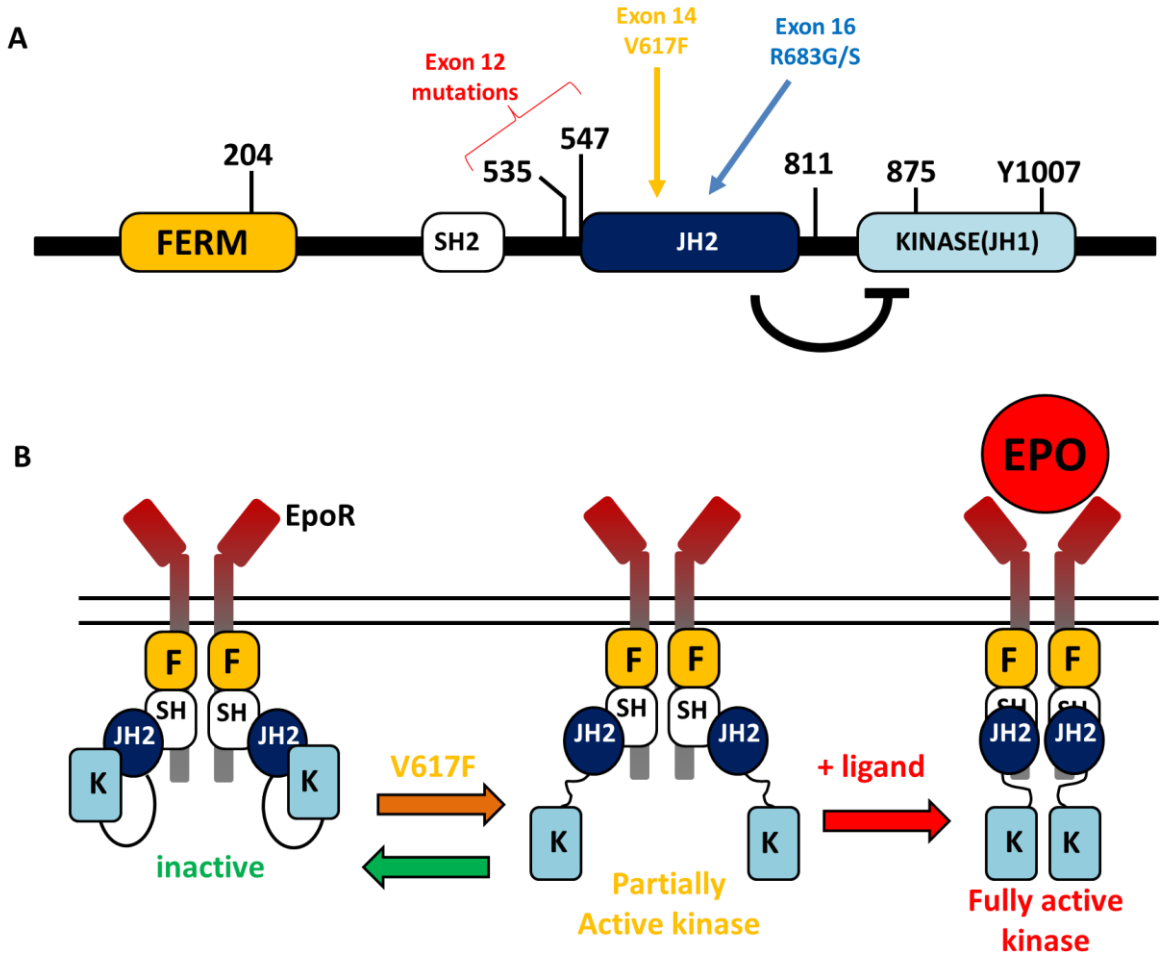

Figure 2. (A) Structural domain of the Jak2 protein. *Numbers* indicate amino acid positions within the Jak2 protein. *Arrows* indicate the positions of the most frequently mutated regions. The auto-inhibitory effect of the JH2 domain is indicated in *yellow*. FERM = N-terminal domain; JH1 (Kinase (K) domain) and JH2 = Jak homology 1 and 2 domains; SH2 = Src homology 2 domain. **(B) Example of the interactions JAK2 protein with the erythropoietin receptor (EpoR).** The V617F mutation in JH2 domain causes constitutive activation of JAK2.

Table 1. Principal *JAK2* mutations in MPNs

	Amino acid substitution	Exon	Protein Domain	Frequency (%)	Ref
PV	V617F	14	JH2	97	17, 19
PV with concurrent V617F mutation	V617F, C618R	14	JH2	<1	59
	V617F, D620E	14	JH2	<1	60
	V617F, L611V	14	JH2	<1	61
PV V617F NEGATIVE	N542_E543del	12	SH2-JH2	40	58
	E543_D544del	12	SH2-JH2	10	62
	F537_K539delinsL	12	SH2-JH2	10	58
	R541_E543delinsK	12	SH2-JH2	10	63
	K539L	12	SH2-JH2	10	64
	H538_K539delinsL	12	SH2-JH2	5	63,64
	I540_E543delinsMK	12	SH2-JH2	5	63
	V536_I546dup	12	SH2-JH2	<1	65
	F537I, K539I	12	SH2-JH2	<1	67
	F537_I546dup10, F547L	12	SH2-JH2	<1	65
	F537_F547dup	12	SH2-JH2	<1	68
	H538_K539delinsF	12	SH2-JH2	<1	67
	H538_K539delinsI	12	SH2-JH2	<1	69
	K539, L545V	12	SH2-JH2	<1	66
	I540T	12	SH2-JH2	<1	67
	I540_E543delinsKK	12	SH2-JH2	<1	70
	R541K, A542_G543del	12	SH2-JH2	<1	71
	R541_E543delinsK	12	SH2-JH2	<1	72
	D544G	12	SH2-JH2	<1	73
	D544_L545del	12	SH2-JH2	<1	66
	547insL, I540_F547dup8	12	SH2-JH2	<1	74
ET	V617F	14	JH2	50-60	17
ET with concurrent V617F mutation	V617F, C618F	14	JH2	<1	59
	V617F, C616C	14	JH2	<1	75
PMF	V617F	14	JH2	50-60	17, 19
	D620E	14	JH2	<1	76
MDS-RARS-T	V617F	14	JH2	50-80	55
CMML	V617F	14	JH2	8	56
AML	V617F	14	JH2	<1	56

Clinical features of JAK2 mutations carriers

In all cases of primary erythrocytosis, i.e., cases that cannot be identified as secondary to acquired diseases (most frequently pulmonary or renal conditions) or, more rarely, congenital diseases (hemoglobinopathies, or alteration of tissue oxygenation protein sensors), the search for mutations in the *JAK2* gene is of fundamental importance for the diagnosis of PV. In fact, virtually 100% of PV patients have the V617F mutation (96%) or one of the more rarely described mutations in *JAK2* exon 12 (3-4%) or exon 14 (really rare) and, consequently, the finding of a clonal *JAK2* mutation has been one of the major criteria for the diagnosis of this pathology since 2008. Mutational analysis of *JAK2* exon 14 (both V617F and more rarely one mutations) is also requiring in the diagnostic process of confirming ET, in patients with thrombocytosis not secondary to other pathological conditions such as inflammatory processes, acute hemorrhage (or, more rarely, chronic blood loss) and iron deficiencies, as well as in the diagnostic confirmation of PMF in cases of leukocytosis (or leukopenia) and/or splenomegaly not attributable to other causes.

Compared to the V617F mutation, mutations of exon 12 (found in no more than 1-2% of all patients with PV), like the even rare ones of exon 14 (1-2% of the total), cause greater ligand-independent activation of the JAK/STAT pathway. Consequently, compared to patients *JAK2*^{V617F}- mutated, those with exon 12 mutations have significantly higher levels of hemoglobin and lower platelets and leukocytes count at diagnosis[58]. However, there are some evidences that the incidence of thrombotic events, leukemic transformation and survival do not differ significantly between the two subgroup of patients[77].

The presence of *JAK2*^{V617F} mutation in ET and PMF not only has a diagnostic significance, but also appears to have clinical and prognostic relevance. Indeed, ET patients with a *JAK2* mutation are older than those with wild-type *JAK2*, have higher levels of hemoglobin but lower platelet counts and a higher risk of venous thromboembolic events, and also a higher degree of leukocytosis[78,79]. Finally, although the probability of transformation to MF is similar in ET patients with or without any *JAK2* mutations, the likelihood of progression to PV is 29% in those with the mutation, while it is negligible in the remaining[80]. As in ET patients, PMF patients with the *JAK2*^{V617F} mutation tend to be older and have higher white blood cell counts at diagnosis than patients with mutations of *CALR* and *MPL*. Moreover, the overall survival of the latter is significantly better than that of patients

with *JAK2*^{V617F} mutation. Finally, the probability of thromboembolic events is higher in PMF patients with the *JAK2*^{V617F} mutation than in PMF patients harboring mutations in *CALR* or *MPL*[79,81].

The determination of *JAK2*^{V617F} AB might be useful for prognostic evaluation of the disease itself, as demonstrated in a prospective study of 338 patients with PV. Indeed, the AB at diagnosis was found to be correlated with hemoglobin concentration, degree of leukocytosis, spleen size and bone marrow cellularity, while it was inversely correlated with platelet count. Moreover, *JAK2*^{V617F} AB seems to be correlated with risk of progression to PPV-MF, but not with the probability of thrombotic events, evolution to acute leukemia or with reduced overall survival[82]. On the contrary, it appears to be of poor prognostic significance in ET, probably because of the small number of patients with high AB, whereas it is more relevant in PMF. In fact, patients with a lower AB have a higher probability to leukemic progression and a lower probability of survival[83].

Finally, *JAK2* mutation might represent a potential target to monitor the effect of therapy. However, the lack of real methodological standardization and the possibility that the mutation is present in a subclone rather than in the whole neoplastic mass and that, therefore, reduction of the AB in response to therapy does not necessarily imply a reduction in tumor mass, have limited the application of this strategy in clinical practice. Two different studies aim to investigate if the mutation burden varies in response to therapy with interferon-α2a and both showed little encouraging results[84,85]. On the opposite, in a recent study the role of quantitative analysis of AB in patients treated with Ruxolitinib has been evaluated, registering a significant reduction of it in the context of clinical response, as well as a correlation with the reduction of spleen volume in individual patients[86].

Nevertheless, to date, the only curative treatment for MF is allogenic stem cell transplantation (ASCT). Determination of *JAK2*^{V617F} AB has been demonstrated to be a useful tool for monitoring effectiveness of the transplant. Studies reported that *JAK2*^{V617F} mutation disappears after ASCT and reappears in the case of ensuring relapse, and the value of this mutation as a minimal residual disease marker to guide donor lymphocyte infusion is well established[87-90].

MPL mutations

Frequency and distribution in MPN

The MPL mutation were discovered in 2006 in patients with a diagnosis of Ph negative MPN who were negative for *JAK2*^{V617F} mutation. These studies showed that 1-10% of patients with ET and PMF have a *MPL* mutation. *MPL*-mutated PV patients have been occasionally reported[91,92].

The *MPL* gene maps to 1p34 and codes for the TPO receptor, the cytokine that regulates megakaryocytopoiesis and the formation of platelets. The majority of identified *MPL* mutations affect exon 10 of the gene and cause a substitution of the amino acid tryptophan in position 515 (W515L, W515K, W515A, W515R). This amino acidic residue belongs to the RWQFP domain, a group of five amino acid of the juxtamembrane portion of the TPO receptor (MPL), which prevents its spontaneous activation in the absence of ligand. The substitution of tryptophan W515 by another amino acid causes the loss of this inhibitory control mechanism and leads to spontaneous activation of the MPL receptor[93]. This mutation has been found also in rare cases of RARS-T[94-95].

Other *MPL* mutations (e.g., L510P, A506T, W519T, S204P) have been found, but they do not appear to have a pathogenic role[63,96]. The S505N mutation, initially identified as a germline mutation in hereditary thrombocytosis[97], has been described some years later as an acquired mutation in rare cases of ET[98].

Clinical features of mutation carriers

The *MPL* mutation load differs between ET and PMF, with the median AB being lower in ET than in PMF (32,8% *vs* 59,4%, respectively) or post-ET myelofibrosis (58,2%), while there does not seem to be any differences in AB between PMF and post-ET myelofibrosis. W515L mutations are typically associated with lower AB than W515K and W515A mutations[99]. Patients with a high AB acquire a loss of heterozygosity of chromosome 1p in granulocytes, with transition to homozygosity, which seems to underlie fibrotic evolution in patients with *MPL*-mutated MPN.

The *MPL* mutations are the third most frequent in Ph-negative MPN, after *JAK2*^{V617F} and *CALR* mutations. Screening for *MPL* mutations is therefore indicated in patients with a

suspicion of ET or MF, especially if *JAK2* and *CALR* mutations have already been excluded. MPL mutations are among diagnostic criteria for ET and PMF in the 2016 revision of the WHO classification[24,29].

Compared to ET patients with wild-type *MPL*, those with *MPL* mutations have higher platelet counts, lower hemoglobin levels, and less total bone marrow and erythroid cellularity[100]. PMF patients with *MPL* mutations are more often female, older, more anemic and have greater transfusion needs compared to patients with wild-type *MPL*. Moreover, they do not show different overall survival and leukemia-free survival compared to patients with *JAK2*-mutated MF[101], but they have a shorter survival than *CALR*-mutated patients[80].

CALR mutations

Frequency and distribution in MPN

Until 2013 approximately one third of cases of ET and PMF did not have a known molecular basis. In 2013 two different research groups, using whole-exome sequencing technique, identified recurrent somatic mutations in *CALR* gene in 20-35% of patients with ET and PMF who were negative for *JAK2* and *MPL* mutations[21,22]. *CALR* mutations are therefore the second most frequent mutations, after the *JAK2*^{V617F} mutation, in Ph-negative MPN. With rare exception, *CALR* and *JAK2* mutations are mutually exclusive[81,102]. *CALR* mutations do not seem to be present in PV; to date, only two isolated cases of *JAK2*^{V617F}-negative PV with *CALR* mutation have been reported in the whole literature[103]. Additionally, *CALR* mutations have been reported rarely in some forms of MDS, CMML, RARS-T and atypical CML[21,22], while no *CALR* mutation have been described in other myeloid/lymphoid malignancies, solid tumors or in healthy controls, indicating a specificity for ET and PMF.

The *CALR* gene maps to 19p13.3-p13.2 and encodes a protein, calreticulin, that is highly conserved from an evolutionary point of view, multi- compartmental and multifunctional protein best known for its role as a Ca^{2+} binding chaperone in the endoplasmic reticulum (ER) lumen. Mature CALR protein consists of three structurally and functionally distinct domains: the globular N-terminal domain (residues 1-180) is lectin binding and consists of a signal sequence for targeting to the ER; the middle proline-rich or P-domain (residues 181–290) contains high affinity, low capacity, binding sites for Ca^{2+}; the C-terminal domain (residues 291–400) is rich in negatively charged residues and contains a number of high capacity, low-

affinity Ca^{2+} binding sites. The acidic C-terminal domain terminates with the KDEL sequence, maybe involved in in the retrieval of CALR protein from the cis-Golgi back to ER (Figure 3).

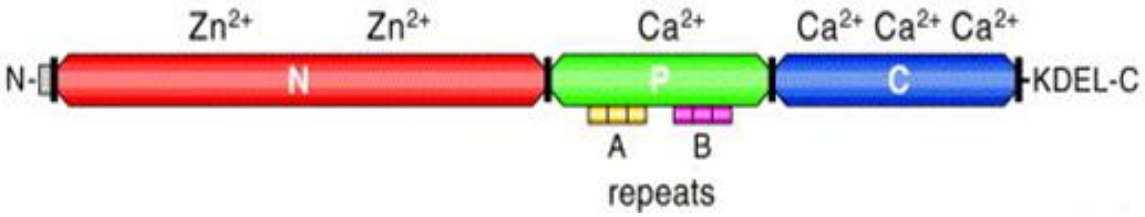

Figure 3. Calreticulin structure and exposure. Linear representation of calreticulin domains is shown. The protein contains an N-terminal amino-acid signal sequence (in grey), N-domain, P-domain, C-domain, and a C-terminal KDEL ER retrieval signal. The N- and P-domains of calreticulin are responsible for the chaperone function of the protein. The C-terminal C-domain contains a large number of negatively charged amino acids and is involved in high-capacity Ca^{2+} storage.

From the functional point of view, calreticulin is involved in a large number of intra- and extra-cellular processes. Within the lumen of ER, and in concert with other chaperone proteins, CALR plays an essential role in ensuring proper protein and glycoprotein folding. Moreover, calreticulin regulates calcium homeostasis, by acting as a binding and deposit protein. In addition to canonical ER related functions, CALR is reported to be found in the cytosol, nucleus, at the cell surface as well as extracellularly, where it accomplishes multiple functions as a critical mediator of physiological and pathological processes, such as the immune response, proliferation and apoptosis, cell phagocytosis, wound healing, and fibrosis[104].

CALR mutations consist of a wide variety of deletions or insertions in exon 9. To date, more than 60 different *CALR* mutations have been identified and classified according to their effect on DNA sequence: deletions have been designated as type 1 or type 1–like, of which c.1092_1143del, L367fs*46 (a 52-bp deletion) is the most common, and insertions as type 2 or type 2– like, of which c.1154_1155insTTGTC, K385fs*47 (a 5-bp insertion), is the most common. Together, these account for 85% of the *CALR* mutations; type 1 mutations are more common in PMF, whereas type 1 and type 2 occur with similar frequency in ET. Various other infrequent insertions and deletions in exon 9 account for up to 15% of *CALR* mutations.

All *CALR* mutations have the same consequence: a 1-bp frame- shift, which removes KDEL motif, and leads to the synthesis of a novel C-terminal peptide sequence, characterized by the acquisition of a minimal 36 amino acid stretch in place of 27 amino acids that are lost from the normal sequence. Remarkably, the C-terminus of the mutant protein contains several positively or neutrally charged amino acids whilst the equivalent region of the wildtype protein is largely negatively charged (Figure 4). This causes reduced capacity of calcium binding and subcellular relocation resulting in abnormal proliferation of cell harboring the mutant protein. There is evidence that mutant CALR binds to the TPO receptor inducing phosphorylation of JAK2 and activating downstream signaling, but the mechanism by which it happens is still to be clarify. Through clonal analysis, *CALR* mutations were shown to occur in a multipotent progenitor capable of both erythroid and myeloid differentiation as well as in hematopoietic stem cells[22]. Finally, by using hierarchical analysis of individual hematopoietic clones the early acquisition of *CALR* mutations was demonstrated, consistent with it being an initiating event in the cases characterized[21,22]. Single nucleotide variants have been reported in rare patients (e.g. E379D and RE398D)[21], however, whether they are true somatically acquired mutations and what significance they carry remain unclear at present.

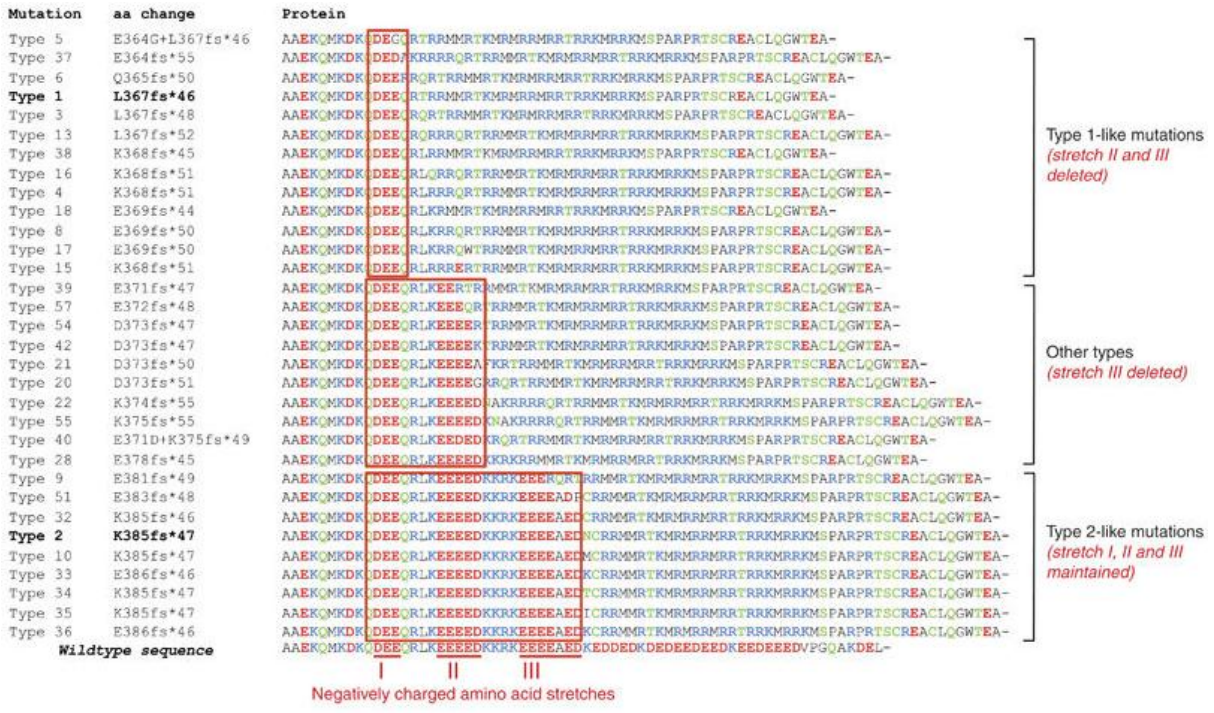

Figure 4. Altered protein reading frame with novel C-terminal associated with *CALR* mutations.

Clinical features of mutation carriers

The impact of *CALR* mutation burden on clinical features and prognosis is still to be elucidated. The median AB of the two most frequent *CALR* mutations was 32% in ET and 50% in post-ET MF, suggesting that disease evolution could be associated with an increased mutation burden[105]. Homozygosity for the *CALR* mutation is a rare event in MPNs, unlike the *JAK2* UPD that is associated with distinct phenotypic traits and more frequent evolution to post-PV/ET-MF. However, similar frequencies of homozygous CALR mutated patients due to acquired 19p UPD were found[21]. Since *CALR* mutations are the second most frequent mutation in MPN after *JAK2*^{V617F} mutation, they have been included among the major diagnostic criteria for ET and PMF in the 2016 revision of WHO classification[24,29].

CALR-mutated ET patients are more often male, are younger, have higher platelet counts, lower white blood cell counts and hemoglobin concentrations, and higher level of serum EPO, compared to those with *JAK2* mutations[80,81]. Despite their marked thrombocytosis, ET patients with *CALR* mutations have a lower risk of thrombotic complications than *JAK2*-mutated ET patients. This may depend on the presence of lower hemoglobin and white blood cells levels but also on reduced leukocyte activation. No differences in terms of risk of disease evolution (both to MF and to leukemia) or overall survival have been found. ET patients with CALR Type 2 mutation or Type 2- like mutations (i.e., CALR mutations that cause a change in amino acid chain similar to that of the type 2 mutation) have a more marked thrombocytosis but a lower risk of thrombosis during follow up, compared to CALR Type 1-mutated ET patients[106]. Patients carried CALR Type 1-like mutations are associated with an increased risk of evolution to MF[107].

Patients with CALR-mutated PMF are younger, have higher platelet counts, lower transfusion requirements, and tend to have a more indolent course with anemia, thrombocytopenia and leukocytosis appearing later in the follow-up. The median survival (about 17 years) is longer than that patients with JAK2- and MPL-mutated MF (about 9 years) and patients with triple-negative (TN) PMF (about 2-3 years)[24,105]. The benefits of CALR mutations in terms of survival are greater for CALR Type 1 and Type 1-like mutations[108].

The additional mutations

Table 2 show the genes most commonly involved in additional mutations in chronic MPN. Approximately 5-30% of patients with chronic MPN have mutations in genes coding epigenetic regulation (*TET2*, *ASXL1*, *IDH1/2*, *EZH2*, *DNMT3A*), in spliceosome genes (*SRSF2*, SF3B1, U2AF1), or in oncogene or tumor suppressor genes (*TP53*, *RUNX1*, *IKZF1*, *NRAS*, *KRAS*)[28,109]. The order of their acquisition contributes to the clinical phenotype of the disease[14,110,111] (Figure 5), but, since these mutations are also commonly present in MDS and other myeloid malignancies, including acute leukemia, they do not have a specific diagnostic value. However, they are particularly associated with patients with PMF, characterized by reduced survival or increased risk of leukemic transformation, thereby acquiring a very specific prognostic significance[112].

897 patients with PMF have been sequenced in a recent study[112], and it was found that the most frequently mutated genes were *ASXL1*, *EZH2*, *TET2*, *SRSF2* and *IDH1/2*. No correlation was found between these genes and a specific clinical phenotype. On the contrary, they correlated with reduced overall survival and increased risk of evolution to acute leukemia. Indeed, the presence of at least one mutated gene among these identifies patients at high molecular risk (HMR). This molecular status is associated with a very poor survival (HR = 2.29, 95%CI, 1.6-3.2) and a high probability of leukemic transformation (HR = 2.96, 95%CI, 1.8-4.7) (Figure 6). Interestingly, mutation burden did not change during follow-up, suggesting that most of the mutations defining HMR status are already present at diagnosis of the disease. For this reason, deep molecular characterization of PMF patients at diagnosis could be of utmost importance to define the prognosis. On the other hand, it could not be excluded the acquisition of additional mutations during follow-up in a subset of patients. Similar results on the impact of the number of mutated genes, including but not limited to the genes that define HMR category, were obtained in a larger study conducted in patients with MPN[113]. In PMF, mutations in *ASXL1* are those with greatest diagnostic impact. Interestingly, it was found that PMF patients with mutations in *ASXL1* without a concurrent *CALR* mutation had a median survival of 2.3 years compared to 10.4 years for those with a *CALR* mutation without any additional *ASXL1* mutations[114] (Figure 7). On the contrary, mutations in splicing genes, such as *U2AF1*, *SRSF2*, *SF3B1*, are rarely associated with *CALR* mutations, leading to think that it may contribute to better survival of these latter mutations[115].

Table 2. Genes most commonly involved in addition mutations in chronic MPN.

Gene	Function	Mutations site	Mutational frequency (%)			Type of mutation	Clinical correlation	Ref
			PV	ET	PMF			
Signaling								
CBL	**JAK2 Signaling**	EX 8-9	rare	rare	5-10	LoF	sAML	116
SH2B3		EX 2-4	1-2	-	rare	LoF	sAML	117
Epigenetic								
TET2	**DNA methylation**	CDS	10-20	5	10-20	LoF	sAML (26%)	116
ASXL1		EX 12	2-5	2-5	15-30	LoF	<OS, <LFS, sAML (19%)	112
EZH2		CDS	1-3	rare	5-10	LoF	<OS, <LFS	112
Splicing								
SRSF2	**Spliceosome**	EX 1	rare	rare	10-20	LoF	<OS, <LFS, sAML	112
SF3B1		EX 12-16	rare	rare	5-10	LoF	>OS	118
U2AF1		EX 2-8	rare	rare	5-15	LoF	<OS	119
Leukemic evolution								
IDH1	**Metabolic regulation**	EX 4	rare	rare	2-5	Neo-Enzyme	<OS, <LFS, sAML (8%)	112
IDH2		EX 4	rare	rare	2-5		<OS, <LFS, sAML (18%)	112
TP53	**Apoptosis, Cell cycling**	CDS	rare	rare	rare	LoF	<OS, <LFS, sAML (20%)	14
DNMT3A	**Chromatin modification**	EX 7-23	5-10	1-5	5-10	LoF	<OS, sAML	120
IKZF1	**Transcriptional factors**	CDS	-	-	rare	Del	sAML (21%)	121
RUNX1		CDS	-	-	rare	LoF	sAML (37%)	121
NRAS	**GTPase, Signaling**	CDS	rare	rare	rare	GoF	sAML (7-13%)	121
KRAS		CDS	rare	rare	rare	GoF	sAML (5-10%)	121

PV: Polycythemia vera; ET:essential thrombocytemia; PMF:primary myelofibrosis; CDS: complete coding sequence; EX: exon; LoF: Loss of Function; GoF: Gain of Function; >: increase; <: decrease; OS: overall survival; LFS: leukemia free survival; sAML: secondary AML.

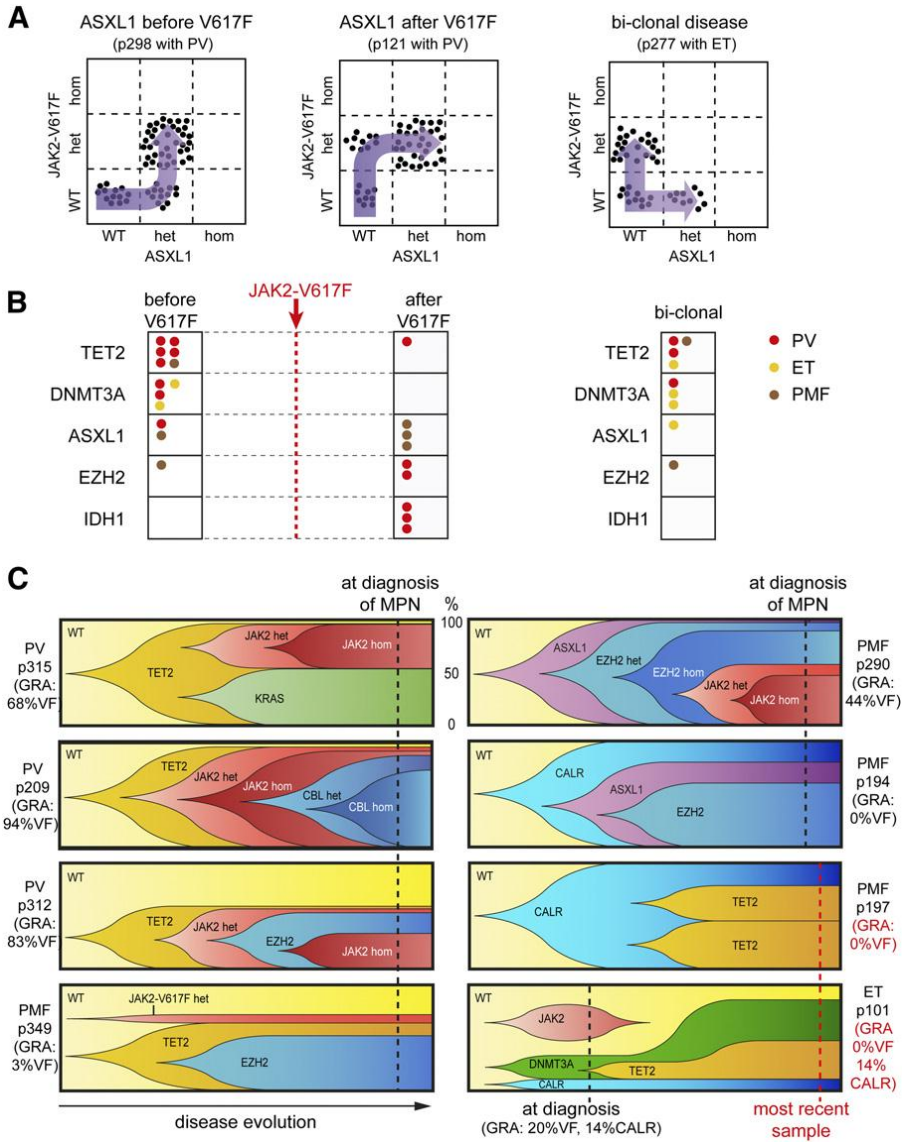

Figure 5. Clonal evolution in MPN patients carrying somatic mutations in epigenetic modifier genes. Single erythroid or granulocytic colonies (BFU-Es and CFU-G) grown in methylcellulose were individually picked and analyzed for the presence or absence of *JAK2*^{V617F} and other somatic mutations. (A) Examples of 3 patients who acquired an ASXL1 mutation before *JAK2*^{V617F} (left panel), after *JAK2*^{V617F} (middle panel), or in a clone separate from *JAK2*^{V617F} (right panel) are shown. Each dot represents a single colony that was genotyped and placed into the corresponding quadrant. (B) Summary of the temporal order of acquisition of mutations in relation to *JAK2*^{V617F}. Each dot represents 1 patient analyzed as shown in panel A and placed into the corresponding quadrant. Events in ET patients are depicted in yellow, PV patients in red, and PMF patients in brown. (C) Patterns of clonal evolution in 8 MPN patients carrying multiple somatic mutations. Dotted lines denote the time of analysis and the y-axis indicates the percentage of the colonies with or without the corresponding somatic mutations. %VF, *JAK2*^{V617F} mutant allele burden in purified granulocytes from peripheral blood. Al- though the order of events depicted can be de- duced from the single-clone analysis (dotted line), the exact timing of the acquisition of the individual mutations and the time needed for the clonal expansion remains unknown and is shown only schematically. GRA, granulocytes (*Lundberg P. et al, Blood. 2014;123(14):2220-2228*).

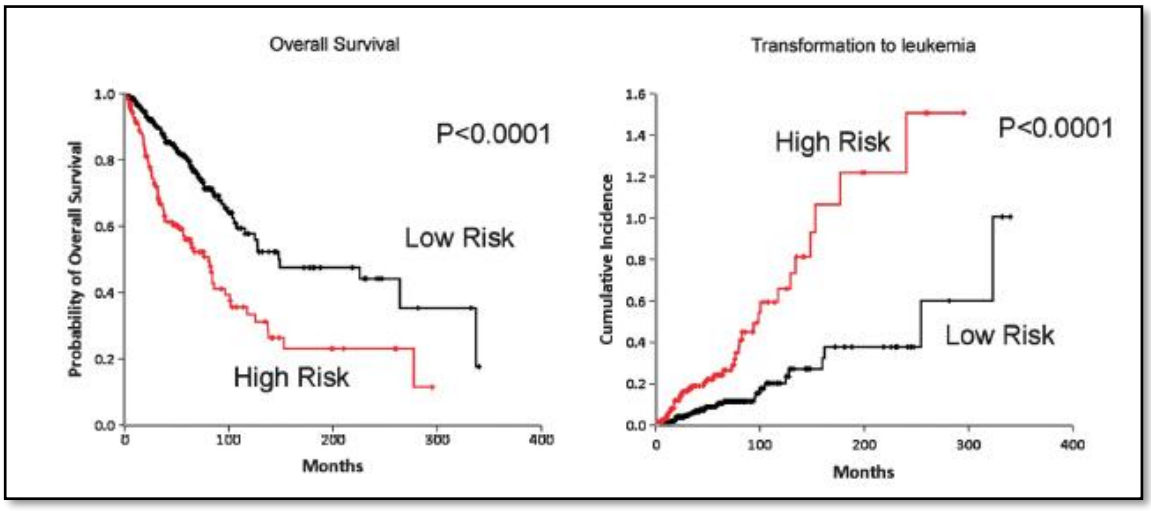

Figure 6. Overall survival and risk of transformation to leukemia of patients with PMF, stratified according to mutational status for EZH2, ASXL1, SRSF2, IDH1/2 (mutations belonging to HMR category) (*Vannucchi et al, Leukemia 2013)*.

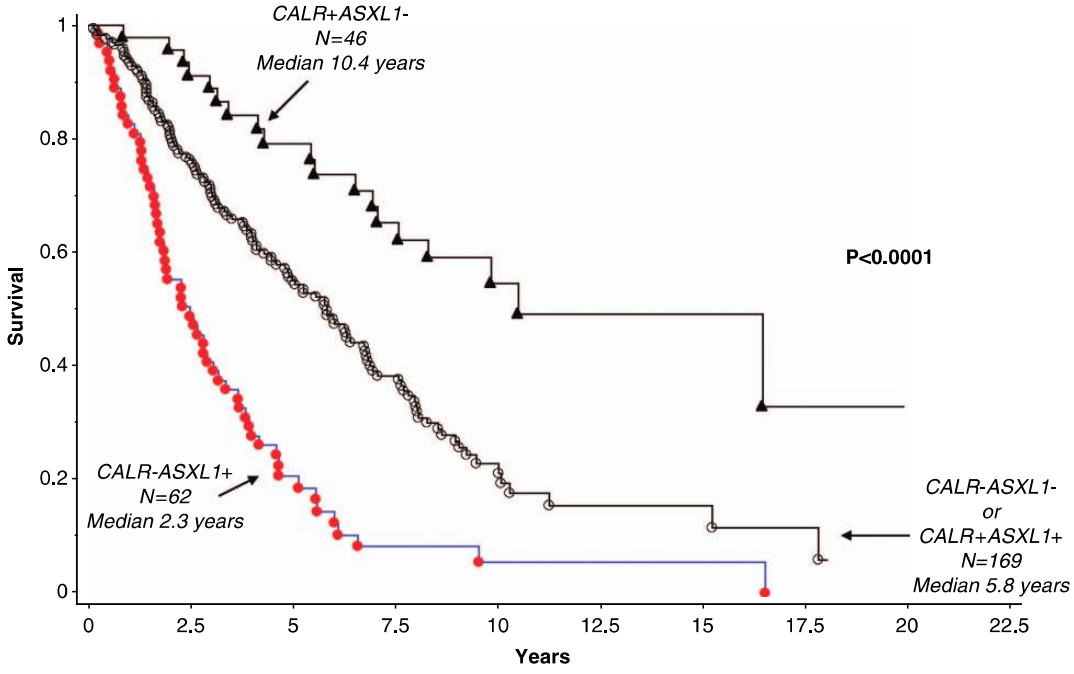

Figure 7. Overall survival of patients with PMF, stratified by the presence or absence of *CALR* and *ASXL1* mutations (*Tefferi A et al., Leukemia 2014).*

Clinical features of mutations carriers

A modern and efficient prognostic model (MIPSS, Molecular International Prognostic Score System) has been recently proposed and validated: it integrates mutational information with clinical data, proving to be more efficient in risk prediction and prognostication than "classical" models, based on clinical variables alone (IPSS and Dinamic IPSS, DIPSS). It remains to clarify whether therapeutic choices based on a prognostic stratification achieved by defining the HMR status might actually result in an improvement of patients' management and overall survival Patients currently classified in the low-risk or intermediate-1 category, according to IPSS or DIPSS, but who have one or more mutations in the genes of HMR category, might particularly take advantages from modern MIPSS. Indeed, since the predicted overall survival for these patients would be comparable to that of higher risk IPSS categories, a more aggressive therapeutic approach, such as stem cell transplantation, could be used. Finally, as regards the impact of additional mutations on response to therapy, no differences have been observed in terms of reduction in splenic volume and improvements of symptoms between high- and low-HMR. Additionally, patients belonging to both risk categories and treated with Ruxolitinib have reached longer survival. Similar results were registered in patients with two or fewer mutated genes (including either driver mutations or additional ones) who showed a nine-fold higher probability of a clinical response, in term of reduction of splenomegaly, than in patients with three or more mutations[122,123]. So that, an extensive molecular profiling of PMF patients may be useful in planning the treatment of patients receiving Ruxolitinib or other JAK inhibitors.

Mutations in most of the genes shown in Table 2, such as *TET2*, *ASXL1*, *IDH1/2*, *TP53*, *SRSF2*, *U2AF1*, *SF3B1*, are present during both the accelerated and the blastic phase of the disease. However the mechanisms through which these mutations contribute to leukemic transformation have to be clarify. More information has been obtained recently about *TP53* mutations[14]. In a study conducted in 22 subjects with secondary AML, it was found that *TP53* mutations were already present during the chronic phase in heterozygous state. After loss of the wild-type allele (loss of heterozygosity, LOH), through mitotic recombination or deletion, the hemi- or homozygous *TP53* clone expanded rapidly and patients transformed to AML. Therefore, *TP53* mutations evaluation could be useful in those cases of suspected evolution to AML, while the predictive role of mutations in chronic phase is still unclear.

Myelofibrosis: the "malignant" inflammatory microenvironment

Besides molecular abnormalities, also the inflammatory microenvironment plays a key-role in MF pathogenesis. Chronic inflammation (CI) is mediated by increased pro-inflammatory cytokines production by both stromal and malignant hematopoietic cells (activated leukocytes and platelets/megakaryocytes) and is an important driver of clonal initiation/evolution[33-37] (Figure 8). Consistently, increased serum levels of pro-inflammatory cytokines significantly correlate with worse survival in MF patients.

Experimental evidences suggest that MF stromal cells are primed by the malignant hematopoietic clone, which, in turn, conditions the stroma to create a favourable microenvironment that nurtures and protect the malignant cells. As a consequence, both pathogenic hypothesis and therapeutic attempts should take into account not just the malignant clone but also the activity of the stromal cell microenvironment. Interestingly, chronic inflammation is an important driver of genomic instability and cancer development/progression[124,125].

Recent findings showed that the HSC/PC actively sense pro-inflammatory factors[126]. However, the role of crucial factors of the inflammatory microenvironment such as extracellular nucleotides (eNTPs)[126,127], selected cytokines (Tumor Necrosis Factor (TNF)-alpha[128] and Tissue Inhibitor of Metalloproteinases (TIMP)-1[129-131]; or circulating microparticles (MP)[132-133] in the phenotype/function of the malignant hematopoietic clone of MF has been poorly (TNF-alpha) or never (eNTPs, TIMP-1, and MP) investigated.

Specifically:

- ✓ eNTPs act through specific receptors (P2YR and P2XR) regulating proliferation and migration of malignant CD34+ stem/progenitor cells.
- ✓ TNF-alpha facilitate clonal expansion of *JAK2*^{V617F}-positive cells.
- ✓ TIMP1, through receptor (CD63) binding, has a role in multiple biological processes, including cell proliferation, survival, inflammation and immune regulation.
- ✓ MP are a membrane- derived vesicles derived from various cells, most notably platelets, but also leukocytes, lymphocytes, erythrocytes, and endothelial cells. MP have a role in intercellular communications and systemic inflammation.

The concept of chronic inflammation as a major driver of disease progression in MPNs opens the avenue for clinical trials in which the two most promising agents within MPNs - IFN and Ruxolitinib - are combined and instituted in the early disease stage according to the early intervention concept[134]. The proof of concept and the rationales for this combination therapy have most recently been published[135]: the ability of IFN to induce deep molecular responses with normalization of the bone marrow, even years after cessation of IFN, and the role of inflammation in the initiation and progression of MPNs make the combination of IFN and ruxolitinib one of the most promising new treatment strategies for patients with MPNs[33,35,36].

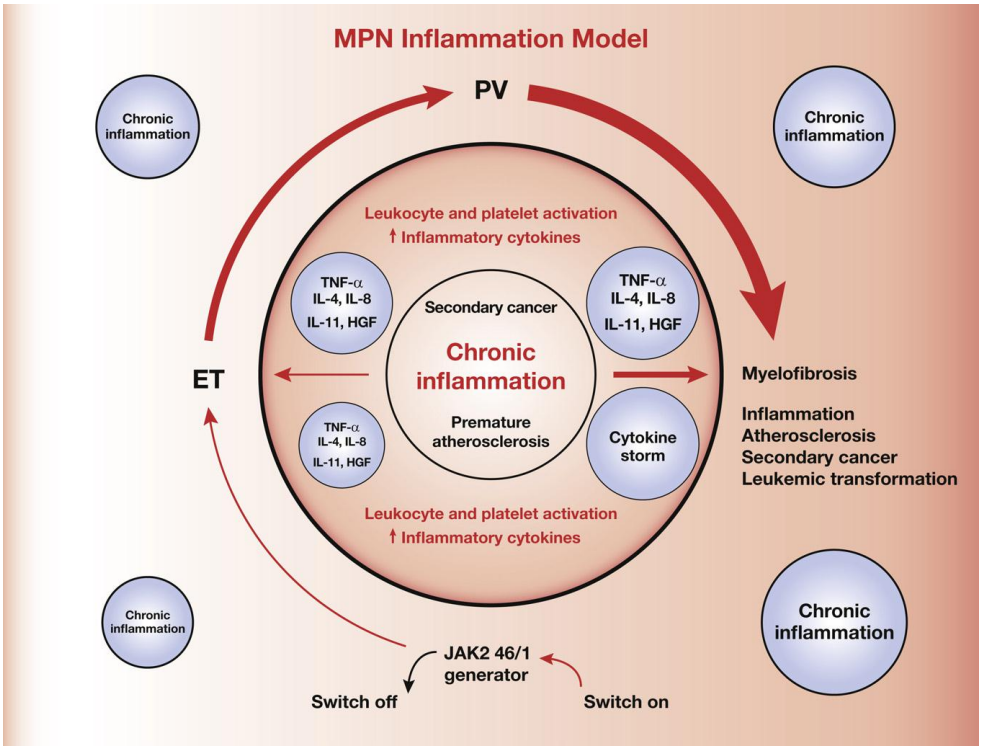

Figure 8. Vicious cycle of inflammation in the biological continuum of ET, PV, and MF. Chronic inflammation is proposed as the trigger and driver of clonal evolution in the biologic continuum from early disease state (ET and PV) to a more progressive disease state (MF). It is possible that combination therapy, using low doses of therapies such as interferon-a, Janus kinase inhibitors, and statins at the early disease state will positively influence the vicious cycle of disease progression. HGF, hepatocyte growth factor; IL, interleukin; MPN, myeloproliferative neoplasms; TNF, tumor necrosis factor (*Hasselbalch HC, Cytokine Growth Factor Rev. 2013; 24: 133–45*).

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AIMS

The overall aim of this study was the molecular characterization of patients diagnosed with Ph- negative Chronic Mieloproliferative Neoplasms and the study of interactions between malignant hematopoietic stem/progenitor cell (HSC/PC) and the inflammatory cell micro-environment.

The specific aims were the followings:

I. Mutational status and outcome of young (≤ 40 years old) ET and early-PMF patients have never been investigated. To address this issue, we report on a relatively large number of WHO-defined ET and early-PMF patients who were younger than 40 years of age at the time of diagnosis.

II. The use of highly sensitive allele-specific assays has significantly increased our ability to detect small *JAK2*^{V617F} mutated clones, with AB below 1%. As a small clonal hematopoiesis has been found also in otherwise healthy subjects, the clinical significance of a low AB has to be clarify. To address this issue, we analyzed clinical and laboratory data of patients with a suspected MPN that presented a low (≤3%) *JAK2*^{V617F} mutation burden.

III. *JAK2*^{V617F} AB monitoring is of utmost importance both during follow-up and post-transplant. In this context, the standardization of molecular techniques is urgently needed. To identify the most robust assay for the standardization of the molecular test and to allow consistent interpretation of individual patient analysis results, we established a network of 19 Italian laboratories and evaluated the analytic performances of 5 different molecular approaches.

IV. The key players linking inflammation and cancer in MF as well as the underlying pathogenetic mechanism are still to be elucidated. Therefore, we aimed to address the functional effects of several pro-inflammatory factors on the in vitro behaviour of HSPCs derived from MF patients.

V. Finally, we evaluated three different Next Generation Sequencing (NGS) gene panels in order to identify the most suitable approach for the molecular characterization of MF patients for both diagnostic and research purpose.

RESULT I

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Mutations and long-term outcome of 217 young patients with essential thrombocythemia or early primary myelofibrosis.

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ABSTRACT

We investigated the influence of molecular status on disease characteristics and clinical outcome in young patients (≤40 years) with World Health Organization (WHO)-defined essential thrombocythemia (ET) or early/prefibrotic primary myelofibrosis (early-PMF). Overall, 217 patients with ET (number 197) and early-PMF (number 20) were included in the analysis. Median follow-up time was 10.2 years. The cumulative incidence of thrombosis, hemorrhages and disease evolution into myelofibrosis/acute leukemia were 16.6%, 8.6% and 3% at 15 years, respectively. No differences were detectable between ET and early-PMF patients, although the latter cohort showed a trend for worse combined-event free survival (EFS). Mutation frequency were 61% for *JAK2*^{V617F}, 25% for *CALR* and 1% for *MPL*W515K, and were comparable across WHO diagnosis; however, *JAK2*^{V617F} allele burden was higher in the early-PMF group. Compared with *JAK2*^{V617F}-positive patients, *CALR*-mutated patients displayed higher platelet count and lower hemoglobin level. *CALR* mutations significantly correlated with lower thrombotic risk (9.1% versus 21.7%, P = 0.04), longer survival (100% *vs* 96%, P = 0.05) and better combined-EFS (86% *vs* 71%, P = 0.02).

However, non-type 1/type 2 *CALR* mutations ('minor' mutations) and abnormal karyotype were found to correlate with increased risk of disease evolution. At last contact, six patients had died; in five cases, the causes of death were related to the hematological disease and occurred at a median age of 64 years (range: 53–68 years). Twenty-eight patients (13%) were unmutated for *JAK2*, *CALR* and *MPL*: no event was registered in these 'triple-negative' patients.

INTRODUCTION

Diagnosis of essential thrombocythemia (ET) or early primary myelofibrosis (early-PMF) in subjects younger than 40 years is relatively uncommon.[1-4] Nonetheless, young patients with ET or early-PMF represent a category of substantial clinical interest, as they are projected to a prolonged survival and also to an extended utilization of medical resources. In addition, uncertainties remain about their best management and long-term outcome, due to the paucity of studies with long follow-up and the absence of specific risk scores.[5] In 2012, Barbui et al.[6] reported on a large series of young patients with ET (n=178) or early-PMF (n=35), with the latter cohort showing higher platelet count and worse combined event free survival (EFS). *JAK2*^{V617F} mutation emerged as a significant predictor for vascular events and disease evolution into MF; however, at that time molecular analysis was limited to the *JAK2*^{V617F} mutation and no data were available on mutation load. In the last 2 years, a plethora of studies have reported the impact of mutations, including those in the *CALR* gene, on main clinical features and outcome in patients with Philadelphia-negative myeloproliferative neoplasms.[7-14] However, no data are yet available on mutational status and outcome in cohorts of young patients with ET or early-PMF. To address this issue, we report on a relatively large number of World Health Organization (WHO)-defined ET and early-PMF patients who were younger than 40 years of age at the time of diagnosis, with particular focus on the impact of molecular alterations on main clinical features and long-term outcome.

SUBJECTS AND METHODS

Study population and definitions

A clinico-pathological database of ET patients was created. A total of 217 WHO-diagnosed[15–17] ET or early-PMF patients ≤40 years at diagnosis followed in five Italian Hematology Centers were included in the study. Inclusion criteria required the availability of demographic, clinical, histological and hematological data at diagnosis and at least one DNA sample to assess mutation status of the three driver genes: *JAK2*, *MPL* and *CALR*. Patients with unmutated *JAK2*, *MPL* and *CALR* were defined as 'triple negative'. All bone marrow biopsies were performed at diagnosis or before treatment start. Histological diagnosis was assessed at a local institution, which also reviewed bone marrow biopsies performed before 2008, for diagnostic accuracy according to WHO criteria. Baseline clinical characteristics and outcome measures (thrombosis, hemorrhages, secondary MF and acute leukemia (AL), second neoplasia, death, overall survival (OS) and EFS) were evaluated. Thrombotic and hemorrhagic events were defined as previously described.[18]

Diagnosis of post ET-MF (PET-MF) was made in accordance with the International Working Group on Myelofibrosis Research and Treatment criteria.[19] Diagnosis of AL was made according to WHO criteria, with a 20% bone marrow blast threshold for diagnosis.[20] The study was approved by the Institutional Review Board of each Institution and was conducted according to the Helsinki declaration.

JAK2, CALR and MPL mutation analysis

JAK2^{V617F} allele burden was assessed in granulocyte DNA by quantitative PCR-based allelic discrimination assay (ipsogen *JAK2* MutaQuant Kit, QIAGEN, Marseille, France) on 7900 HT Fast Real Time PCR System (Applied Biosystem, Life Technologies, Carlsbad, CA, USA).[21] *CALR* exon 9 sequencing was performed by next-generation sequencing approach with GS Junior (Roche-454 platform, 454 Life Sciences, a Roche company, Branford, CT, USA); analysis was carried out with AVA Software (GRCh38 as reference, 454 Life Sciences, a Roche company).[22] Rare *CALR* mutations identified by next-generation sequencing were confirmed by Sanger sequencing. *MPL* exon 10 mutations were evaluated using the Ipsogen *MPL*W515L/K MutaScreen Kit (QIAGEN). *MPL* exon 10 S505N mutations were evaluated by Sanger sequencing.[23]

Cytogenetic analysis

Chromosome banding analysis was performed on bone marrow cells by standard banding techniques according to the International System for Human Cytogenetic Nomenclature.[24] At least 20 metaphases were required.

Statistical analysis

Numerical variables have been summarized by their median and range, and categorical variables by count and relative frequency (%) of each category. Comparisons of quantitative variables between groups of patients were carried out by the non-parametric Wilcoxon rank-sum test.

Association between categorical variables (two-way tables) was tested by the Fisher's exact test.

All survival curves were calculated by the Kaplan–Meier method from the date of ET/early-PMF diagnosis to the date of first appearance of the event. Specifically, OS was estimated from the date of ET/early-PMF diagnosis to the date of death or last contact, whichever came first. Evolution-free survival was calculated to the first documentation of PET-MF or AL. Combined EFS included all events, explicitly: thrombosis, hemorrhages, disease evolution into PET-MF/AL or death, whichever came first. Survival curves of different subgroups were compared by the log-rank test.

Multivariate analysis of survivals were carried out by Cox regression. All P-values were considered statistically significant when ≤ 0.05 (two-tailed). Statistical analyses were performed using Graphpad (Graphpad Software Inc., La Jolla, CA, USA) and SPSS software (PASW Statistics for Windows, Version 18.0., Chicago, IL, USA).

RESULTS

Disease phenotype, type of treatment and outcome measures according to WHO diagnosis

Overall, 197 WHO-defined ET and 20 early-PMF (age range: 16–40, median 34 years) were included in the study. Table 1 provides a comparative illustration of presenting clinical/laboratory features and outcome measures according to WHO diagnosis. No significant differences were seen in hematological and clinical data at presentation, except for higher frequency of elevated serum lactate dehydrogenase levels in early-PMF patients. A total of 6 out of 161 (3.7%) evaluable patients had an abnormal karyotype, specifically:

46,XY,inv(9)(p11q13); 46,XX,inv(16) (q21q23); 46,XX,t(2;3)(q13;p25); 47,XY,+9 (2 patients); 45,XX,-9. Median follow-up was 10.2 years (range: 0.5–37.5). Cytoreductive therapy was required in 132 patients (61%), 122 patients with ET (61%) and 10 with early-PMF (50%) (P = 0.34). The use of cytoreductive and antiplatelet therapy was based on physician's decision, according to local standard clinical practice. Reasons for treatment start were as follows: extreme thrombocytosis (94 patients, 71%), thrombotic or hemorrhagic event (18 patients, 14%) or persistence of microvessel disturbances despite antiplatelet therapy (20 patients, 15%). The use of cytoreductive and anti- platelet therapy was comparable in the ET and early-PMF cohorts. During follow-up, ET and early-PMF patients showed comparable incidences of thrombosis, hemorrhages, disease evolution into PET-MF or AL and death. However, a trend for a worse combined EFS at 15 years was noted in early-PMF patients (65.6% versus 80.9% in ET patients, P = 0.27).

Table 1. Baseline features and outcome measures of 217 young patients with ET or early-PMF subdivided according to their WHO diagnosis

	ET (n = *197*)	*Early-PMF* (n = *20*)	P-*value*
Male/female, no (male %)	64/133 (32%)	7/13 (35%)	0.80
Median age, years (range)	34 (16–40)	35 (22–40)	0.12
Median leukocyte, $x10^9$/l (range)	8.6 (4–19.6)	9.1 (3.2–31.4)	0.87
Median platelets, $x10^9$/l (range)	850 (467–2741)	876 (451–2513)	0.29
Median hemoglobin, g/dl (range)	14.2 (10.3–17.5)	14 (11–16.1)	0.60
Palpable splenomegaly, no. of patients (%)	33 (17%)	6 (30%)	0.06
Previous hemorrhages, no. of patients (%)	4 (2%)	0	1.00
Previous thrombosis, no. of patients (%)	16 (8%)	2 (10%)	0.67
Elevated lactate dehydrogenase, no. of patients (% on evaluable patients)	9/137 (6.5%)	9/16 (56%)	< 0.001
Cytogenetic abnormalities, no. of patients (% on evaluable patients)	5/143 (3.5%)	1/18 (5.5%)	0.51
Cardiovascular comorbidities, no. of patients (%)	105 (53%)	8 (40%)	0.34
*JAK2*V617F, no. of mutated patients (%)	124 (63%)	8 (40%)	0.055
CALR, *no. of mutated patients (%)*			
All mutations	47 (24%)	7 (35%)	0.28
Type 1 mutation	16 (34%)	5 (71%)	0.09
Type 2 mutation	23 (49%)	2 (29%)	0.43
'Minor' mutations	8 (17%)	0	0.57
MPL, no. of mutated patients (%)	3 (2%)	0	1.00
Triple negative, no. of mutated patients (%)	23 (12%)	5 (25%)	0.09
Cytoreductive therapy, no. of pts (%)	122 (61%)	10 (50%)	0.34
IPSET thrombosis,[35] no. of patients (%)			
High	8 (4%)	0	1.00
Intermediate	68 (31%)	4 (20%)	0.31
Low	121 (61.5%)	16 (80%)	0.14
Thrombosis-free survival, % at 15 years (95% CI)	86.1% (80.7%–94.5%)	65.6% (43%–88.1%)	0.12
Hemorrhage-free survival, % at 15 years(95% CI)	93.6% (86.5%–97.8%)	88.9% (45%–98%)	0.69
Evolution-free survival, % at 15 years (95% CI)	94.7% (87.5%–98%)	100%	0.46
OS, % at 15 years (95% CI)	99.3% (92.8%–100%)	100%	0.67
CombinedEFS, % at 15 years (95% CI)	80.9% (72%–87%)	65.6% (28%–88.5%)	0.27
Median follow-up, years (range)	10.3 (0.5–37.3)	7 (0.5–27.4)	0.06

Abbreviatons: CI, confidence interval; EFS, event-free survival; ET, essential thrombocythemia; IPSET, international prognostic score in WHO ET; OS, overall survival; PMF, prefibrotic primary myelofibrosis; WHO, World Health Organization.

Disease phenotype according to mutational status

Among the 217 patients included in the analysis, mutational frequencies were 61% for *JAK2*^{V617F}, 25% for *CALR*, 1% for *MPL*W515K and 13% for triple negative.

Within the 54 *CALR*-mutant patients, 21 (39%) had the 52-bp deletion (c.1099_1150del52, L367fs*46, type 1 mutation), 25 (46%) had the 5-bp insertion (c.1154_1155insTTGTC, K385fs*47, type 2 mutation) and 8 (15%) carried other anomalies ('minor' mutations). Two (25%) of these 'minor' mutations were 46-bp deletions (p.L367fs*48: c.1100_1145del46; c.10944T,c.1100_1145del46), whereas one was a 48-bp deletion (c.1113_1160del48); in one patient, a non-type one 52-bp deletion was detected (c.1092_1142del52, p. E364fs*46).

The last four patients showed various substitution, deletions or insertions of few base pairs (c.1147_11544TGTC; c.1151_11544 TTTGTC; c.1129_11394CTCTGCCTCT; c.1129_11394CTCTGTC). 'Minor' mutations were detected only in ET patients. Table 2 provides the details of baseline clinical and laboratory features according to mutational status and WHO diagnosis. Given the low number of *MPL*-mutated patients, these three cases were omitted from the analysis. Compared with the *JAK2*^{V617F}-mutant population, *CALR*-mutant patients had higher platelet count that was probably responsible for higher administration of cytoreductive therapy over the follow-up. In addition, *CALR*-mutant patients had lower baseline hemoglobin and hematocrit levels, and presented with a lower incidence of thrombosis before ET/early-PMF diagnosis.

Table 2. Demographic and clinical features at diagnosis of 186 young patients with ET or early-PMF subdivided according to their molecular marker

	JAK2V617F-mutant (n = *132)*	*CALR-mutant* (n-*54)*	P-*value*
Male/female, no. (male %)	45/87 (34%)	19/35 (35%)	1.00
Median age, years (range)	34 (16–40)	34 (16–40)	0.94
Median leukocyte, x10^9/l (range)	9.1 (3.2–19.6)	8.2 (4.8–17.7)	0.10
Median platelets (range)	812 (467–1540)	1105 (451–2741)	< 0.001
Platelets > 1000x10^9/l, no. of patients (%)	28 (21%)	29 (54%)	< 0.001
Median hematocrit, % (range)	44 (32–51)	41.8 (31.7–52)	0.001
Median hemoglobin, g/dl (range)	14.6 (10.3–17.5)	13.6 (11.4–15.8)	< 0.001
Palpable splenomegaly, no. of patients (%)	22 (17%)	12 (22%)	0.41
Elevated Lactate dehydrogenase, no. of patients (% on evaluable patients)	7/112 (6%)	11/41 (27%)	0.001
Previous hemorrhages, no. of patients (%)	2 (2%)	1 (2%)	0.58
Previous thrombosis, no. of patients (%)	15 (11%)	1 (2%)	0.04
Cardiovascular comorbidities, no. of patients (%)	73 (55%)	25 (46%)	0.33
Cytoreductive therapy, no. (%)	71 (54%)	43 (80%)	0.001
Antiplatelet therapies, no. (%)	106 (80%)	44 (81%)	1.00
IPSET thrombosis,[35] no. (%)			
High	8 (6%)	0	0.10
Intermediate	72 (55%)	0	< 0.001
Low	52 (39%)	54 (100%)	< 0.001
Median follow-up, years (range)	10.9 (0.7–33.5)	9.8 (0.5–37.3)	0.91

Abbreviatons: ET, essential thrombocythemia; IPSET, international prognostic score in WHO ET; PMF, prefibrotic primary myelofibrosis. The 3 MPL-mutated patients are not reported.

Impact of mutation load on disease phenotype

A systematic comparison was performed between ET and early- PMF cohorts in relation to the allele burden of driver mutation (*CALR* or *JAK2*^{V617F}). Overall, median *JAK2*^{V617F} and *CALR* mutations allele burden were 21.5% (range: 0.5–97.1%) and 45% (range: 10–63%), respectively, (P < 0.001). Twenty out of 54 (37%) *CALR*-mutated patients and 13 out of 132 (10%) *JAK2*^{V617F}-mutated patients had an allele burden ≥ 50% (P = 0.68). Notably, early-PMF patients showed a *JAK2*^{V617F} mutation load significantly higher than ET patients, with a median allele burden of 44.5% (versus 21% in ET patients, P<0.001), while *CALR* allele burden was comparable in the two cohorts (P = 0.19).

Consequently, no significant differences in allele burden of *CALR* and *JAK2* was detected in early-PMF patients (P = 0.74) (Figure 1). In addition, *CALR* mutation load was comparable across the different types of CALR mutations. Patients with a *JAK2*^{V617F} allele burden ≥ 50% showed significantly higher hemoglobin levels compared with patients with lower mutation load (P<0.001), while platelet and leukocyte count were comparable in the two cohorts. On the contrary, *CALR* mutation load did not influence hematological features at diagnosis.

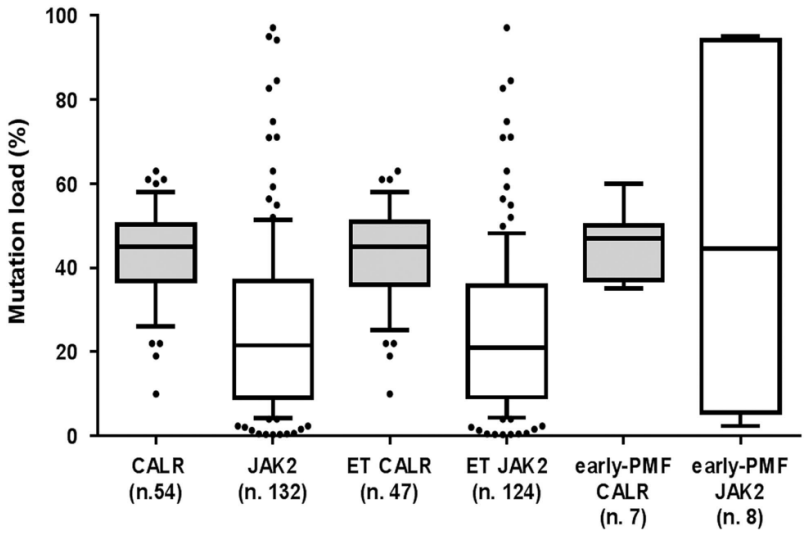

Figure 1. Mutation load in WHO-defined ET and early-PMF patients. The box plot depicts the 10% and the 90% percentiles with median value; dots represent outside values. *JAK2*^{V617F} allele burden was significantly higher in the early-PMF cohort compared with ET cohort. Median *CALR* allele burden was 40% for type 1 mutations, 47% for type 2 mutations and 48.5% for 'minor' mutations (P>0.05).

Cumulative risk of thrombosis and hemorrhages according to mutational status and allele burden

Overall, 22 patients (10%) experienced a total of 31 thrombotic events (arterial: 32%), with an incidence rate of 0.91% patients per year. Details concerning thrombotic events are reported in Table 3. The 10-year and 15-year cumulative incidence of thrombosis were respectively 11% and 21.7% in patients carrying *JAK2*^{V617F}, and 3.5% and 9.1% in those with *CALR* mutation (P = 0.042; Figure 2a). Moreover, patients with a *JAK2*^{V617F} allele burden higher than 50% had a higher thrombotic risk compared with all other patients (the thrombosis-free survival at 15 years was 64.2% versus 97.3% in the other patients, P=0.009). No impact of *CALR* mutations allele burden on thrombotic risk was observed. In multivariate Cox analysis, previous thrombosis, male sex and *JAK2*^{V617F} mutation remained significant (P = 0.039, 0.025 and 0.05, respectively).

Twelve hemorrhagic events occurred in 11 patients (1 patient with early-PMF, 5%, and 10 patients with ET, 5%) with an incidence rate of 0.39% patients per year. The 10-year cumulative incidence of hemorrhages was 7.8% in patients carrying *JAK2*^{V617F} and 5.2% in those with *CALR* mutations. No correlation was detectable.

Table 3. Thrombotic events occurred during follow-up

	Thrombotic events
No. of patients with events (%)	22 (10%)
No. of events	31
Site of thrombosis	
Arterial	10 (32%)
Ictus/transient ischemic attack	4 (13%)
Acute myocardial infarction	4 (13%)
Arterial peripheral	2 (7%)
Venous	21 (68%)
Deep vein thrombosis	10 (32%)
Thrombophlebitis	6 (19%)
Splanchnic vein thrombosis	5 (16%)
Median leukocyte count at event, x10^9/l (range)	7.4 (3.5–20.2)
Median platelet count at event, x10^9/l (range)	525 (360–1000)
Cytoreductive therapy at the time of the event, no. of patients (%)	12 (55%)
Antiplatelet therapy at the time of the event, no. of patients (%)	20 (91%)
Median time from diagnosis to first thrombosis, years (range)	8.8 (0.2–16.2)
Median time from first to second thrombosis, years (range)	3.5 (1–16)

Six patients had more than one thrombotic event. All patients with recurrent thrombosis were positive for the *JAK2*V617F mutation. After the first thrombosis, the 10 previously untreated patients started a cytoreductive treatment. In one case, the thrombosis was fatal (acute myocardial infarction).

Disease evolution and second neoplasia according to mutational status and allele burden

After a median follow-up of 10.2 years, an evolution to MF was observed in 11 patients (5.5%) and to AL in 1 patient (0 5%). No progressions were observed in early-PMF patients. The overall cumulative incidence of disease progression into MF/AL was 3% and 13% at 15 and 20 years, respectively. The incidence rate was 0.43% patients per year. A relationship with the type of mutation, *JAK2* or *CALR*, and with allele burden was not detectable. The 15-year cumulative incidence of disease evolution was 6.4% and 5.8% in *JAK2*^{V617F} and *CALR*-mutated, respectively (P=0.68). Interestingly, four of the eight patients carrying 'minor' *CALR* mutations, evolved to PET-MF/AL, while disease transformation was not observed in the 46 patients with type 1 or type 2 *CALR* mutations. In addition, 2 out of 6 patients with abnormal karyotype developed a PET-MF, while among the 155 patients with normal karyotype disease evolution was observed in 9 cases (P=0.04). Ten patients (4.6%) developed a second neoplasia, after a median time of 13 years from ET/early-PMF diagnosis, specifically: breast cancer (2), prostate cancer, colon-rectal cancer, tymoma, thyroid cancer (2), lung cancer, glioblastoma, non-melanoma skin cancer. Considering the type of neoplasia, no increase of the risk was detectable. Moreover, all the patients survived the second neoplasia, that was not a cause of death.

OS and combined EFS according to mutation status

At last contact, six (2.7%) patients had died, at a median age of 61 years (range: 20–71), for an OS of 98% at 15 years (Figure 2b). Causes of death were related to the myeloproliferative neoplasia in all patients but one (four patients died because of disease evolution into MF/AL, one patient because of acute myocardial infarction and one patient with uneventful and untreated ET died in a car accident). In multivariate analysis, only *JAK2*^{V617F} mutation was found as a negative predictor of survival.

The 15-year cumulative incidence of composite outcomes (thrombosis, bleeding, evolution to PET-MF/AL and death) were respectively 33% in patients carrying *JAK2*^{V617F} and 15.1% in those with *CALR* mutation (Figure 2c). *JAK2* and *CALR* mutations allele burden did not significantly influence OS or combined-EFS (P>0.05).

Characteristics and outcome of 'triple-negative' patients

A total of 28 patients were negative for *JAK2*^{V617F}, *MPL* and *CALR* mutations ('triple negative'). WHO diagnosis was early-PMF in 5 out of 28 patients (18%) and ET in 23 patients. Presenting clinical and hematological characteristics were fully comparable to those of *CALR*-mutated patients. With respect to the *JAK2*^{V617F}-positive patients, triple-negative patients displayed higher platelet count and lower hemoglobin levels at presentation. During a median follow-up of 9.7 years (range: 12–32.9), no cases of thrombosis, hemorrhages, disease evolution or death occurred in this cohort, for a combined EFS of 100%. In addition, no second solid neoplasia was registered.

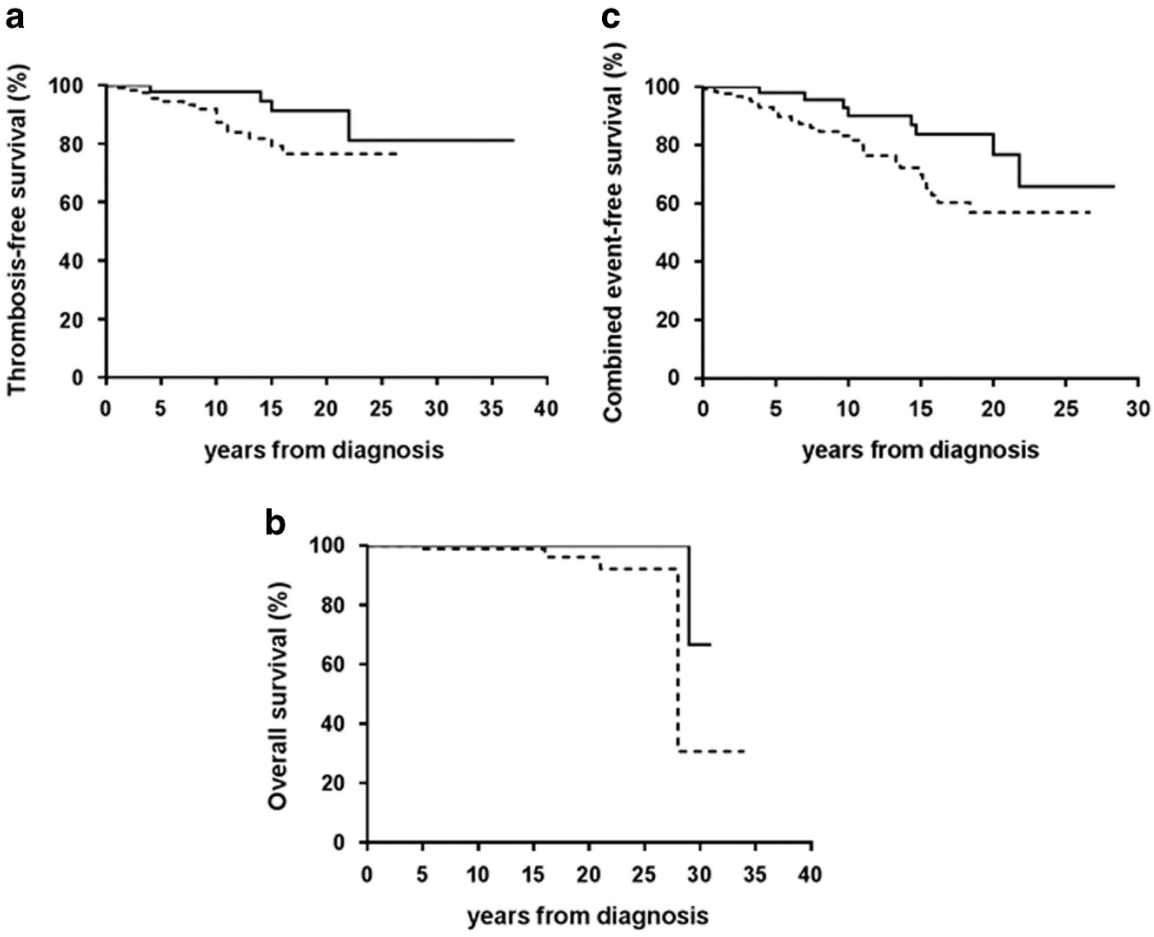

Figure 2. Outcome measures according to mutational status of JAK2 and CALR. (a) At 15 years, thrombosis-free survival was 91.3% (95% confidence interval (CI): 76–96%) and 77.9% (95% CI: 66–84%) in *CALR*- and *JAK2*^{V617F}-mutated patients, respectively (P = 0.042)). (b) Overall survival at 20 years was 96% in *JAK2*^{V617F}-mutated patients and 100% in *CALR*-positive patients (P = 0.05). (c) Combined event-free survival (Combined-EFS) at 15 years was 86.3% (95% CI: 78–94%) for *CALR*-mutated patients and 69% (95% CI: 65–80%) for *JAK2*^{V617F}-positive patients (P = 0.02). *CALR*-mutated patients (n = 54): continuous line. *JAK2*^{V617F}-mutated patients (n = 132): dotted line.

DISCUSSION

It is recognized that an accurate distinction between ET and early- PMF according to WHO criteria carries significant prognostic implications; it is also acknowledged that an extended molecular evaluation provides further information.[6,25–27] However, little data are available on selected cohorts of young individuals with an ET/early-PMF diagnosis.

We hereby report the molecular features, clinical findings and long-term outcome of a large series of patients with WHO-defined ET (n=197) or early-PMF (n=20), followed for a median time longer than 10 years and quantitatively evaluated for *JAK2*^{V617F}, *MPL* and *CALR* mutations. Presenting characteristics and frequency of mutations were comparable between ET and early-PMF cohort; however, the *JAK2*^{V617F} mutation load was significantly higher in early-PMF patients. In addition, despite the relatively small number of early-PMF patients, which limits the statistical potency of the comparison, a clear trend for significance in combined-EFS was observed between ET and early-PMF (80.9% versus 65.6%, respectively).

CALR-mutated patients were significantly different from *JAK2*^{V617F}-mutated patients in terms of hematologic and clinical features. At presentation, *CALR*-positive patients were characterized by higher platelet count and lower hemoglobin levels, while no significant differences in hematological parameters were noted between ET and early-PMF carrying the same mutation. Despite the higher platelet count, the incidence of thrombosis was significantly lower in *CALR*-mutated compared with *JAK2*-positive patients. Although these data might reinforce the opinion that platelets have an ancillary role in the pathogenesis of thrombotic complications,[28,29] the more frequent use of cytoreductive therapy in *CALR*-mutated patients may provide a contributory explanation.

In addition, mutation load was found to influence disease phenotype and outcome, as *JAK2*^{V617F} allele burden higher than 50% identified patients with a more myeloproliferative disorder and significantly predicted higher risk of major thrombotic events. Notably, allele burden was higher for *CALR* mutations compared with *JAK2*^{V617F}. However, none of the *CALR*-positive patients had mutant allele burden higher than 75%. This observation confirms prior reports and underpins the hypothesis that *CALR*-positive diseases are characterized by the gradual growth of a heterozygous clone rather than by progressive acquisition of homozygosis,[8,10] as demonstrated in the *JAK2*^{V617F} mutation.[30]

Despite the young age of the study cohort, 20 patients (9%) presented disease evolution or a second solid neoplasia (12 patients had overt MF/AL and 10 patients had a second solid neoplasia, with 2 patients experiencing both events). Although the time interval between ET/early-PMF diagnosis and these events was relatively long (median: 15.8 years; range: 3–35), in most cases patients were younger than 55 years at the time of the disease evolution/second cancer. The association of abnormal karyotype with disease evolution may reflect an underlying genome instability that favors the accumulation of genetic lesions and the development of additional neoplastic events.

Notably, *CALR* mutations other than type 1 and type 2 ('minor' mutations) seemed to correlate with higher probability of disease progression into overt MF/AL. Calreticulin has multiple physiological functions, mainly control of calcium homeostasis and glycoprotein folding, but also involving immune response, cellular adhesion and proliferation, phagocytosis and apoptosis.[31,32] *CALR* mutants seem to result in enhanced JAK-STAT signaling so that a shared mechanism of pathogenesis of *JAK2* and *CALR* mutations seems plausible. However, the exact role of *CALR* mutations in myeloproliferative neoplasms has still to be fully elucidated; to this regard, the present data point out the possibility that certain subtypes of *CALR* mutations could have an unfavorable prognostic role.[33,34]

Despite presenting characteristics were comparable to *CALR*- mutated patients, in our study the outcome of triple-negative patients was remarkably favorable, with no registered event after a median follow-up of 10 years. Ongoing studies are aimed at identifying the molecular basis of ET/early-PMF with unmutated *JAK2, CALR* or *MPL*; however, the possibility that some of these patients may not have a clonal thrombocytosis cannot be excluded.

With the limitations due to the relatively low number of early- PMF patients, the present analysis shows that mutational frequencies are comparable in the two groups, although *JAK2*^{V617F} allele burden is higher in early-PMF. The assessment of the molecular status is of utmost importance in these cohorts of patients, as both thrombosis and survival were significantly influenced by mutation type and burden. Finally, despite a low number of events throughout the follow-up, causes of death were mostly related to the hematological malignancy, pointing out the substantial impact that this generally indolent disease may acquire in young adults.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

FP designed the research, analyzed data and wrote the paper. RL, NP, AT, MC and BM collected the data and gave the final approval. MP and EO performed molecular analysis. NT performed cytogenetic analysis. FM, FA, GA, MC, GM, LC, MB and NV reviewed the paper and gave the final approval.

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RESULT II

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The relevance of a low *JAK2*^{V617F} allele burden in clinical practice: a monocentric study

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Keywords: *JAK2, V617F mutation, allele burden, myeloproliferative neoplasms, MPN*

ABSTRACT

Since low *JAK2*^{V617F} allele burden (AB) has been detected also in healthy subjects, its clinical interpretation may be challenging in patients with chronic myeloproliferative neoplasms (MPNs). We tested 1087 subjects for *JAK2*^{V617F} mutation on suspicion of hematological malignancy. Only 497 (45.7%) patients were positive. Here we present clinical and laboratory parameters of a cohort of 35/497 patients with an AB ≤ 3%.

Overall, 22/35 (62.9%) received a WHO-defined diagnosis of MPN and in 14/35 cases (40%) diagnosis was supported by bone marrow (BM) histology (''Histology- based'' diagnosis). In patients that were unable or refused to perform BM evaluation, diagnosis relied on prospective clinical observation (12 cases, 34.3%) and molecular monitoring (6 cases, 17.1%) (''Clinical-based'' or ''Molecular-based'' diagnosis, respectively). In 11/35 (31.4%) patients, a low *JAK2*^{V617F} AB was not conclusive of MPN. The probability to have a final hematological diagnosis (ET/PV/MF) was higher in patients with thrombocytosis than in patients with polyglobulia (73.7% vs 57.1%, respectively). The detection of AB ≥ 0.8% always corresponded to an overt MPN phenotype. The repetition of *JAK2*^{V617F} evaluation over time timely detected the spontaneous expansion (11 cases) or reduction (4 cases) of *JAK2*^{V617F}-positive clones and significantly oriented the diagnostic process.

Our study confirms that histology is relevant to discriminate small foci of clonal hematopoiesis with uncertain clinical significance from a full blown disease. Remarkably, our data suggest that a cut-off of AB ≥ 0.8% is very indicative for the presence of a MPN. Monitoring of the AB over time emerged as a convenient and non-invasive method to assess clonal hematopoiesis expansion.

INTRODUCTION

In 2008, the World Health Organization (WHO) classification indicated the positivity of the *JAK2*^{V617F} mutation as a major criterion for the diagnosis of chronic myeloproliferative neoplasms (MPNs), specifically Essential Thrombocytemia (ET), Polycythemia Vera (PV) and Myelofibrosis (MF) [1-7]. The *JAK2*^{V617F} mutation is detected in around 50-60% of ET and MF patients and in most (95%) patients with PV [8,9]. Also, the *JAK2*^{V617F} mutation may be found in other hematological malignancies. Infrequent occurrence of this unique *JAK2* mutation has been reported in chronic myelomonocytic leukemia (CMML), atypical or unclassified myeloproliferative disorder (MPD), myelodysplastic syndrome (MDS), systemic mastocytosis (SM), and chronic neutrophilic leukemia (CNL) [10-16]. A *JAK2*^{V617F} allele burden (AB) above 50% identifies patients with a higher thrombotic risk, both in ET and in PV [17-22]. Conversely, a low AB seems to correlate with significantly shortened survival and leukemia-free survival in MF [23-25]. As a result, the determination of the mutation load is becoming a standard diagnostic procedure in most molecular laboratories, though WHO criteria do not specify a cut-off value for the diagnosis of a MPN. In the last years, the extensive and generalized use of molecular techniques with high sensitivity, specifically allele-specific Real-Time Quantitative Polymerase Chain Reaction (RQ-PCR), has significantly increased our ability to detect small mutated clones, with AB below 1% [26-32]. Many recent studies have shown that a small clonal hematopoiesis may be present also in otherwise healthy subjects at low level (0.03-1%) [33-38]. Accordingly, a study of the Myeloproliferative Neoplasms and Related Disorders European Network (MPN&MPNr-EuroNet) on 36 subjects carrying low *JAK2*^{V617F} AB has further suggested that the detection of a small *JAK2*^{V617F}-mutated clone cannot represent a sufficient evidence to establish malignant myeloproliferation [39]. As a result, the clinical interpretation of a low AB of the *JAK2*^{V617F} mutation may be challenging.

Here, we analyzed the results of *JAK2*^{V617F} molecular tests performed at our Hematology Department in subjects with a suspected hematological disease over a 2-year period.

Specifically, we analyzed clinical and laboratory data of patients with a suspected MPN that presented a low (≤3%) *JAK2*^{V617F} mutation burden, with the aim to define the frequency and the significance of a low AB in the everyday clinical management.

RESULTS

Study plan

We tested 1087 subjects for *JAK2*^{V617F} mutation due to clinical suspicion of hematological malignancy. A total of 716 (65.9%) out of 1087 tests were performed due to a suspect of classical MPN, including ET (299 cases, 41.8%), PV (272 cases, 38%), MF (133 cases, 18.6%) and MPN underling atypical splanchnic vein thrombosis (12 cases, 1.6%). The remaining 371 (34.1%) out of 1087 tests were performed on suspicion of Myeloproliferative Disease (MPDs; 23 cases) or other causes/not specified (348 cases) (data not shown).

Figure 1 depicts the study population and the study plan. Overall, 497 (45.7%) of the 1087 subjects that were tested for the *JAK2*^{V617F} mutation resulted positive for the mutation.

A total of 455/497 (91.5%) patients had an AB >3% (IC 95%: 88.75-93.71, p =0.05): 52 (11.4%) were tested because of erythrocytosis, 186 (40.9%) because of thrombocytosis and 217 (47.7%) for other causes (specifically 94 suspicion of MF, 3 MPN underling atypical thrombosis and 120 others/not specified). Overall, the final diagnosis of these patients was: WHO-defined ET (91/186, 48.9%), probable ET (95/186, 51.1%), PV (26/52, 50%), probable PV (50%).

The remaining 42/497 (8.5%) patients had a low (0.1-3%, median 0.59%) AB (IC 95%; 0.62-1.15, p =0.05). In most cases (30 patients, 71.4%), AB was below 1%, while only 8 (28.6%) patients had an AB above 2%. Overall, the final diagnosis of these patients was: WHO-defined MPDs (7 cases), WHO-defined ET/PMF-0 (11/19, 57.9%), probable ET (3/19, 15.8%), reactive thrombocytosis (5/19, 26.3%), PV (8/14, 57.1%), secondary erythrocytosis (6/14, 42.9%).

Here we present clinical and laboratory parameters of this cohort of 42 patients with an AB ≤3%.

Low AB patients suspected of MPD

By bone marrow (BM) histology, 7/42 (16.7%) patients had a confirmed diagnosis of a hematological disease that not included classical MPNs, specifically: refractory cytopenia with unilineage dysplasia (RCUD, 2 cases); refractory cytopenia with multilineage dysplasia (RCMD, 1 case); refractory anemia with excess blasts-1 (RAEB-1, 1 case); MDS with isolated del(5q) (1 case); chronic myelomonocytic leukemia (CMML, 2 cases). Median AB of these patients was 0.46% (range, 0.12-2.79%). No additional mutations in *JAK2* exon 14, *CALR* and *MPL* genes were detected in these 7 patients.

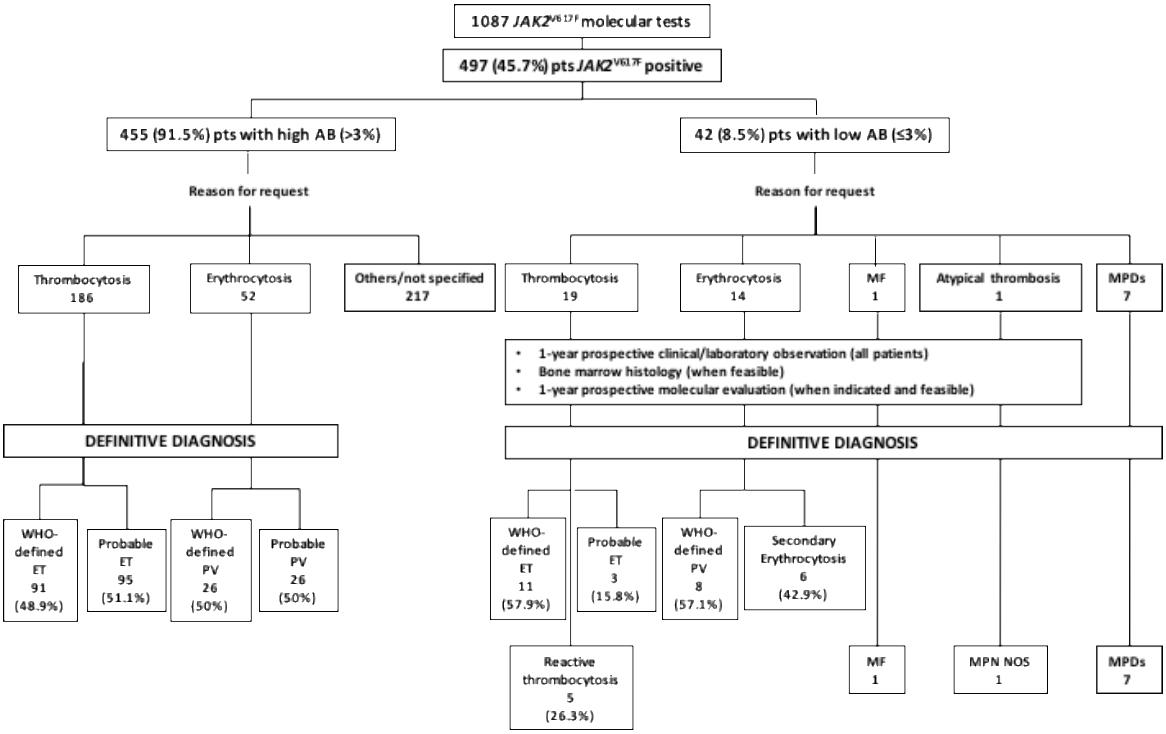

Figure 1: Schematic representation of the study population and the study plan. ET: Essential Thrombocythemia; PV: Polycythemia Vera; MF: primary Myelofibrosis; MPN NOS: Myeloproliferative Neoplasm Not Otherwise Specified; MPDs: Myeloproliferative Diseases.

Low AB patients suspected of MPN

Diagnostic workflow of the 35/42 patients with low AB and referred to our Institutions with a suspicion of classical MPNs is shown in Figure 2. Specifically, 19 patients were evaluated for suspected ET, 14 for probable PV, 1 for probable MF and 1 for MPN underling atypical thrombosis. Consequently, they were prospectively followed over time with clinical/laboratory data. In 2/35 cases, BM biopsy confirmed the suspect of a primary MF or MPN not otherwise specified (NOS) in one subject tested after a splanchnic vein thrombosis.

Prospective molecular monitoring was performed in 15/35 low AB patients at a 12-month follow-up A significant increase of *JAK2*^{V617F} AB over time was observed in 11 cases ($p < 0.05$). Indeed, the median value of *JAK2*^{V617F} AB at diagnosis and during follow-up was 0.49% (range, 0.12-2.98) and 1.3% (range, 0.28-9.2), respectively. In the remaining 4 cases, a slight decrease of *JAK2*^{V617F} AB over time was registered (Figure 3).

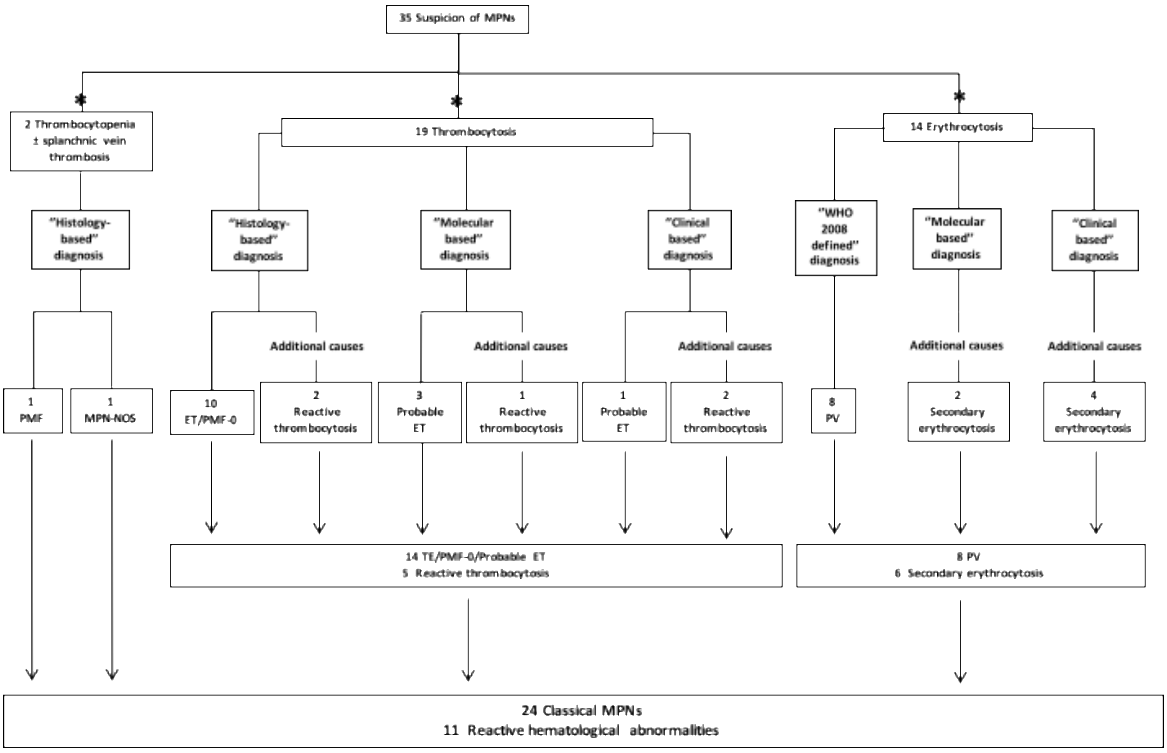

Figure 2: Diagnostic work ow of patients with suspected MPN and low *JAK2*^{V617F} allele burden. *Main hematological abnormality motivating the *JAK2*^{V617F} evaluation. ''Histology-based'' diagnosis was made when BM histology was available. In patients that were unable or refused to perform BM evaluation, prospective clinical observation and prospective molecular monitoring were crucial to direct diagnostic uncertainty, allowing to define a ''Clinical based'' or ''Molecular based'' diagnosis, respectively.

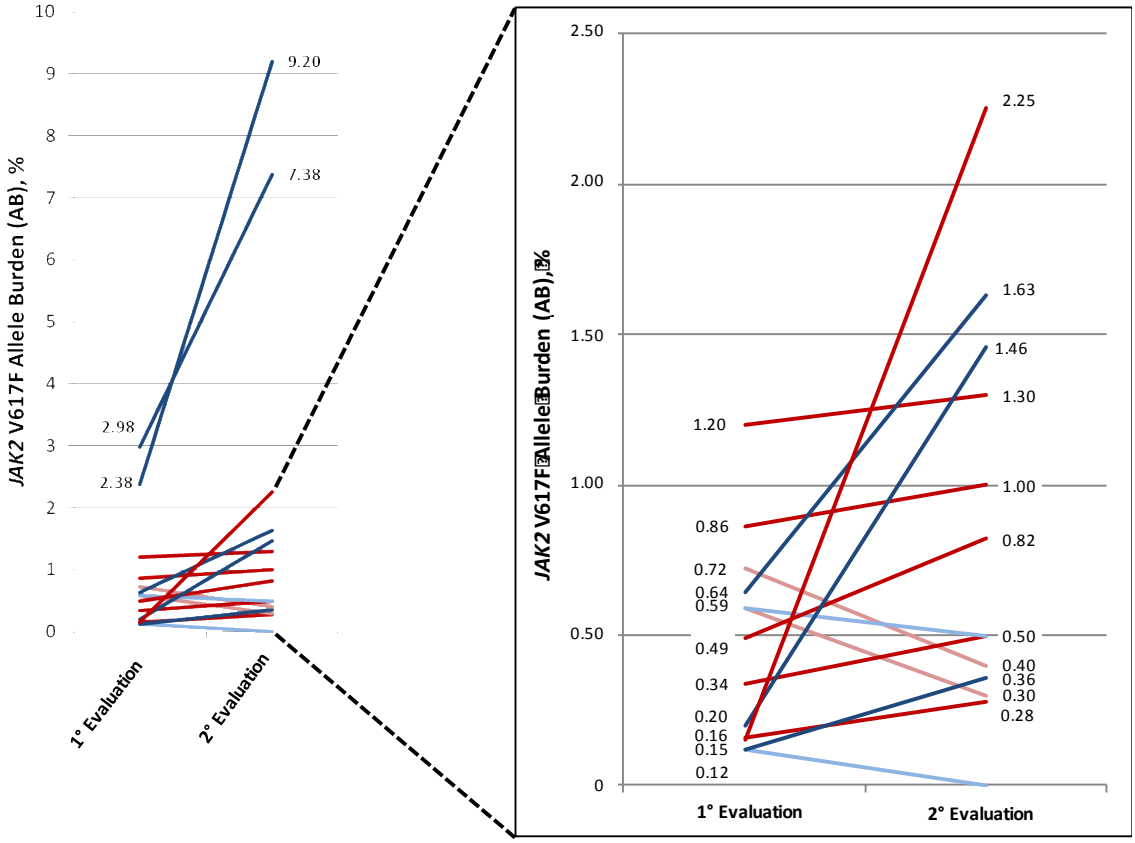

Figure 3: *JAK2*^{V617F} allele burden over time in patients with suspected Essential Thrombocytemia and Polycythemia Vera. Fifteen patients received the second evaluation of *JAK2*^{V617F} allele burden after a period of 12 months from the first mutational test. Dark red line: final diagnosis of PV. Light red line: final diagnosis of secondary polyglobulia. Dark blue line: final diagnosis of ET. Light blue line: final diagnosis of secondary thrombocytosis.

Among the 19/35 (54.3%) subjects with low AB that received *JAK2*^{V617F} evaluation for thrombocytosis, 14 (73.7%) patients finally had a clinical diagnosis of MPN (Table 1). In 10 cases, the diagnosis was confirmed by BM histology (patients T1-T8, T10 and T12). In one case, an additional Type 1 *CALR* mutation was detected with an AB of 42% (patient T9). Conversely, three additional patients refused or were unable to perform BM biopsy, therefore were classified as ''Probable ET'' because presented a persistent thrombocytosis in absence of other causes (patients T11,T13-T14). In two of these patients (T11 and T14), *JAK2*^{V617F} mutation load was reassessed after a 12-month follow-up and an increased AB was observed, corroborating the diagnosis of MPN (Table 1 and Figure 3); in the remaining case (T13), prospective molecular evaluation was not performed because cytoreductive therapy was already ongoing. Indeed, despite the absence of BM biopsy, cytoreductive therapy was administered according

to standard criteria for treatment start (e.g.: age>60 years and/or previous thrombosis and/or massive thrombocytosis). In the remaining 5 patients (patients T15-T19), thrombocytosis was transitory and was finally considered as secondary to an inflammatory disease (erysipelas, 1 case; rheumatoid arthritis, 2 cases) or to iron deficiency (2 cases). In two of these cases, BM biopsy excluded a hematological disease (patients T18 and T19). Accordingly, a decrease of AB was observed at second evaluation in patients T17 and T18.

The 14 patients with a final diagnosis of MPN (including WHO-defined ET and "probable ET") showed higher hemoglobin level (median (range): 13.6 (10.9-16.2) *vs* 10.3 (7.2-12.1) g/dl) and lower leukocyte count (median (range): 7.4 (4.2-13.3) *vs* 11.3 (8.2-21) x109/l) compared to the 5 patients with reactive thrombocytosis ($p < 0.001$ and $p = 0.05$, respectively). Conversely, platelet count was similar in the two groups (median (range): 650 (161-1000) *vs* 587 (490-701) x109/l; p =N.S.) (data not shown). The mutation load was significantly higher in MPN patients than in subjects finally diagnosed with reactive thrombocytosis (median (range): 1.18 (0.15-2.98) *vs* 0.34 (0.12-0.61) %; $p = 0.02$). Overall, no patient with reactive thrombocytosis showed an AB ≥0.64% (Table 1).

Among the 14/35 (40.0%) patients with low AB that performed mutational analysis due to erythrocytosis, 8 (57.1%) had a final diagnosis of MPN (Table 2). PV diagnosis was sustained by absence of other causes of polyglobulia, *JAK2*^{V617F} positivity and reduced baseline erythropoietin (EPO) levels (patients E1-E8). In patients E1-E3 and E6-E8, the diagnosis was also confirmed by the detection of an increased *JAK2*^{V617F} AB at a 12-month molecular follow-up. In the remaining 6 cases, the hematological abnormality was finally classified as secondary erythrocytosis (patients E9-E14) due to kidney cancer with increased endogenous EPO levels (patient E9) and chronic obstructive pulmonary disease (COPD, 5 patients: E10-E14). In two of these patients, the diagnosis was supported by a decrease in the *JAK2*^{V617F} AB over time (patients E13 and E14) (Table 2 and Figure 3). In the majority of these patients with diagnosis of secondary erythrocytosis, phlebotomy and/or aspirin were administered after consideration of the causes of polyglobulia and the overall thrombotic risk of the patients [40-42]. No additional mutations were found in none of the patients investigated for erythrocytosis.

The 8 patients with a diagnosis of PV showed higher hemoglobin level compared to the 6 patients with secondary polyglobulia (median (range): 18.8 (17.4-20.1) *vs* 16.9 (16.2-19.7) g/dl; $p = 0.04$), but presented comparable leucocyte (median (range): 7.3 (5.4-10.9) *vs* 6.2 (4.5-

9.4) x109/l; p =N.S.) and platelet counts (median (range): 250 (186-703) *vs* 235 (166-283) x109/l; p =N.S.) (data not shown). Median *JAK2*^{V617F} AB was also similar in the two groups (median (range): 0.42 (0.16-2.25) vs 0.49 (0.12-0.74) %; p =N.S.). Nonetheless, 75% of PV patients carried a mutation load above the median value of 0.44% (*vs* 16.7% in patients with secondary erythrocytosis, p =0.03) (Table 2).

As expected, low AB patients investigated for a suspected PV showed significantly higher median hemoglobin levels (median (range): 18.2 (16.2-20.1) *vs* 12.9 (7.2-16.2) g/dl; p <0.001) and lower median platelet count (median (range): 238 (166-703) *vs* 638 (161-1000) x109/l; p <0.001) compared to patients with suspected ET (data not shown). Conversely, median *JAK2*^{V617F} burden was similar in the two cohorts both considering all evaluated patients (median (range): 0.44 (0.12-2.25) *vs* 0.64 (0.12-2.98) %; p =N.S.) and only patients with final diagnosis of PV and ET/early-PMF/MPN NOS/probable ET (median (range): 1.18 (0.16-2.25) *vs* 0.42 (0.15-2.98) %; p =N.S.) (Table 1 and 2).

Overall, in 11 out of 35 (31.4%) patients the detection of a low *JAK2*^{V617F} AB was considered insufficient to make a diagnosis of MPN (Figure 2). In 6 cases, the exclusion of MPN relied only on clinical monitoring over time, with the observation that the hematological abnormalities were transient and dependent on other contributing factors. In 2 cases BM histology revealed no signs of MPNs (patients T18 and T19), and in 3 additional patients the *JAK2*^{V617F} mutation load spontaneously decreased over time (patients T17, E13 and E14). The probability to have a final hematological diagnosis was higher in patients tested for thrombocytosis who received a diagnosis of ET/early-PMF in 73.7% of the cases (vs 57.1% of patients with polyglobulia finally diagnosed with PV). In the former cohort, BM biopsy was fundamental for diagnosis in 62.9% of the cases; also, the detection of a concomitant *CALR* mutation was decisive in confirming ET diagnosis in one patient. Conversely, in patients tested for polyglobulia BM biopsy was never performed, thus limiting the diagnostic accuracy. However, in 15 cases the repetition of the *JAK2*^{V617F} mutation load over time was of remarkable help in the diagnostic process. Nonetheless, in the low AB cohort, the probability to carry a hematological disease directly correlated with a higher mutation load, since all patients with a *JAK2*^{V617F} AB >0.8% were finally diagnosed with a MPN. In order to exclude under-estimation of the *JAK2*^{V617F} AB caused by hampering correct primer or probe annealing, additional mutations in *JAK2* exon 14 were investigated; however, they were not detected in any patient.

Finally, regarding clinical outcome, the frequency of thrombosis was not significantly different according to *JAK2*^{V617F} AB, in both ET and PV. In addition, when we analyzed the distribution of low and high AB patients according to risk categories, we did not find any significant correlation between the two parameters (data not shown).

Patient	Age, gender	Prior Thrombosis (Y/N)	Plt count (>1000/ul)	BM histology	*JAK2*^{V617F} AB, % 1st evaluation	*JAK2*^{V617F} AB, % 2nd evaluation	CALR	*Fulfiled 2008 WHO diagnostic criteria (Y/N)*	Final diagnosis	Therapy	Status at last follow-up
T1	20, F	N	Y	Y	0.12	0.36	WT	Y	ET	IFN	PLT < 400
T2	46, M	Y	Y	Y	0.79	-	WT	Y	ET	IFN	PLT < 400
T3	33, F	N	Y	Y	0.20	1.46	WT	Y	ET	-	PLT < 1000
T4	25, F	N	Y	Y	2.37	-	WT	Y	ET	ASA	PLT <1000
T5	62, M	Y	Y	Y	2.98	7.38	WT	Y	ET	HU, ASA	PLT < 600
T6	17, M	N	Y	Y	3.00	-	WT	Y	ET	-	PLT <1000
T7	50, F	N	Y	Y	0.43	-	WT	Y	Early-PMF	IFN	PLT < 400
T8	31, M	N	Y	Y	2.36	-	WT	Y	Early-PMF	IFN	PLT < 400
T9	46, M	N	Y	N.A.	0.56	-	Type1 (42%)	N (absence of BM biopsy)	ET	HU	PLT < 600
T10	38, F	N	Y	Y	0.41	-	WT	Y	ET	HU	PLT < 1000
T11	57, F	N	Y	N.A.	0.64	1.63	WT	N (absence of BM biopsy)	Probable ET	ASA	PLT < 1000
T12	66, M	N	Y	Y	1.05	-	WT	Y	ET	HU, ASA	PLT < 600
T13	71, F	N	Y	N.A.	1.56	-	WT	N (absence of BM biopsy)	Probable ET	ASA	PLT < 400
T14	91, F	Y	Y	N.A.	2.38	9.20	WT	N (absence of BM biopsy)	Probable ET	HU, ASA	PLT <400
T15	72, F	Y	Y	N.A.	0.32	-	WT	N (absence of BM biopsy, evidence of reactive thrombocytosis)	Reactive (reumatoid arthritis)	-	PLT < 600
T16	81, M	N	Y	N.A.	0.34	-	WT	N (absence of BM biopsy, evidence of reactive thrombocytosis)	Reactive (reumatoid arthritis)	-	PLT < 600
T17	43, F	N	Y	N.A.	0.59	0.50	WT	N (absence of BM biopsy, evidence of reactive thrombocytosis)	Reactive (iron deficency)	IRON THERAPY	PLT < 400
T18	25, F	N	Y	Y (normal)	0.12	WT	WT	N (normal BM histology, evidence of reactive thrombocytosis)	Reactive (erysipelas)	-	PLT < 400
T19	35, F	N	Y	Y (normal)	0.61	-	WT	N (normal BM histology, evidence of reactive thrombocytosis)	Reactive (iron deficency)	-	PLT < 600

Table 1. Main baseline characteristics and clinical outcome of patients investigated for thrombocytosis. Patients with persistent thrombocytosis in absence of other causes that did not perform BM biopsy for histological confirmation were classified as ''Probable ET''. PLT: platelet (x10^9/l); BM: bone marrow; N.A.: not available; ET: Essential Thrombocythemia; Early-PMF: early-primary myelofibrosis; WT: wild-type; HU: hydroxyurea; IFN: interferon-alpha; ASA: low-dose aspirin. Patient 19 had received splenectomy for a previous diagnosis of immune thrombocytopenia. Only in 5 cases, lactate dehydrogenase (LDH) was elevated. No patients had splenomegaly.

Patient	Age, gender	Prior Thrombosis (Y/N)	Hb ≥ 18.5 g/dl (males) or Hb ≥ 16.5 g/dl (females) (Y/N)	*JAK2*^{V617F} AB, % 1st evaluation	*JAK2*^{V617F} AB, % 2nd evaluation	Reduced EPO levels	*Fulfilled 2008 WHO diagnostic criteria (Y/N)*	Final diagnosis	Therapy	Status at last follow-up
E1	54, M	N	Y	0.15	2.25	Y	Y	PV	ASA, PHLEBOTOMY	Hct control < 45%
E2	63, M	N	Y	0.16	0.28	Y	Y	PV	ASA, PHLEBOTOMY	Hct control < 45%
E3	58, M	Y	Y	0.34	0.50	Y	Y	PV	NONE	Hct control < 45%
E4	50, M	Y	Y	0.16	-	Y	Y	PV	HU, ASA, PHLEBOTOMY	Hct control < 45%
E5	70, M	N	Y	2.23	-	Y	Y	PV	HU, ASA, PHLEBOTOMY	Hct control < 45%, PLT< 600
E6	81, M	Y	N	1.20	1.30	Y	No (Hb ≤ 18.5)	PV	HU, ASA, PHLEBOTOMY	Hct control < 45%
E7	65, M	N	N	0.86	1.00	Y	No (Hb ≤ 18.5 & EPO levels not available)	PV	ASA, PHLEBOTOMY	Hct control < 45%
E8	41, M	Y	N	0.49	0.82	Y	No (Hb ≤ 18.5 & EPO levels not available)	PV	ASA, PHLEBOTOMY	Hct control < 45%
E9	59, M	N	Y	0.12	-	N	No (increased EPO levels)	Secondary (kidney carcinoma)	ASA, PHLEBOTOMY	Deceased (kidney carcinoma)
E10	67, M	Y	N	0.16	-	N	No (Hb ≤ 18.5 & normal EPO levels)	Secondary (COPD)	ASA, PHLEBOTOMY	Hct control < 48%
E11	54, M	Y	N	0.39	-	N.A.	No (Hb ≤ 18.5 & EPO levels not available)	Secondary (COPD)	PHLEBOTOMY	Hct control < 48%
E12	82, F	N	N	0.74	-	N.A.	No (Hb ≤ 18.5 & EPO levels not available)	Secondary (COPD)	NONE	Hct control < 48%
E13	77, M	Y	N	0.59	0.30	N	No (Hb ≤ 18.5 & normal EPO levels)	Secondary (COPD)	ASA, PHLEBOTOMY	Hct control < 48%
E14	72, M	N	N	0.72	0.40	N	No (Hb ≤ 18.5 & normal EPO levels)	Secondary (COPD)	ASA, PHLEBOTOMY	Hct control < 48%

Table 2. Main baseline characteristics and clinical outcome of patients investigated for erythrocytosis. Hb: hemoglobin (g/dl); Hct: hematocrit; N.A.: not available; HU: hydroxyurea; ASA: low-dose aspirin; COPD: chronic obstructive pulmonary disease.

DISCUSSION

The present study investigated the role of a low *JAK2*^{V617F} AB in a cohort of subjects that received the molecular test in the suspect of MPN. All mutational studies were performed according to international recommendations over a 1-year period and patients were homogeneously followed at a single Institution. BM histology was always decisive to direct diagnosis. When histology was unavailable, molecular monitoring together with clinical observation were utmost importance.

Among the 42 patients with low (0.1-3%) *JAK2*^{V617F} AB, 7 had a confirmed diagnosis of non-classical MPNs by BM histology, 11 were classified as reactive hematological abnormalities due to the presence of additional causes of thrombocytosis (5 cases) or polyglobulia (6 cases) whereas 24 received a diagnosis of classical MPNs. *JAK2*^{V617F} mutation represents a non-driver and subclonal event in non-classical MPNs, occurring most frequently with low mutation burden [11]. As a consequence, the percentage of subjects with an AB ≤3 that were finally diagnosed as MPDs was higher (7/23, 30.4%) compared to MPNs 24/716 (3.3%).

Also, the present study demonstrates that within low AB patients a higher mutation load is associated with a higher probability to receive a hematological diagnosis, with an AB ≥0.8% always corresponding to an overt MPN phenotype. Moreover, while higher hemoglobin levels significantly correlated with a diagnosis of WHO-defined PV, platelet count was similar in patients with or without a final diagnosis of ET/early-PMF, and was therefore not indicative per se of a hematological disorder. Another interesting observation is that histology, when performed, was diagnostic for a full-blown disease regardless of the *JAK2*^{V617F} AB. This not only further confirms the central role of histology in MPN diagnosis, distinguishing true MPNs by small foci of clonal hematopoiesis that are not of clinical significance, but also indicate that the morphologic pattern is not strictly driven by the molecular aberrancy. As a result, evaluation of BM histology could be useful especially for patients with a suspicion of PV and an AB below 0.8% to confirm MPNs diagnosis. Accordingly, revised WHO classifications for myeloproliferative neoplasm indicated BM morphology as one of three major diagnostic criteria, also in PV [43]. Nonetheless, BM biopsy is not feasible in all patients, because of older age and/or patients' refusal; also, the material may result inadequate for a correct histology evaluation. In these cases, the repetition of the molecular test over time timely detected the spontaneous expansion (11 cases) or reduction (4 cases) of the *JAK2*^{V617F} -positive clones and significantly oriented the diagnostic process. Finally, the co-existence of additional *CALR/MPL* or *JAK2* exon 14 and exon 12 mutations was routinely excluded in all cases of thrombocytosis or polyglobulia, respectively, that present with a low *JAK2*^{V617F} AB [44]. Accordingly, many recent studies have demonstrated that *CALR* and *MPL* mutations may co-exist with *JAK2* mutations in chronic MPNs/MDS [45,46].

From the biological point of view, our clinical results seem to sustain the hypothesis that a low *JAK2*^{V617F} AB reflects the presence of a small mutated clone within an overall polyclonal hematopoiesis. Indeed, it was recently demonstrated that patients with early stage hematological malignancy may harbor distinct clones, and that such clones may arise independently [47]. Alternatively, we can also draw the assumption that, depending on whether the oncogenic hit marks the stem or the progenitor's compartments, the mutation load might be more or less enlarged. In any case, this mutational event represents an early molecular onset and is probably not sufficient per se to induce the malignant MPN phenotype. Consequently, the detection of low AB in one single occasion is likely not appropriate to determine the diagnosis. Therefore, the molecular monitoring over time may allow knowing

whether this is a temporary clone (a condition which is likely to occur in healthy subjects) or may expand and give origin to the disease.

In conclusion, our results highlight that the detection of a *JAK2*^{V617F} mutation at low levels is difficult to be interpreted in everyday clinical practice, since not all positive patients received a hematological diagnosis. However, all patients with an AB ≥0.8% finally received a diagnosis of MPN; therefore, a mutation load above this cut-off may be considered very indicative for the presence of a myeloproliferative disease. Additionally, the study identified the prospective evaluation of *JAK2*^{V617F} mutation load as a convenient and non-invasive method to evaluate patients with small mutated clones in order to timely detect the expansion of clonal hematopoiesis and diagnose a full blown disease. The study should require validation in larger cohorts of patients prospectively examined with standardized molecular methods.

MATERIALS AND METHODS

Study population

Between January 2013 and January 2015, 1087 *JAK2*^{V617F} mutational studies were performed at the Institute of Hematology "L. e A. Seràgnoli", Bologna. The clinical suspicions that motivated the request for *JAK2*^{V617F} evaluation were: essential thrombocytemia (ET) (299 cases, 27.5%); polycythemia vera (PV) (272 cases, 25.1%); myelodysplasia (MDS) (23 cases, 2.1%); atypical thrombosis (12 cases, 1.1%); myelofibrosis (MF) (133 cases, 12.2%); others/not specified (348 cases, 32.0%) (Figure 1). Clinical and laboratory data of patients with a suspected ET, PV or MF and an allele burden ≤3% were prospectively monitored for 1 year. All patients provided an informed written consent in accordance with the Declaration of Helsinki for the use of remnant DNA for investigational purposes. The study was approved by the local Ethics Committee.

Patient samples

Polymorphonuclear cells were isolated from peripheral blood samples by density centrifugation with Polymorphoprep (Axis-Shield, Scotland) [48-50]. Genomic DNA was extracted using the QIAamp DNA Blood Mini kit (QIAGEN-Werfen) on QIAcube (QIAGEN GmbH, Hilden, Germany) and was quantified with the NanoDrop spectrophotometer (Wilmington, DE, USA).

Molecular evaluations

Molecular analyses were assessed at diagnosis or before treatment start on DNA obtained from granulocytes. When clinical, laboratory and/or histological data were not decisive for the diagnosis of MPN, mutational status was prospectively evaluated at a 12-month interval. A second *JAK2*^{V617F} molecular test was not performed in patients that had already started cytoreductive therapy due to high thrombotic risk. *JAK2*^{V617F} mutation was evaluated with ipsogen *JAK2* MutaQuant Kit, which is based on allele specific real time quantitative polymerase chain reaction (qPCR) technology on 7900 HT Fast Real Time PCR System (Applied Biosystem) [21]. The percentage of mutant *JAK2*^{V617F} allele was expressed as the ratio of *JAK2*^{V617F} copies to total copy number (CN) of *JAK2* (CN of *JAK2*^{V617F} + CN of *JAK2* wild type). Even if the lower detection limit (LOD) of the assay was 0.01% (See Supplemental File), we identified the 0.1% as cut-off of positivity as also suggested by several studies [23-24, 27-29]. All samples were tested in duplicate with both qPCR and also with digital PCR (ddPCR) to confirm the evaluation [51-52] (See Supplemental File). In addition to *JAK2*^{V617F} mutation, *CALR* and *MPL* mutations were screened for all patients to obtain a comprehensive molecular characterization and to exclude the co-existence of additional mutations [44-46, 53-54]. *CALR* exon 9 sequencing was performed by Next Generation Sequencing (NGS) approach with GS Junior (Roche 454 platform); analysis was carried out with AVA software (GRCh38 as references). *CALR* mutations identified by NGS were confirmed by Sanger sequencing. *MPL* mutations were investigated by ipsogen *MPL*W515K/L MutaScreen Kit and by Sanger sequencing (for *MPL*S505N and other secondary exon 10 mutations), as previously described [55]. Additional masking mutations in *JAK2* exon 14 were investigated by Sanger sequencing [39]. In case of clinical suspicion of PV, *JAK2* exon 12 mutations were also tested by Denaturing High Pressure Liquid Chromatography (DHPLC) and confirmed by Sanger sequencing [55-58]. Diagnoses of all hematological diseases were made according to the WHO2008 criteria [1].

Statistical methods

Numerical variables have been summarized by their median and range, and categorical variables by count and relative frequency (%) of each category. Comparisons of quantitative variables between groups of patients were carried out by the nonparametric Wilcoxon rank-sum test. Association between categorical variables (2-way tables) was tested by the Fisher

exact test or χ2, as appropriate. All p values were two-sided and statistical significance was defined as $p < 0.05$. All statistical analyses were computed with SPSS software (SPSS Inc., Chicago, IL, USA).

AUTHOR CONTRIBUTIONS

F.P., M.P., N.P., G.M. designed the study and wrote the paper. L.C., D.F., G.S., C.P., M.C., N.V. contributed to the data collection and interpretation. M.P., E.O., E.Z., E.F. performed molecular evaluations. E.S. performed bone marrow analysis. All Authors revised the manuscript critically, and gave final approval to submit for publication.

CONFLICT OF INTEREST

There is no conflict of interest.

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Supplementary Data

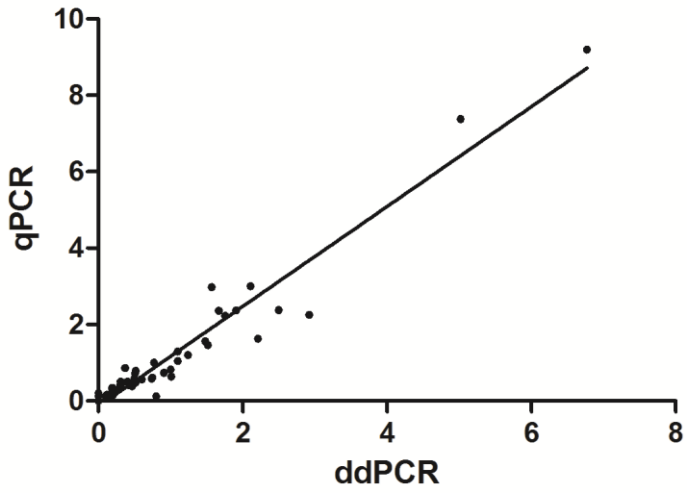
The relevance of a low *JAK2*^{V617F} allele burden in clinical practice: a monocentric study

RESULTS

Detection and quantitation of *JAK2*^{V617F} AB by both qPCR and ddPCR

Overall, 48 samples (18 at diagnosis and 15 both at diagnosis and during the follow-up) were tested for *JAK2*V617F with both qPCR and ddPCR. The median mutation burden obtained was 0.59% (range: 0–9.2%) and 0.52% (range: 0–6.77%), respectively.

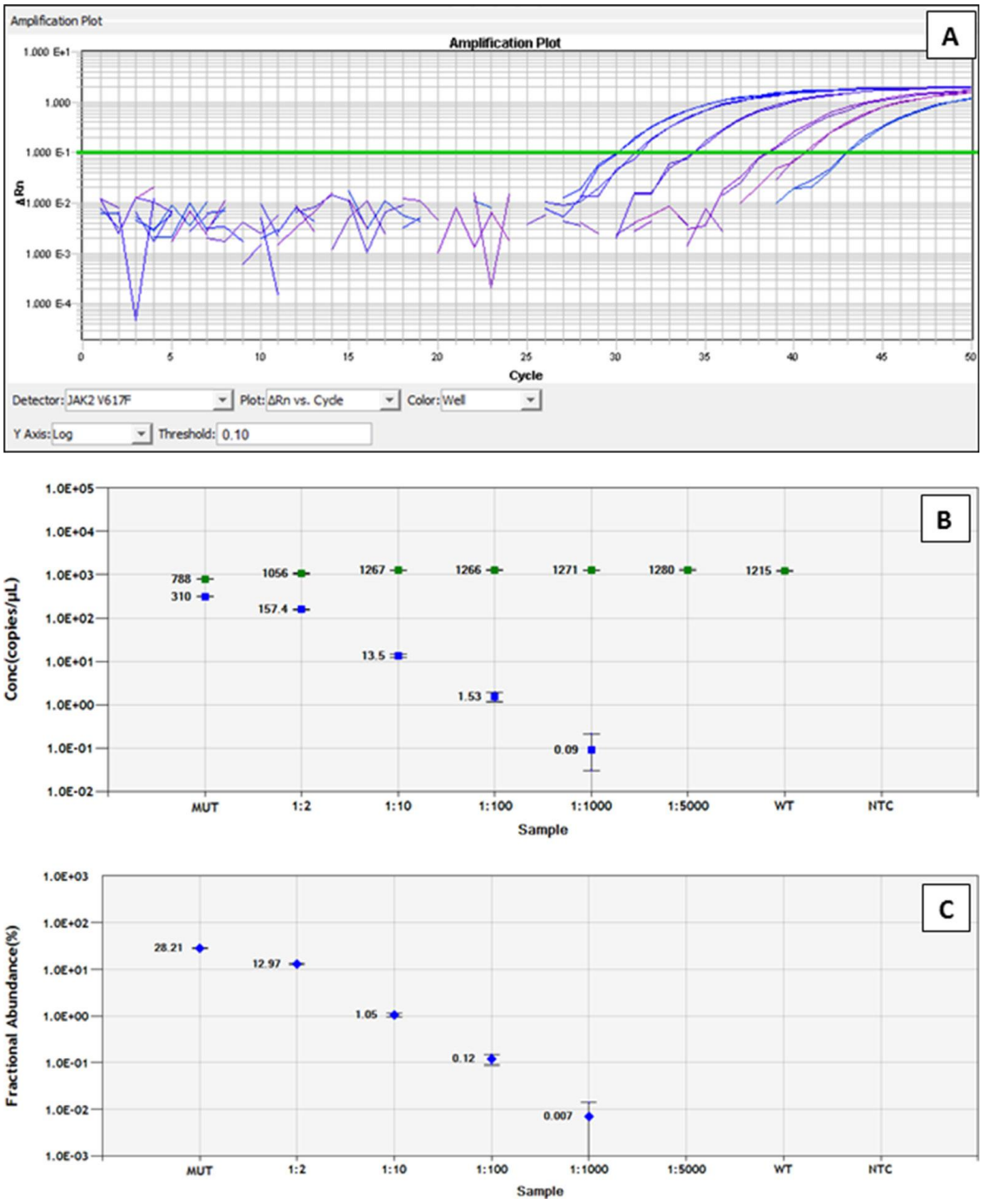
As the AB did not follow a normal distribution, Spearman's rho test was used for correlation analysis. As shown in Figure S1, there was a high degree of correlation between data obtained with the two methods (R = 0.924; p < 0.0001).



Supplementary Figure S1- Correlation between qPCR and ddPCR. Each point represents a sample where *JAK2*^{V617F} AB has been evaluated, according to qPCR (Y-axis) and ddPCR (X-axis). The calculated Spearman's rho value is 0,924 (p <0,0001).

Limit of detection (LOD) determination

As shown in Supplementary Figure 2, both the qPCR and the ddPCR achieved a sensitivity of 0.01% .



Supplementary Figure S2- Limit Of Detection (LOD) determination. Ipsogen JAK2 MutaQuant kit (qPCR) (A) and ddPCR (B-C) sensitivity was assessed by quantifying a serial dilution of a mutant genomic DNA into a wild-type DNA. For simplicity, only selected dilutions of the entire standard curve (prepared as described in the Methods) are shown. (A) Amplification plot of mutant genomic DNA serial dilutions (Mut, 1:2, 1:10, 1:100, 1:1000, and WT, respectively). (B) The blue dots indicate the number of mutated copies/µl; the green dots indicate the number of wild-type copies/µl. (C) Fractional abundance plot shows the frequency of the mutant DNA into total genomic DNA.

MATERIALS AND METHODS

Digital PCR

In order to demonstrate repeatability of qPCR results, all samples were tested also with digital PCR (ddPCR) assay. ddPCR was performed using the specific PrimePCR ddPCR Mutation Detection Kit Assay for JAK2 wild-type and the V617F mutation on the QX200 platform (BioRad, Milan, Italy). The data analysis was performed using the QuantaSoft analysis software and results were expressed as the fractional abundance (FA) of mutant (a) to wild type (b) template (a/a+b).

A comparison between qPCR and ddPCR was performed through a nonparametric correlation analysis (Spearman's rho).

Analytic sensitivity test

The Limit Of Detection (LOD) is defined as the lowest mutant concentration that can be consistently detected above the limit of blank.

In order to compare the ability of both qPCR and ddPCR of detecting diluited mutant alleles, a sensitivity curve was performed. We prepared serial dilutions (1:2, 1:10, 1:100, 1:1000, 1:5000, 1:10000) of an already quantitated *JAK2*^{V617F} mutant genomic DNA into a genomic wild-type DNA. We include also "WT only" control wells and no template control (NTC) wells. All the dilutions were tested in duplicate.

RESULT III

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Assessment of the interlaboratory variability and robustness of *JAK2^{V617F}* mutation assays: A study involving a consortium of 19 italian laboratories

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ABSTRACT

To date, a plenty of techniques for the detection of *JAK2*^{V617F} is used over different laboratories, with substantial differences in specificity and sensitivity. Therefore, to provide reliable and comparable results, the standardization of molecular techniques is mandatory.

A network of 19 centers was established to 1) evaluate the inter- and intra- laboratory variability in *JAK2*^{V617F} quantification, 2) identify the most robust assay for the standardization of the molecular test and 3) allow consistent interpretation of individual patient analysis results. The study was conceived in 3 different rounds, in which all centers had to blindly test DNA samples with different *JAK2*^{V617F} allele burden (AB) using both quantitative and qualitative assays.

The positivity of samples with an AB < 1% was not detected by qualitative assays. Conversely, laboratories performing the quantitative approach were able to determine the expected *JAK2*^{V617F} AB. Quantitative results were reliable across all mutation loads with moderate variability at low AB (0.1 and 1%; CV = 0.46 and 0.77, respectively). Remarkably, all laboratories clearly distinguished between the 0.1 and 1% mutated samples.

In conclusion, a qualitative approach is not sensitive enough to detect the *JAK2*^{V617F} mutation, especially at low AB. On the contrary, the ipsogen *JAK2* MutaQuant CE-IVD kit resulted in a high, efficient and sensitive quantification detection of all mutation loads. This study sets the basis for the standardization of molecular techniques for *JAK2*^{V617F} determination, which will require the employment of approved operating procedures and the use of certificated standards, such as the recent WHO 1st International Reference Panel for Genomic *JAK2*^{V617F}.

INTRODUCTION

The *JAK2*^{V617F} mutation represents a hallmark of Philadelphia (Ph)- negative myeloproliferative neoplasms (MPNs), fulfilling a 2008 World Health Organization (WHO) major criterion for the diagnosis of MPNs [1–2]. The *JAK2*^{V617F} mutation is an acquired, somatic mutation carried by almost all patients (approximately 95%) with polycythemia vera (PV) and in more than half (approximately 50–60%) of those with essential thrombocythemia (ET) or primary myelofibrosis (PMF) [3, 4].

The assessment of the *JAK2*^{V617F} allele burden (AB) is a common practice either at diagnosis, for prognostic information, or during treatment as a means to assess minimal residual disease [5]. Indeed, *JAK2*^{V617F} AB seems to be correlated with an increased risk of thrombosis and evolution in a secondary myelofibrosis in PV (PPV-MF) and, possibly, in ET (PET-MF) [6,7]. Additionally, low AB is associated with a reduced survival in PMF [8–11]. With regard to drug therapy, several studies showed that interferon-alpha, and the most recent telomerase inhibitors (Imetelstat), significantly reduces *JAK2*^{V617F} mutation burden, whereas, *JAK* inhibitors and hydroxyhurea (HU) did not have any significant effects [12–21]. Moreover, *JAK2*^{V617F} quantification has been incorporated as a potentially useful tool to predict relapse in those patients who underwent allogeneic stem-cell transplantation (alloHSCT). In this setting of patients, early monitoring of the AB (1, 3 and 6 months post alloHSCT) is crucial to predict overall survival and risk of relapse and might guide therapeutic decisions [22–24].

To date, a plenty of techniques for *JAK2*^{V617F} determination is used over different laboratories, with substantial differences in specificity and sensitivity [5, 25–31]. The extensive and worldwide use of molecular techniques with high sensitivity has significantly increased our ability to detect small mutated clones, with low AB (i.e. <1% of mutation loads) [5, 26, 32].

Additionally, many recent studies have shown that a small clonal hematopoiesis may be present also in otherwise healthy subjects at low level (0.03–1%) [27, 32–38]. In the context of highly sensitive allele-specific assays and low mutant AB in the peripheral blood, the possibility of both false-positive and false-negative test results is not negligible [5, 32].

Therefore, to provide a reliable and comparable molecular results, the standardization of molecular techniques is urgently needed. In a recent study by European LeukemiaNet/MPN&MPNr-EuroNet group, nine different *JAK2*^{V617F} quantitative assays were evaluated by the 12 participant laboratories, with the aim to identify the most robust one for routine diagnostic purpose and also for post alloHSCT monitoring [39]. Therefore, a network of 19 Italian laboratories was established with the aim 1) to evaluate the inter- and intra-laboratory variability in *JAK2*^{V617F} quantification in these 19 centers, 2) to identify the most robust assay for the standardization of the molecular test and 3) to allow consistent interpretation of individual patient analysis results.

RESULTS

Between 2014 and 2015, a network of 19 Italian laboratories, routinely involved in the molecular diagnosis of MPNs, was established. The study was coordinated by the Institute of Hematology "L. e A. Seràgnoli", Bologna, and conceived in 3 different rounds in which seven, ten and nineteen laboratories were included over time, respectively (Figure 1). Overall, one quantitative (ipsogen *JAK2* MutaQuant kit, QIAGEN) and four qualitative assays were evaluated. Of these latter, two were commercial (ipsogen *JAK2* MutaSearch kit, QIAGEN, and GeneQuality JAK-2, AB Analitica) and two were built "in-house" methods: allele specific polymerase chain reaction (AS-PCR) and Amplification-refractory mutation system (ARMS) analysis [25, 30].

I Round: proficiency test

In order to obtain information about the variability in *JAK2*^{V617F} quantification between different centers, seven laboratories were employed to evaluate several DNA samples with their own established *JAK2*^{V617F} qualitative and/or quantitative method. Precisely, four DNA samples derived from granulocytes of patients with a diagnosis of MPNs were analyzed. All laboratories using the quantitative assay (ipsogen *JAK2* MutaQuant kit) were able to determine the expected

JAK2^{V617F} AB, as summarized in Table 1. Only in one case (i.e. DNA sample 1), the Center 3 obtained a false positive result. Indeed, the sample was found to be positive with an AB of 0.13%. On the contrary, two Centers (i.e. 6 and 7), using a qualitative approach, were not able to detect the positivity of the DNA sample 2 (with an expected AB of 0.15%).

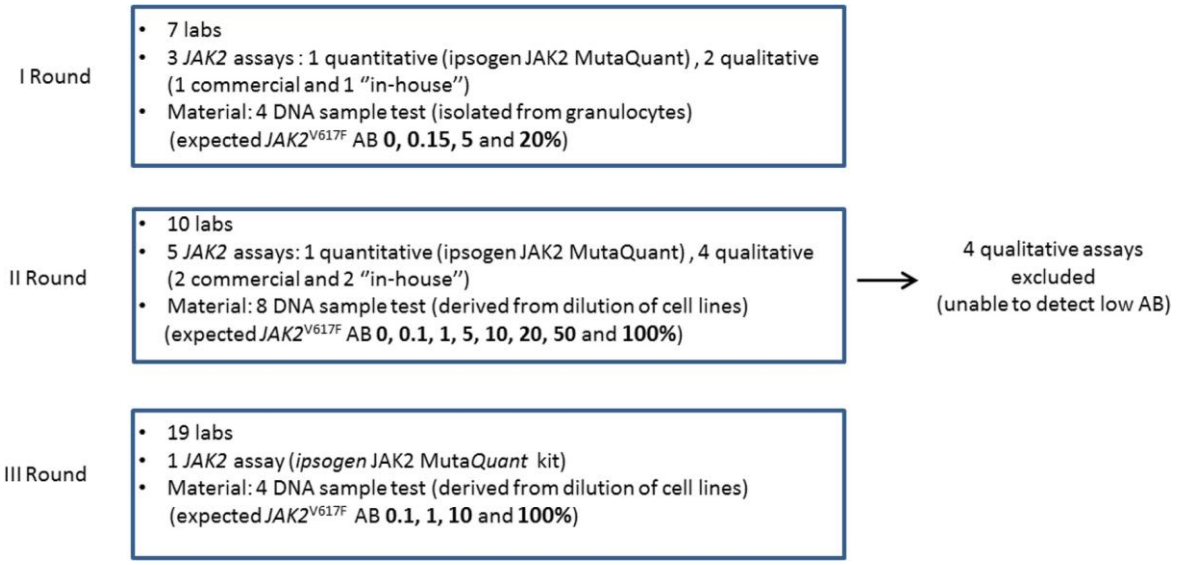

Figure 1: Design of the study. A network of 19 Italian Centers was established and the study was conceived in 3 different rounds, in which seven, ten and nineteen laboratories participated, respectively. Each laboratory had to blindly test DNA samples with different *JAK2*V617F allele burden (AB). Overall, one quantitative and four qualitative assays were evaluated.

Sample	Expected JAK2V617F AB (%)	C1	C2	C3	C4	C5	Min	Median	Max	CV (Coefficent of Variation)	C6	C7
DNA 1	0.005	0.0056	0.00013	0.13	0	0.0007	0	0.00315	0.13	2.1	Neg	Neg
DNA 2	0.15	0.18	0.13	0.19	0.15	0.15	0.13	0.15	0.19	0.15	Neg	Neg
DNA 3	5	7.84	4.19	6.2	8.22	6.25	4.19	6.225	8.22	0.24	Pos	Pos
DNA 4	20	23.17	25.95	20.24	19.44	19.82	19.44	20.03	25.95	0.08	Pos	Pos

Table 1: I Round *JAK2*V617F mutation burden and summary statistics (minimum, maximum, calculation of median, coef cient of variation) of results obtained by the seven participating laboratories, using their everyday analysis method. The expected *JAK2*^{V617F} allele burden (AB, %) was 0.005 (DNA 1), 0.15 (DNA 2), 5 (DNA 3) and 20 (DNA 4). Centers 1–5 performed quantitative assay (ipsogen *JAK2* MutaQuant kit) whereas Centers 6 and 7 used a qualitative approach: allele specific polymerase chain reaction (AS-PCR) and ipsogen *JAK2* MutaSearch kit, respectively.

II Round: comparison between molecular assays

With the aim to further investigate on the inter-laboratory variability in quantifying *JAK2*^{V617F} mutation, especially at low mutation burden, a second standardization round was developed and three additional laboratories were included. Eight DNA samples, derived from dilution of cell lines negative and positive for the *JAK2*^{V617F} mutation, were tested by each laboratory with both ipsogen *JAK2* MutaQuant kit and their own routine qualitative or quantitative method.

We first examined the methods sensitivity, and the detection ability of the ipsogen *JAK2* MutaQuant kit at low-positive samples (i.e. 0.1 and 1%) was compared to those of qualitative *JAK2* commercial and validated "in- house" methods. Overall, the ARMS-PCR "in-house" method and the ipsogen *JAK2* MutaSearch kit were able to detect the positivity of the sample with AB of 1%, whereas none of the laboratories using any qualitative methods were able to detect the low-positive sample (i.e. AB < 0.1%). Remarkably, laboratories using the quantitative approach clearly defined the positivity of both 1% and 0.1% mutated samples. Specifically, 10 out of 16 *JAK2*^{V617F} determinations were clearly defined positive, with an AB ≥ 0.091% which is the Limit Of Detection (LOD) of the ipsogen *JAK2* MutaQuant kit. In the remaining 6 cases, the *JAK2*^{V617F} mutation percentage was found between Limit of Blank (LOB = 0.014%) and LOD.

Additionally, the inter-laboratory variability evaluation was restricted to the ipsogen *JAK2* MutaQuant kit, as six out of 10 participating laboratories already used this assay in their routine practice. The data from two laboratories (i.e. Centers 3 and 8) were excluded from statistical analysis, as Negative Controls of *JAK2*^{V617F} mutation (NC-VF) were found to be positive (> 0.1%) for each run, and considered as invalid runs. This was mainly due to either operator error or to instrumentation suitability, instead of an intrinsic bias of the kit. Overall, quantitative results between the laboratories were reliable as summarized in Table 2 and showed in Figure 2. A small variability was observed especially at low AB (0.1 and 1%, CV = 0.42 and 0.24, respectively).

Sample	Expected *JAK2*^{V617F} AB (%)	Min.	Median	Max	CV (Coefficent of Variation)
DNA G	0	0.002	0.008234	0.02	0.73
DNA E	0.1	0.03	0.09874	0.17	0.42
DNA C	1	0.39	0.8471	1.12	0.24
DNA B	5	2.38	3.643	4.66	0.18
DNA F	10	6.04	8.794	17.65	0.38
DNA H	20	13.12	15.2	19.35	0.11
DNA D	50	30.54	42.62	46.76	0.12
DNA A	100	99.76	99.92	99.97	0.0005

Table 2: II Round *JAK2*V617F mutation burden and summary statistics (minimum, maximum, calculation of median, coefficient of variation) of results obtained by the ten participating laboratories with ipsogen *JAK2* MutaQuant kit. The expected *JAK2*^{V617F} mutation burden of the eight DNA samples was 0, 0.1, 1, 10, 20, 50 and 100%.

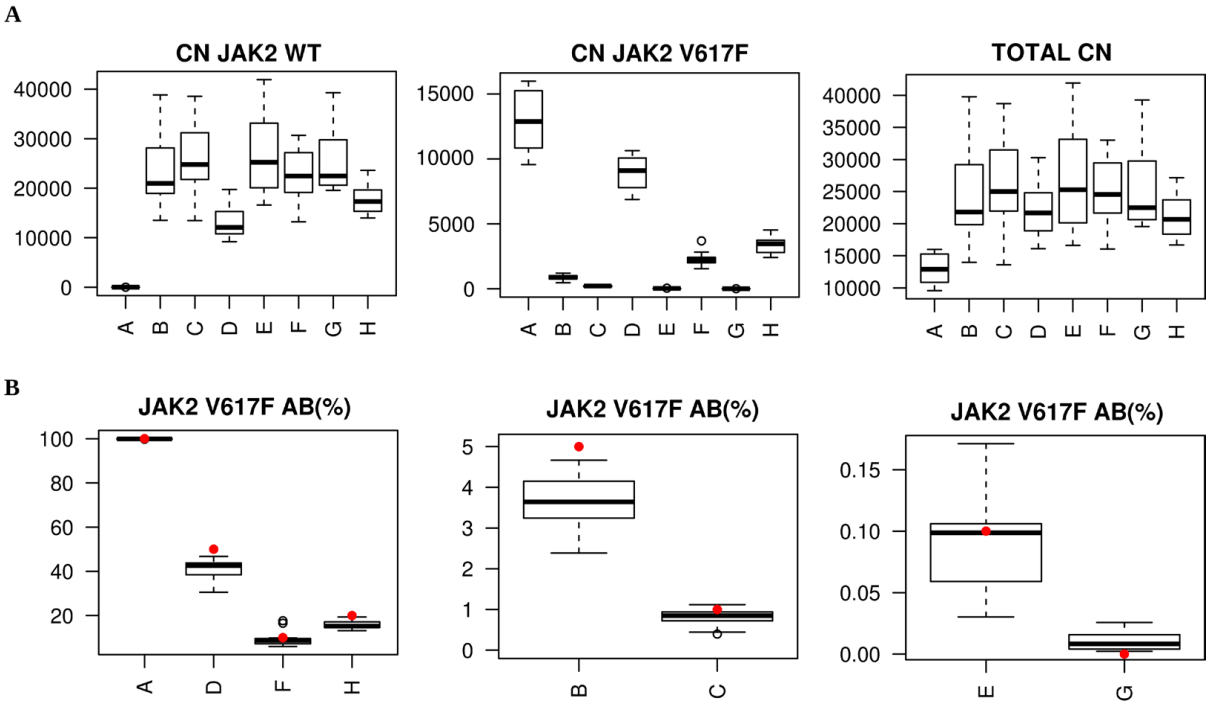

Figure 2: II Round. ipsogen *JAK2* MutaQuant kit Copy number (CN) boxplot of *JAK2* wild-type (WT), *JAK2* mutated (V617F) and *JAK2* total (A) and *JAK2*V617F mutation percentage boxplot (B) for each of the 8 DNA samples. Red dots are expected values.

III Round: inter-laboratory standardization by ipsogen JAK2 MutaQuant kit

The study was extended to 9 additional centers to confirm the robustness of the ipsogen *JAK2* MutaQuant kit in a larger cohort. Each participating laboratory had to test four DNA samples, derived from dilution of cell lines as described above, only with ipsogen *JAK2* MutaQuant kit.

The quantification data from Center 11 were excluded from statistical analysis, as NC-VF was found to be positive (> 0.1%) for each run and Positive Control of *JAK2*^{V617F} mutation (PC-VF) did not reach the recommended value (> 99.9%). Moreover, Center 9 failed to perform correctly both runs due to instrument failure. Of note, both Center 9 and Center 11 did not assess *JAK2*^{V617F} AB in their own routine practice. Among the remaining laboratories, Centers 2, 6 and 17 did not reach the minimum number of *JAK2* total copy number required (10.000 copies) in five different determinations (two at 0.1% AB sample, two at 1% and one at 10%, respectively), and, therefore, these points were not included in the analysis. Quantitative results were reliable across all mutation loads, as reported in Table 3 and showed in Figure 3. All the 17 laboratories were able to quantify the 0.1% AB sample with the same variability observed in the previous II round (CV = 0.46 and 0.42, respectively). Specifically, 23 out of 32 *JAK2*^{V617F} determinations were clearly defined positive, with an AB > 0.091% (LOD). In the remaining 9 cases, the *JAK2*^{V617F} mutation percentage was found between LOB and LOD. Surprisingly, a higher variability between laboratories was observed at 1% of AB (CV = 0.77, *vs* 0.24 in the II round). More importantly, all laboratories clearly distinguished between the 0.1 and 1% mutated samples.

We also evaluated the robustness of the quantitative approach in terms of amplification efficiency. It is well known that amplification efficiency in PCR measures the amount of template converted into amplified product during each cycle of the exponential phase of the reaction. At 100% efficiency, the quantity of product exactly doubles each cycle, thus an efficiency close to 100% is the best indicator of a robust, reproducible assay. An amplification efficiency of 90–105% is recommended for each assay. In our study, the mean value of amplification efficiency obtained, with respect to all runs performed, was of 92% with a CV value of 0.065, confirming the sensitivity and the robustness of the ipsogen *JAK2* MutaQuant kit.

Sample	Expected *JAK2*^{V617F} AB (%)	Min.	Median	Max	CV (Coefficent of Variation)
S02	0.1	0.07	0.11	0.27	0.46
S04	1	0.64	1.05	4.90	0.77
S01	10	6.67	10.04	24.37	0.37
S03	100	99.79	99.92	99.97	0.0005

Table 3: III Round *JAK2*V617F mutation burden and summary statistics (minimum, maximum, calculation of median, coefficient of variation) of results obtained by the nineteen participating laboratories with ipsogen *JAK2* MutaQuant kit. The expected *JAK2*^{V617F} mutation burden of the four DNA samples was 0.1, 1, 10 and 100%.

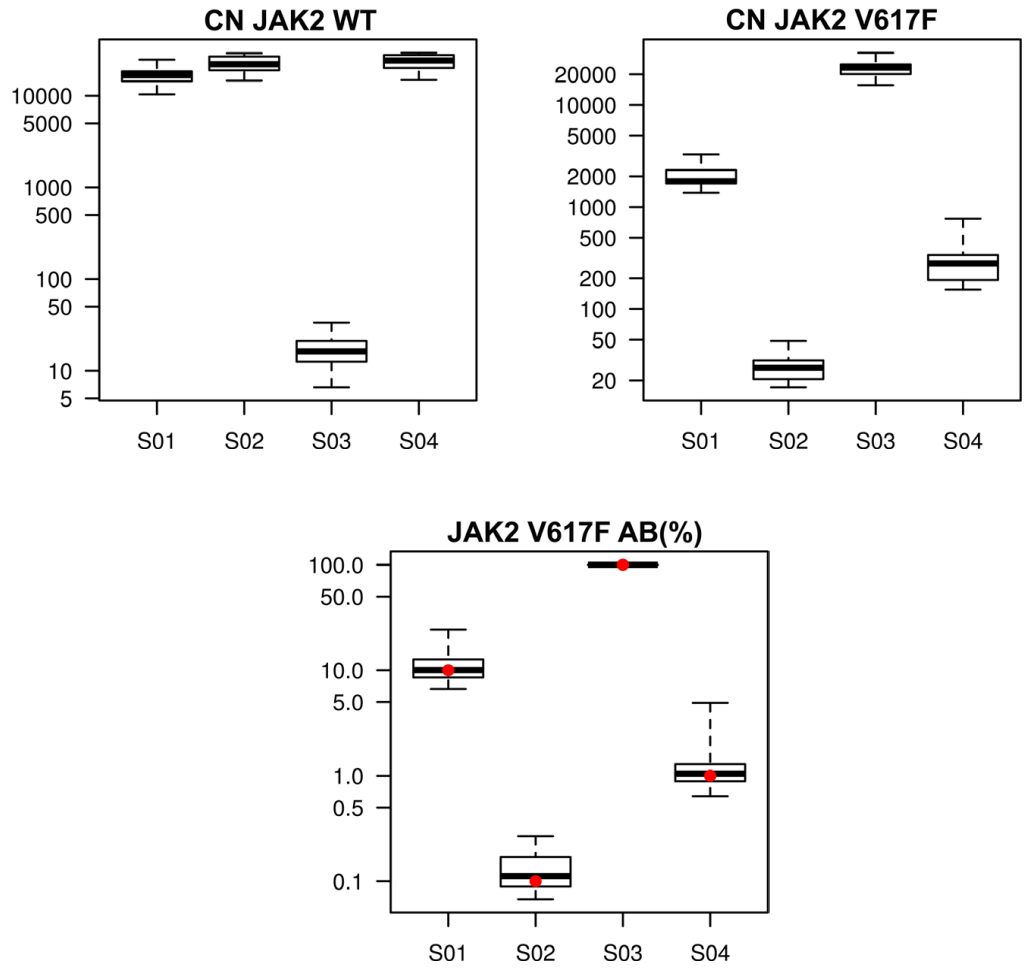

Figure 3: III Round. ipsogen *JAK2* MutaQuant kit Copy number (CN) boxplot of *JAK2* wild-type (WT), *JAK2* mutated (V617F) and *JAK2*V617F mutation percentage boxplot for each of the 4 DNA samples. Red dots are expected values.

DISCUSSION AND CONCLUSIONS

In this study we demonstrated that a qualitative approach is not sensitive enough to detect *JAK2*^{V617F} mutation at low mutation burden (i.e. < 1%). Conversely, the quantitative approach proved to be highly efficient and sensitive, although a modest variability was observed between all participating centers, both in the II and in the III round (CV = 0.42 and 0.46, respectively). Interestingly, only the qualitative ARMS-PCR methods (both "in-house" and ipsogen *JAK2* MutaSearch kit) and the quantitative approach were able to detect the positivity of samples with and AB of 1%. An acceptable variability was observed at this AB in the II round (CV = 0.24) whereas a higher inter-laboratory variability was registered in the III one (CV = 0.77). With regard to samples with AB > 1%, a lower inter-laboratory variability was observed, as demonstrated by CV which ranges from a minimum of 0.0005 to a maximum of 0.38.

Overall, the quantitative ipsogen *JAK2* MutaQuant kit assay performed consistently across different platforms, affirming itself as a robust method to obtain comparable results. This is confirmed by optimal amplification efficiency obtained from each laboratory involved in the study. Indeed, the mean efficiency obtained in this study was of 92% with a CV of 0.065. The observed variability can be explained with both differences in laboratory experience in *JAK2*^{V617F} quantitative determination and intrinsic instrumental bias, as happened in our study, with some laboratories experiencing technical issues. This, together with the variability observed at low AB samples, highlights the need for the standardization of practices, including both pre-analytical and analytical phases. To this aim, the WHO 1st International Reference Panel for Genomic *JAK2* V617F (WHO document WHO/ BS/2016.2293) was established in 2016 by the Expert Committee on Biological Standardization of the World Health Organization [40]. The availability of *JAK2*^{V617F} primary standards should improve the quality of MPN genomic diagnostics by enabling the calibration of assays and kits, and the derivation of secondary standards for routine diagnostic use in determining testing accuracy and sensitivity, thus providing inter-laboratory comparison towards the harmonization of *JAK2*^{V617F} testing.

Moreover, we are considering evaluating digital PCR (dPCR). This emerging technology may improve the ability to detect rare mutations and/or low-positive samples due to higher sensitivity and precision, especially during follow-up to assess minimal residual disease or to monitor patients post alloHSCT [41–42]. But further studies are needed on this technology to

reach the level of standardization of real-time qPCR.

In conclusion, this study sets the basis for the standardization of molecular techniques for *JAK2*^{V617F} determination which will require the employment of approved operating procedures and the use of certificated standards to calibrate *JAK2*^{V617F} quantitative assays.

MATERIALS AND METHODS

The study was conceived in 3 different rounds, in which 19 Italian laboratories were employed. Centers 9 and 16 did not perform *JAK2*^{V617F} molecular testing in their routine. Of the remaining centers, 8 used only a quantitative approach (2 "in-house" and 1 commercial assays), 7 performed only a qualitative evaluation (1 "in-house" and 3 commercial assay) of *JAK2*^{V617F} mutation whereas 2 laboratories used both qualitative and quantitative approaches (2 "in-house" and 1 commercial assays). Regarding real-time PCR instruments used during the study: most (13 out of 19) of the laboratories used Applied Biosystem platforms (ABI7300/7500/7900; Applied Biosystem, Foster City, CA, USA); two laboratories used a Lightcycler LC480 platform (Roche Applied Science, Penzberg, Germany), and the remaining four laboratories used a Rotor-Gene Q 2plex/MDx 5plex HRM instrument (QIAGEN GmbH, Hilden, Germany) (Table 4).

I Round

In the I round, we aimed to investigate the inter- laboratory variability on different mutation loads. In this first step, seven laboratories were involved (Center 1–7). Four of them routinely performed quantitative analysis of *JAK2*^{V617F} with ipsogen *JAK2* MutaQuant Kit (QIAGEN), two used a qualitative approach for *JAK2*^{V617F} evaluation (1 "in-house" method, 1 ipsogen *JAK2* MutaScreen Kit-QIAGEN) whereas one laboratory (Center 5) assessed both qualitative and quantitative assays. Each center had to test four DNA samples (DNA 1–4) with the method routinely employed in their own laboratory. DNA samples were isolated from granulocytes of patients with diagnosis of MPNs. The expected mutation burden of the 4 DNA samples was 0.005 (DNA 1), 0.15 (DNA 2), 5 (DNA 3) and 20% (DNA 4), as previously quantified by Bologna's laboratory with ipsogen *JAK2* MutaQuant Kit.

All patients provided an informed written consent in accordance with the Declaration of Helsinki for the use of remnant DNA for investigational purposes. The study was approved by the local Ethics Committee.

ID Center	Method		Instrumentation
1	Quantitative	ipsogen JAK2 MutaQuant kit	Applied Biosystem 7900HT Fast* Real-Time PCR System
2	Quantitative	ipsogen JAK2 MutaQuant kit	Applied Biosystems 7300 Real-Time PCR System
3	Quantitative	ipsogen JAK2 MutaQuant kit	Rotor-Gene Q 2plex HRM System Rotor-Gene Q MDx 5plex HRM System (3rd Round)
4	Quantitative	ipsogen JAK2 MutaQuant kit	Applied Biosystems 7300 Real-Time PCR System
5	Qualitative home-made Quantitative	ARMS PCR (Chen et al., 2007) **[30]** ipsogen JAK2 MutaQuant kit	7500 Real Time PCR System Life Technologies
6	Qualitative home-made	Allele-specific PCR (Baxter et al, Lancet 2005) **[25]**	LightCycler 480 (2nd and 3rd Round)
7	Qualitative	ipsogen JAK2 MutaSearch	Applied Biosystems 7300 Real-Time PCR System
8	Qualitative	AB Analitica GeneQuality JAK-2 kit	Applied Biosystems 7300 Real-Time PCR System
9	N.A.	N.A.	LightCycler 480 (2nd and 3rd Round)
10	Quantitative	ipsogen JAK2 MutaQuant kit	Applied Biosystems 7500 Fast* Real-time PCR System
11	Qualitative home-made	Allele-specific PCR (Baxter et al, Lancet 2005) **[25]**	Rotor-Gene Q MDx 5plex HRM (3rd Round)
12	Quantitative	ipsogen JAK2 MutaQuant kit	Rotor-Gene Q MDx 5plex HRM
13	Quantitative home-made	Allele-specific primers (0,1-25%) Allele-specific hydrolysis probes (10–90%)	ABI PRISM 7900 HT Real-Time PCR System
14	Qualitative home-made Quantitative	Allele-specific PCR (Baxter et al, Lancet 2005) **[25]** ipsogen JAK2 MutaQuant kit	Applied Biosystems 7500 Real-time PCR System
15	Qualitative	ipsogen JAK2 MutaScreen	Applied Biosystems 7500 Fast* Dx PCR System
16	N.A.	N.A.	Applied Biosystems 7500 Fast* Real-time PCR System
17	Quantitative home-made	Allele-specific PCR (Søren Germer et al, Genome Res. 2000)	ABI PRISM 7900 HT SDS
18	Qualitative	ipsogen JAK2 MutaScreen	Applied Biosystems 7500 Real-time PCR System
19	Qualitative	ipsogen JAK2 MutaScreen	StepOnePlus Real-Time PCR System Rotor-Gene Q MDx 5plex HRM (3rd Round)

Table 4: Summary of participating centers, methods used in their routine practice for *JAK2*V617F detection and instrumentation used in their routine and/or in the study (specified when different).

*Fast mode was not used, as the ipsogen *JAK2* MutaQuant kit is not compatible with "Fast" mode.

II Round

To further investigate the inter-laboratory variability on low-positive samples, a II round was developed. The two main objectives of this round were to assess inter- laboratory variability across the 10 participating clinical centers and to compare the low-positive sample detection ability of the ipsogen *JAK2* MutaQuant kit with the *JAK2* validated "in-house" methods. Compared to the first round, three additional centers (Centers 8–10) were included in this step: only Center 8 and 10 routinely performed *JAK2*^{V617F} evaluation (1 with a qualitative and 1 with a quantitative method, respectively). Eight DNA test samples (DNA Samples A-H) were manufactured by QIAGEN and were centrally distributed by Werfen. The DNA samples were derived from dilution of cell lines: K562 (*JAK2*^{V617F} negative) and MUTZ-8 (*JAK2*^{V617F} positive). The ipsogen *JAK2* MutaQuant kits and associated master-mix were provided by QIAGEN and Werfen. Each center had to blindly test the DNA samples in four different runs: 2 runs were performed with the ipsogen *JAK2* MutaQuant kit and 2 runs with their validated qualitative or quantitative method. The expected mutation burden of the DNA samples was 0, 0.1, 1, 10, 20, 50 and 100%.

III Round

The III round was intended to confirm the robustness of the ipsogen *JAK2* MutaQuant kit. Qualitative methods were therefore excluded and the study was extended to 9 additional laboratories (Centers 11–19). Centers 12, 13 and 17 routinely assessed quantitative evaluation of *JAK2*^{V617F} mutation (2 "in-house" and 1 commercial methods), 4 laboratories (Centers 11, 15, 18 and 19) used qualitative assays (1 "in-house" and 1 commercial methods), Center 14 performed both qualitative ("in-house") and quantitative (ipsogen *JAK2* MutaQuant kit) analysis whereas Center 16 did not perform *JAK2*^{V617F} molecular testing in its routine. Four DNA test samples (DNA Samples S01-S04), provided from the same batches as II round's DNA Samples, were centrally distributed by Werfen. The ipsogen *JAK2* MutaQuant kits and associated master-mix were provided by QIAGEN and Werfen. Each laboratory had to blindly test the DNA samples in 2 runs with the ipsogen *JAK2* MutaQuant kit. The expected mutation burden of the four DNA samples was 0.1, 1, 10 and 100%.

Moreover, amplification efficiency (E) was calculated from the slope of the standard curve using the following formula: $E = 10^{-1/slope}$. Amplification efficiency was expressed as a percentage, that is the percent of template that was amplified in each cycle. To convert E into a percentage we used the following formula: % Efficiency = (E – 1) × 100%.

Data collection and run validity check

Raw data were collected and run validity was checked according to manufacturer's instructions in the kit's handbook.

Statistical method

Statistical analysis was carried out by QIAGEN and by Bologna University. Wild type and mutation copy numbers together with mutation percentage were summarized by mean, median, first and third quartiles, standard deviation and coefficient of variation and plotted by sample for the ipsogen *JAK2* MutaQuant kit.

Inter-laboratory variability: II round

Fisher test was performed by sample to compare variance in order to conclude on the acceptability of the inter-laboratory variability.

Inter-laboratory variability: III round

Shapiro-Wilk normality test was performed to check for data normality and quantile-quantile normal plots were employed for data visual inspection. Kruskall-Wallis test was applied and multiple comparison post-hoc test (Wilcoxon test) was carried out to identify the significant differences. False discovery rate correction was applied to avoid increase in type I error (false positive) because of multiple testing following the Benjamini and Hochberg procedure.

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CONFLICTS OF INTEREST

L. Orlandi, F. Cassavia and F. Orsini are employed by Werfen; V. Laloux is employed by QIAGEN.
None of the co-authors, participating laboratories or institutions received any payments for being involved in this study.

Authors' contribution

M.P., F.P.,G.M. designed the study and wrote the paper. M.P, E.O.,M.A., L.B., E.B., M.D., D.G., P.Z., S.M., V.M., S.T., G.D.M., S.D.Z., M.F., M.A.F., C.B., G.M., F.N., D.G., L.T., P.G., B.G., A.T., S.B., M.L.S., M.M., A.R.S., N.G., D.V., M.B., P.A., C.S., V.F., M.G., E.T., M.S., F.G. performed experiments. L.O., V.L., M.M., F.C., F.O. contributed to the data collection and interpretation. All Authors revised the manuscript critically, and gave final approval to submit for publication.

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RESULT IV

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Crucial factors of the inflammatory microenvironment (IL-1β/TNF-α/TIMP-1) promote the maintenance of the malignant hemopoietic clone of myelofibrosis: an in vitro study

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ABSTRACT

Along with molecular abnormalities (mutations in *JAK2*, Calreticulin (*CALR*) and *MPL* genes), chronic inflammation is the major hallmark of Myelofibrosis (MF). Here, we investigated the in vitro effects of crucial factors of the inflammatory microenvironment (Interleukin (IL)-1β, Tumor Necrosis Factor (TNF)-α, Tissue Inhibitor of Metalloproteinases (TIMP)-1 and ATP) on the functional behaviour of MF-derived circulating CD34+ cells.

We found that, regardless mutation status, IL-1β or TNF-α increases the survival of MF-derived CD34+ cells. In addition, along with stimulation of cell cycle progression to the S-phase, IL-1β or TNF-α ± TIMP-1 significantly stimulate(s) the in vitro clonogenic ability of CD34+ cells from *JAK2*^{V617F} mutated patients. Whereas in the *JAK2*^{V617F} mutated group, the addition of IL-1β or TNF-α + TIMP-1 decreased the erythroid compartment of the *CALR* mutated patients. Megakaryocyte progenitors were stimulated by IL-1β (*JAK2*^{V617F} mutated patients only) and inhibited by TNF-α. IL-1β + TNF-α + C-X-C motif chemokine 12 (CXCL12) ± TIMP-1 highly stimulates the in vitro

migration of MF-derived CD34+ cells. Interestingly, after migration toward IL-1β + TNF-α + CXCL12 ± TIMP-1, CD34+ cells from *JAK2*^{V617F} mutated patients show increased clonogenic ability.

Here we demonstrate that the interplay of these inflammatory factors promotes and selects the circulating MF-derived CD34+ cells with higher proliferative activity, clonogenic potential and migration ability. Targeting these micro-environmental interactions may be a clinically relevant approach.

INTRODUCTION

Myelofibrosis (MF) is a life-threatening chronic myeloproliferative neoplasia (MPN) of the hematopoietic stem/progenitor cell (HSPC) clinically characterized by progressive anemia, splenomegaly and constitutional symptoms and by an increased risk to develop acute leukemia (AL). It can arise de novo (primary MF; PMF) or can evolve from Polycythemia Vera (PV; PPV MF) or Essential Thrombocythemia (ET; PET MF) [1–3].

Approximately 50 to 60% of MF patients carry a mutation in the Janus kinase 2 (*JAK2*) gene, while 20–25% of patients show recurrent mutations in the Calreticulin (*CALR*) and an additional 5 to 10% have activating mutations in the myeloproliferative leukemia virus oncogene (*MPL*) gene. Around 10% of patients have non-mutated *JAK2*, *MPL* and *CALR* genes ("triple negative"). Regardless of molecular status, all patients have a deregulation in the JAK/STAT signalling [4–9].

Besides molecular abnormalities, the inflammatory microenvironment has emerged in the last few years as a key-player in MF pathogenesis [10]. Abnormal expression and activity of several cytokines involved in inflammation and immunoregulation are associated with MF [11] and correlate with more severe marrow fibrosis [12, 13], worsening systemic symptoms [14] and decreased survival [15]. Also, the constitutive mobilization of CD34+ cells into the peripheral blood has been associated with profound alterations in the CXC chemokine receptor 4 (CXCR4)/ C-X-C motif chemokine 12 (CXCL12) axis [16–18]. Up-regulated production of proinflammatory cytokines by HSPCs and surrounding stromal cells generates a microenvironment that selects for the malignant clone [11, 19–23].

Interestingly, HSPCs actively sense pro-inflammatory factors [24]. However, the key players linking inflammation and cancer in MF are still to be defined. Particularly, the plasma

levels of Interleukin (IL)-1β, Tumor Necrosis Factor (TNF)-α and Tissue Inhibitor of Metalloproteinases (TIMP)-1 are increased in MF patients [5, 15, 25], but their contribution to disease pathogenesis in MF has been poorly [26] or never investigated. This is also true for the extracellular ATP nucleotide [27]. Under inflammatory conditions, IL-1β stimulates leukocytosis and thrombocytosis by inducing various cytokines (i.e. Granulocyte-Colony Stimulating Factor, IL-6) that are overexpressed in MF; also, IL-1β regulates the survival/proliferation of AL cells [27–30]. IL-1β has been recognized as the main trigger for neural damage and Schwann cell death caused by bone marrow mutant HSPC. Notably, mutant-HSPC- driven niche damage seems to critically contribute to MPN pathogenesis [31]. TNF-α promotes survival of human quiescent bone marrow-derived CD34+ Burst Forming Unit-Erythrocyte (BFU-E) and facilitates the clonal expansion of *JAK2*^{V617F} -positive cells in MPNs [26,32]. TIMP-1, through receptor (CD63) binding, promotes cell survival, differentiation and migration; also, TIMP-1 displays cytokine-like features in the HSPC compartment [33–35]. It was initially found to enhance the proliferation of erythroid cells [36]; also, we recently demonstrated that TIMP-1 increases the clonogenic efficiency of normal CB-derived progenitor cells [37]. Finally, extracellular nucleotides, mainly ATP, are important mediators in inflammation and modulation of cell proliferation, migration and death, including AL CD34+ stem/progenitor cells [24, 37–41].

Here, we addressed the functional effects of these pro-inflammatory factors on the *in vitro* behaviour of HSPCs derived from MF patients, with the aim to investigate their putative role in disease pathogenesis.

RESULTS

Regardless of mutational status, the plasma levels of IL-1β, TNF-α and TIMP-1 are increased in MF patients

To evaluate the pro-inflammatory pro le, selected plasma cytokines were measured. Compared with controls, IL-1β, TNF-α and TIMP-1 plasma levels were significantly increased in MF patients (regardless of IPSS risk stratification values) (Figure 1A, 1B, 1C). We found a trend, albeit not statistically significant (*p = 0.06*), toward increased IL-1β plasma levels in *CALR* mutated patients. Targeting TNF-α and TIMP-1, no significant differences were observed between *JAK2*^{V617F} and *CALR* mutated groups.

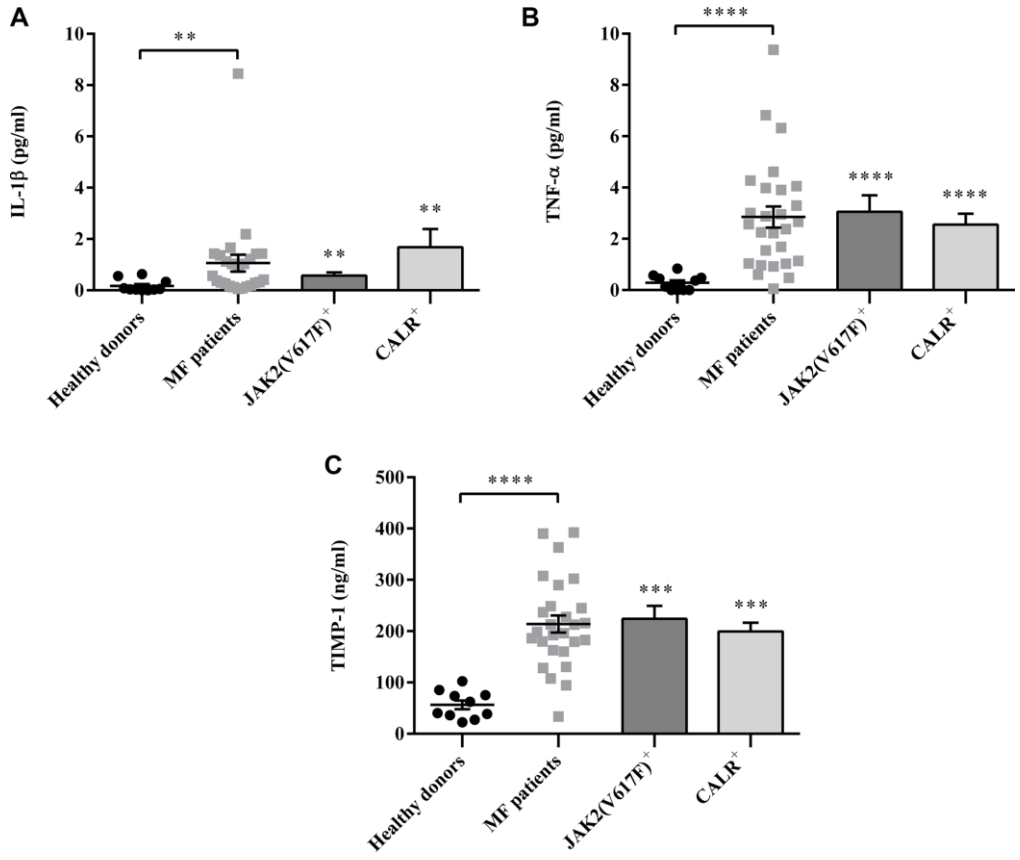

Figure 1: Regardless mutation status, the plasma levels of IL-1β, TNF-α and TIMP-1 are increased in MF patients. IL-1β (**A**), TNF-α (**B**) and TIMP-1 (**C**) plasma levels were measured by ELISA in MF patients. (*n* = 26; *JAK2*^{V617F} positive *n* =16; *CALR* positive *n* = 10) and healthy controls (*n* = 15). Compared with controls, cytokines plasma levels were significantly increased in MF patients. Of note, there was no signi cant difference between *JAK2*^{V617F} or *CALR* mutated patients. All data are presented as mean ± SEM (**$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$).

Selected subsets of circulating HSPCs are expanded in MF patients

To determine the extent of the circulating HSPCs compartment according to mutations, we phenotypically analyzed the whole blood of MF patients.

Irrespective of mutation status, the mean number of circulating CD34+ cells was significantly higher in MF patients than in controls *($p \leq 0.0001$)*. No significant differences were observed between the two mutated groups (Figure 2A). Of note, the number of CD34+ cells correlated with IPSS risk in *JAK2*^{V617F} mutated patients (r = 0.88; *$p = 0.02$*; data not shown).

Along with CD34+CD38- and CD34+CD133+ cells (Figure 2B, 2C), circulating CD34+ cells co-expressing adhesion molecules (CD49d, CD47 and CD44; Figure 2D–2F) were also significantly increased in MF patients. Once again, no significant difference was observed between the two mutated groups.

The median number of circulating MF-derived CD34+ cells co-expressing the TIMP-1 (CD63) or the CXCL12 receptor (CD184; CXCR4) was significantly higher (*$p \leq 0.001$* and *$p \leq 0.01$*, respectively) than the CB counterparts (Figure 2G, 2H). *CALR* mutated patients showed increased number of circulating CD34+CD63+ and CD34+CD184+ cells compared to *JAK2*^{V617F} mutated patients (*$p \leq 0.01$* for CD34+CD63+) or the CB-counterparts (*$p \leq 0.01$* and *$p \leq 0.05$,* respectively). CD34+CD63+ cells of *JAK2*V617F mutated patients were also increased compared with the CB-derived cells (*$p \leq 0.05$*).

As shown in Figure 2I, circulating megakaryocyte (MK) progenitors (CD34+CD41+) were also significantly increased (*$p \leq 0.01$*). *CALR* mutated patients showed increased number of CD34+CD41+ cells compared to *JAK2*^{V617F} mutated patients (*$p \leq 0.01$*) or the CB-counterparts (*$p \leq 0.001$*).

Of note, except of the decreased expression of CD184 in MF cells, the analysis of mean fluorescence intensity (MFI) of CD133, CD63,CD41, CD49d, CD44 and CD47 antigens on the CD34+ cells did not reveal any difference between patients and controls or between the two mutated groups (data not shown).

These data demonstrate that in MF, irrespective of mutation status, there is an *in vivo* expansion of the HSPCs compartment. However, the *CALR* mutated patients show an increased number of circulating CD34+CD63+ and CD34+CD41+ cells compared to the *JAK2*^{V617F} mutated counterparts.

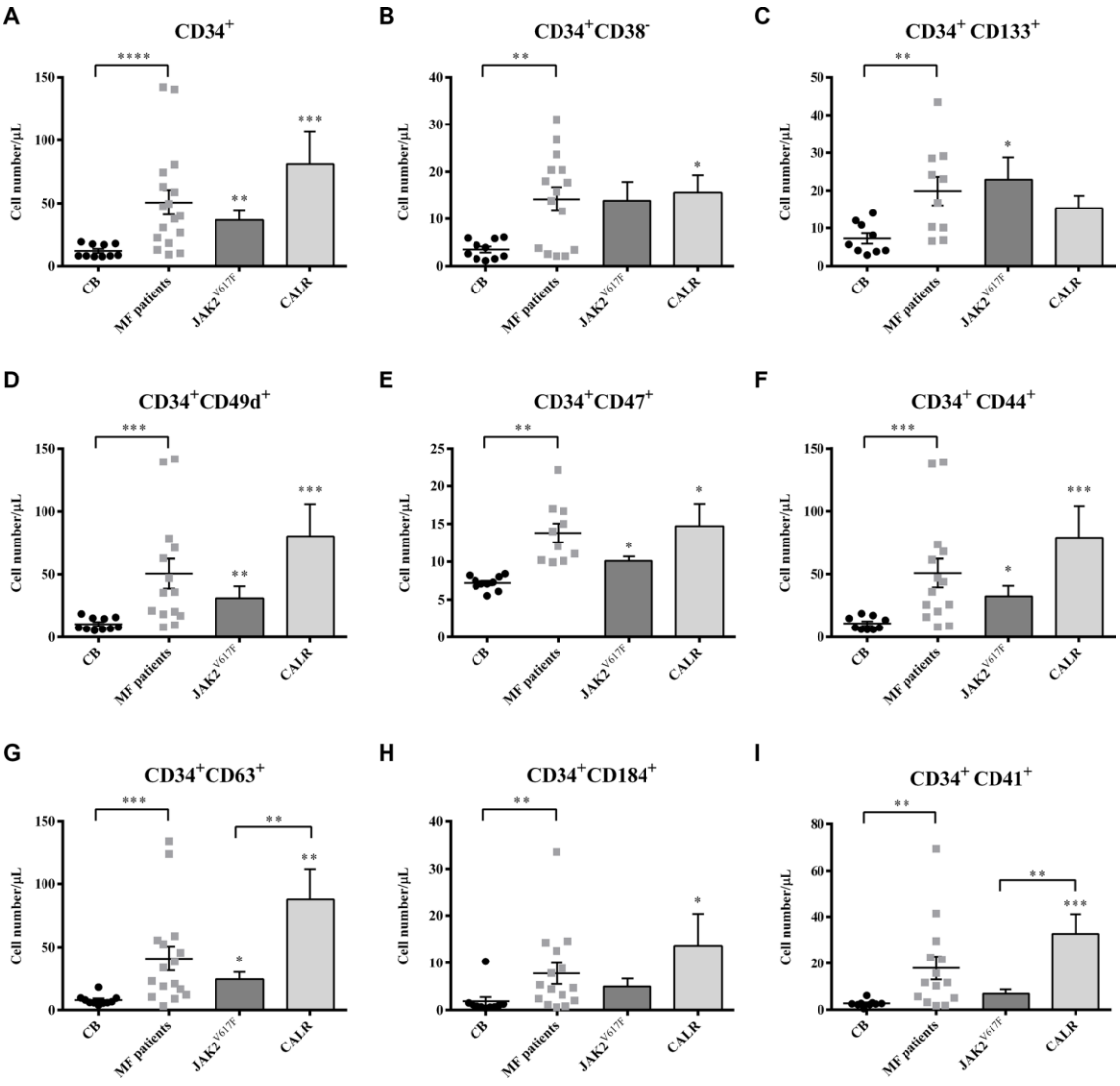

Figure 2: Selected subsets of circulating HSPCs are expanded in MF patients. The circulating absolute number of MF (total (*n* = 30) and subdivided into *JAK2*^{V617F} (*n* = 20) or *CALR* (*n* = 10) mutated groups) and CB (*n* = 10) CD34+ cells coexpressing the CD133, CD49d, CD47, CD44, CD63, CD184 and CD41 antigens together with the CD34+ CD38− subset are shown (A–I). All subsets were increased in MF patients as compared with the CB counterparts. No significant differences were observed between the two mutated groups, except the CD34+ CD63+ and the CD34+ CD41+ cells of *CALR* mutated patients which were significantly increased as compared with the *JAK2*^{V617F} counterparts. All data are presented as mean ± SEM (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$).

Survival of $CD34^+$ cells from MF patients is increased by IL-1β and TNF-α

To investigate whether inflammatory signals may regulate the survival of HSPCs, CD34+ cells from MF patients or CB were *in vitro* cultured with the selected pro-inflammatory factors, alone or in combination, at concentrations previously shown to be effective in dose-response experiments (Supplementary Figure 1).

We firstly assessed the effects of factors alone on the *in vitro* survival of CD34+ cells. As shown in Figure 3A, the survival of CD34+ cells from MF patients was significantly promoted by IL-1β or TNF-α as compared with the CB CD34+ cells ($p \leq 0.01$ *and* $p \leq 0.05$, respectively) or with the untreated MF cells ($p \leq 0.001$ and $p \leq 0.01$, respectively). No significant differences in survival were observed between the two mutated groups in all tested conditions (data not shown).

As shown in Supplementary Figure 2, the combinations of factors two-by-two significantly promoted the MF-derived CD34+ cells survival as compared with untreated cells. However, no significant differences in cell viability were observed as compared with factors alone. Interestingly, the two by two combined factors did not significantly enhance the survival of CB-derived CD34+ cells, except for IL-1β + TNF-α ($p \leq 0.01$). Comparing MF vs CB-derived cells, the survival of MF CD34+ cells was significantly enhanced by IL-1β + TIMP-1 ($p \leq 0.01$) and IL-1β + ATP ($p \leq 0.01$).

When multiple factors were combined no significant differences were observed between MF and CB-derived CD34+ cells. Only TNF-α + TIMP-1 + ATP significantly promoted the survival of $JAK2^{V617F}$ CD34+ cells as compared with the *CALR* ($p \leq 0.01$) or CB counterparts ($p \leq 0.001$) (data not shown).

When we analyzed the CD34+ CD38- cells (Figure 3B), we found that multiple factors combinations (particularly those including IL-1β) significantly stimulated the cell survival of MF and CB-derived cells as compared with untreated cells. However, no significant differences were observed between patients/ controls (Figure 3B) or the two mutated groups (data not shown)

Taken together these data demonstrate that, regardless of mutation status, the survival of MF-derived CD34+ cells is highly stimulated by the in vitro treatment with IL-1β or TNF-α. Combinations of pro-inflammatory factors do not have synergistic effects.

Clonogenic output of circulating MF-derived CD34+ cells is positively enhanced by IL-1β + TNF-α ± TIMP-1 combinations

To analyze the functional role of the selected pro-inflammatory cytokines on HSPCs, we investigated their effects on the clonogenic output of circulating MF and CB-derived CD34+ cells.

Factors alone did not induce a significant CFU-C growth from MF CD34+ cells (data not shown). However, when MF-derived CD34+ cells were tested in the presence of combinations of factors two-by-two, the IL-1β + TIMP-1 combination was the only one effective in stimulating the CFU-C growth as compared with untreated cells ($p \leq 0.05$) or CB-derived CD34+ cells ($p \leq 0.05$) (Figure 4A). IL-1β + TNF-α and IL-1β + TIMP-1 significantly promoted the BFU-E growth of the MF-derived CD34+ cells as compared with the untreated samples and the CB counterparts. The CFU-GM growth was positively enhanced by IL-1β + TIMP- 1 (Supplementary Figure 3A, 3B). Of note, when combinations of multiple factors were tested, only IL-1β + TNF-α + TIMP-1 significantly promoted the CFU-C growth ($p \leq 0.05$) of CD34+ cells from CB (data not shown).

As shown in Figure 4B, when the growth of MK progenitors was investigated in the presence of inflammatory factors alone, we found that, at variance with CB, the MF-derived CFU-MK growth was significantly inhibited by TNF-α. By contrast, IL-1β has stimulatory activity on MK colony formation. Factors in combination did not significantly modify the growth of patients/CB pure CFU-MK as compared with factors alone.

We also examined the cell-cycle pro le of MF- derived and CB-derived CD34+ cells after *in vitro* exposure to the cytokines. We found that most of the untreated CD34+ cells from MF patients were in a dormant state. Factors alone did not significantly increased the percentage of CD34+ cells in S phase as compared with untreated cells, both in MF patients and CB (data not shown). Conversely, in MF patients, irrespective of mutation status, cell-cycle progression was observed in presence of various cytokines combinations, with the notable exception of ATP + TNF-α + TIMP-1 (Figure 4C, 4D).

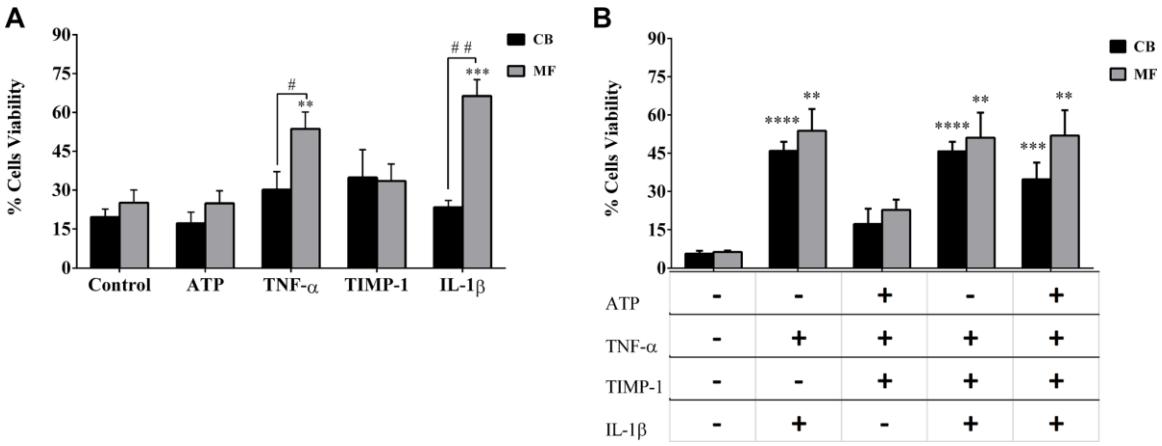

Figure 3: Survival of CD34+ cells from MF patients is increased by IL-1β and TNF-α. (**A**) CD34+ cells from MF patients (*n* = 20) or CB (*n* = 8) were *in vitro* treated for 4 days with factors alone and the percentage of cell viability was assessed after Annexin V/PI staining, as described in Methods. At variance with CB-derived cells, TNF-*α* and IL-1*β* alone significantly stimulated the survival of MF-derived CD34+ cells as compared with untreated cells and the CB-derived counterparts. Conversely, ATP and TIMP-1 were ineffective in normal and diseased cells.(**B**) In selected experiments, before Annexin V/PI staining, MF- (*n* = 10) and CB- (*n* = 6) derived CD34+ cells were also labeled with a MoAb against the human CD38 antigen and the CD34+ CD38− cells were gated and cell viability was analyzed. Once again, multiple combinations of cytokines with IL-1β or TNF-α significantly stimulated the survival of MF- and CB-derived CD34+ CD38− cells. Notably, this was not true for ATP+ TNF-α+ TIMP-1. No differences were observed between MF patients and CB. All data are presented as mean ± SEM. (***p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001 vs untreated cells (CTR)*) (*#p ≤ 0.05; ##p ≤ 0.01 vs CB*).

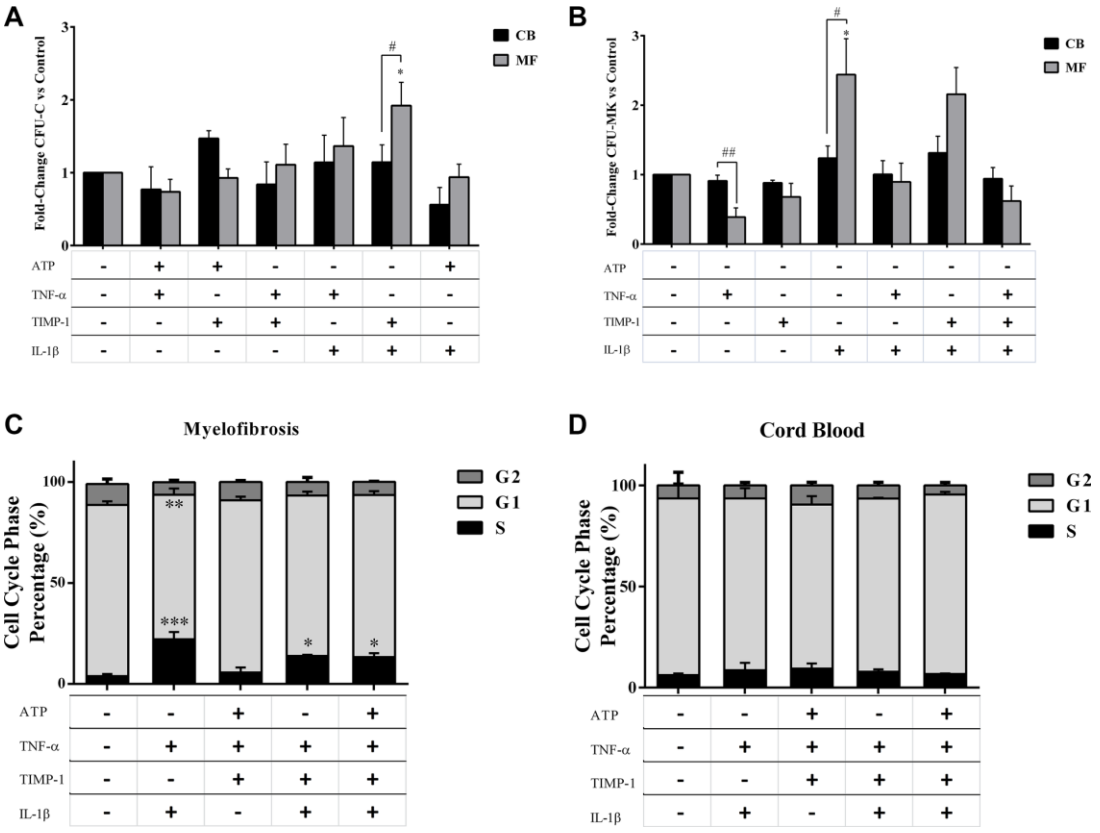

Figure 4: Proliferation of circulating MF-derived CD34+ cells is positively enhanced by IL-1β + TNF-α ± TIMP-1 combinations. Circulating CD34+ cells were isolated from MF patients (*n* = 20) and CB units (*n* = 8) and cultured in the presence of the selected two-by-two pro-inflammatory factors. After 14 days, the total CFU-C output was assessed as described in Methods (A). Circulating CD34+ cells were isolated from MF patients (*n* =10) and CB units (*n* =8) and cultured in the presence or absence of inflammatory factors alone or combined. After 12 days, the CFU-MK growth was assessed as described in Methods (B). The results are expressed as growth fold change versus untreated CTR samples. (A) The clonogenic output of the MF-derived CD34+ cells was significantly stimulated by the IL-1β + TIMP-1 combination as compared with untreated cells or the CB-derived counterparts. No other combinations of factors two-by- two were effective. The mean number of colonies in MF-derived and CB-derived untreated samples was 59 ± 8 and 63 ± 6, respectively. (B) The MF-derived CFU-MK growth was significantly inhibited by TNF-α. By contrast, IL-1β has stimulatory activity on MK colony formation. Factors in combination did not significantly modify the growth of patients/CB CFU-MK as compared with factors alone. Factors alone or in combination did not significantly modify the CFU-MK growth of the CB counterparts. The mean number of CFU-MK in MF- and CB-derived untreated samples was 26 ± 11 and 46 ± 10, respectively. In (C and D) are shown the results of cell-cycle analysis of MF-derived (*n* =10) and CB-derived (*n* =8) CD34+ cells after *in vitro* incubation for 24 hours in the presence or absence of various combinations of pro-inflammatory factors. Results are expressed as the percentage of cells in different phases of the cell cycle. IL-1β plus TNF-α highly promote cell cycling of CD34+ cells from MF patients. IL-1β + TNF-α + TIMP-1 and IL-1β + TNF-α + TIMP-1 + ATP were also effective (C). Conversely, no significant differences were observed when CB-derived cells were analyzed (D). All data are presented as mean ± SEM. (**p ≤0.05; **p ≤0.01; ***p ≤0.001 vs untreated cells*) (*#p ≤0.05; ##p ≤0.01 vs CB*).

Opposite effects of pro-inflammatory cytokines on clonogenic potential of CD34+ cells from *JAK2*^{V617F} or *CALR* mutated patients

When clonogenic potential was analyzed according to mutation status and in the presence of pro-inflammatory factors alone, no differences were observed between the two mutated groups. Colony composition analysis demonstrated that only IL-1β enhanced the erythroid compartment of the *JAK2*V617F mutated group (Supplementary Figure 4A and 4B).

By contrast, (Figure 5A), the combination of IL-1β + TIMP-1 and IL-1β + TNF-α significantly promoted the CFU-C growth of *JAK2*^{V617F} mutated patients compared with the *CALR* mutated counterparts. Similar results were obtained when CFU-GM and BFU-E growth were distinctly analyzed (data not shown).

When colony composition was analyzed according to mutation status (Figure 5B), both mutated groups showed reduced BFU-E growth of the untreated samples as compared with the CB counterparts. Interestingly, in the *JAK2*^{V617F} mutated group, the addition of different combinations of pro-inflammatory factors enhanced the erythroid compartment as compared with untreated samples. Conversely, some cytokines combinations significantly decreased BFU-E growth in *CALR*-mutated patients.

Multiple combinations did not significantly modify the clonogenic activity and colony composition of the two mutated groups (data not shown).

When we analyzed the percentage of *JAK2*^{V617F} and *CALR* mutant colonies in the absence or presence of IL- 1β + TNFα, we found that the percentage of *JAK2*^{V617F}, but not *CALR*, mutated colonies was increased (data not shown).

MK progenitors of *JAK2*^{V617F} mutated patients were highly stimulated by IL-1β alone. By contrast, TNF-α significantly inhibited the CFU-MK growth of both *JAK2*^{V617F} and *CALR* mutated patients as compared with CB counterparts (Supplementary Figure 5).

Taken together these results demonstrate that the hemopoietic function of MF-derived CD34+ cells is highly promoted by the IL-1β or TNF-α ± TIMP-1 combinations, even though TNF-α alone show inhibitory effects on MK progenitors. Interestingly, whereas in the *JAK2*^{V617F} mutated group, the addition of various combinations of growth factors decreased the erythroid compartment of the *CALR* mutated patients.

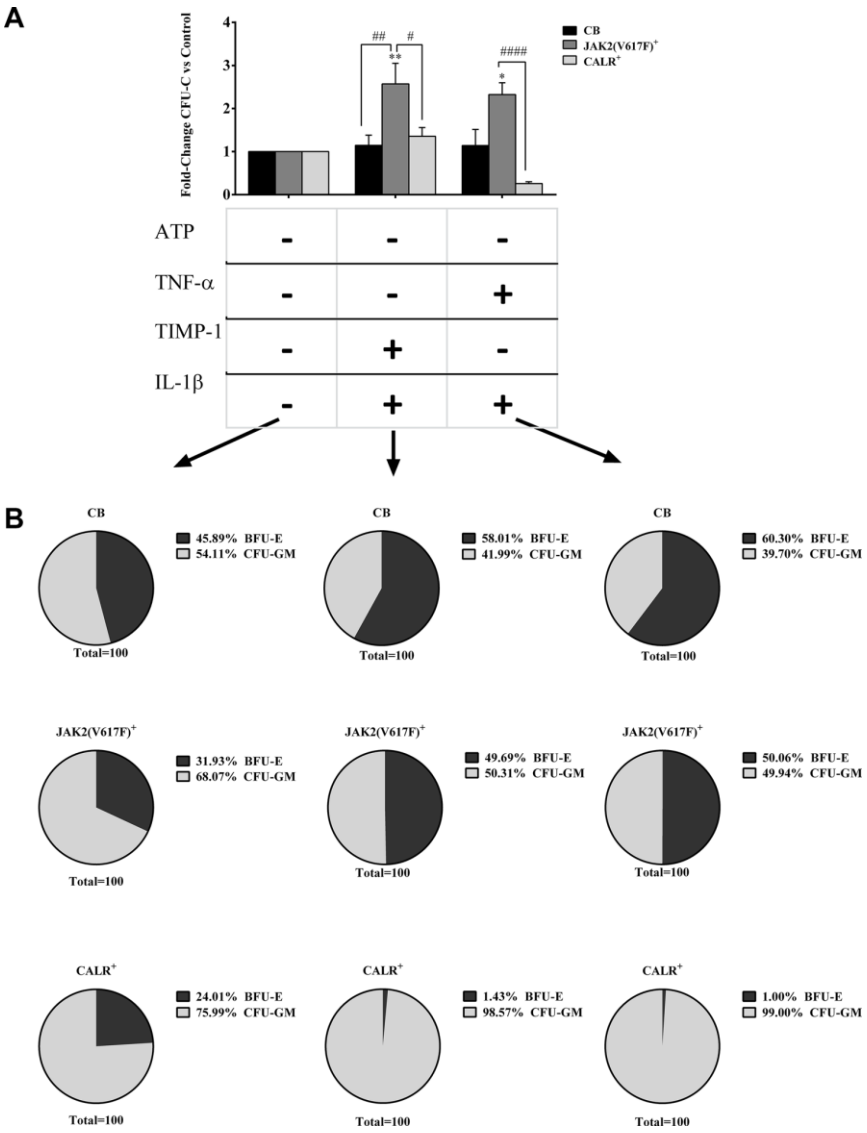

Figure 5: Opposite effects of pro-inflammatory cytokines on cells from *JAK2V617F* or *CALR* mutated patients. (A) When clonogenic activity was analyzed according to mutation status, the CFU-C growth of *JAK2*^{V617F} mutated patients was significantly up- regulated by IL-1β + TIMP-1 and IL-1β + TNF-α as compared with untreated control samples, the *CALR* mutated counterparts and the CB-derived cells (only IL-1β + TIMP-1). The results are expressed as growth fold change versus untreated CTR samples. All data are presented as mean ± SEM. (**p ≤ 0.05; **p ≤ 0.01 vs untreated cells*) (*#p ≤ 0.05; ##p ≤ 0.01; ####p ≤ 0.0001 vs CB-derived cells*) (B) When colony composition was analyzed according to mutation status, we found that the erythroid compartment of the untreated samples was reduced in both mutated groups as compared with the CB counterparts. However, no significant differences were observed between the two mutated groups. Interestingly, in the *JAK2*^{V617F} mutated group, the addition of IL-1β + TIMP-1 and IL-1β + TNF-α enhanced the erythroid compartment as compared with untreated samples. Conversely, some cytokines combinations significantly impaired BFU-E growth in *CALR* mutated patients. The results are expressed as mean percentage of CFU-GM/BFU-E as compared with the total CFU-C count.

IL-1β and TNF-α significantly promote migration of MF-derived CD34+ cells showing enhanced clonogenic ability after migration in *JAK2^{V617F}* mutated patients

To evaluate whether selected pro-inflammatory factors may differentially regulate the migratory ability of HSPCs from MF patients, we firstly analyzed the plasma concentration of CXCL12. CXCL12 plasma level was markedly higher in patients than in controls, either total ($p \leq 0.05$) or subdivided according to mutation status (*JAK2*^{V617F} $p \leq 0.05$; *CALR* $p \leq 0.05$). Conversely, no significant differences were observed between the two mutated groups (Figure 6A).

To mirror the *in vivo* pattern of MF, we set up *in vitro* migration experiments in the presence of the identified inflammatory factors and CXCL12. The migration rate of MF- or CB-derived CD34+ cells toward inflammatory factors alone (without CXCL12) was not significantly different from that of untreated cells (data not shown).

As shown in Figure 6B, CXCL12 significantly increased the migratory behaviour of MF-derived CD34+ cells as compared with CB counterparts ($p \leq 0.05$). The addition of IL-1β or TNF-α + CXCL12 shows a trend toward increased migration of CD34+ cells from MF patients, but doesn't reach statistical significance. At variance with CB derived cells, the addition of both cytokines significantly increased the migratory potential of CD34+ cells from MF patients ($p \leq 0.01$). No differences were observed between the two mutated groups (data not shown).

The addition of TIMP-1 and ATP alone or inflammatory factors two by two in the presence of CXCL12 did not significantly increase the migration ability of MF-derived CD34+ cells as compared with CXCL12 alone (data not shown). The migratory behavior of the MF-derived CD34+ cells toward multiple combinations of factors was significantly enhanced as compared with the CB-derived counterparts in all tested combinations. Conversely, CB-derived CD34+ cells were almost insensitive (Supplementary Figure 6).

To evaluate whether migrated cells toward various pro-inflammatory gradients show different hemopoietic function, we also tested the clonogenic potential of CD34+ cells from MF patients or CB after migration toward CXCL12 alone or CXCL12 plus various combinations of factors (Figure 7A, 7B). Interestingly, at variance with the CFU-C growth of unmigrated HSPCs from MF patients, IL-1β + TNF-α + CXCL12 and IL-1β + TNF-α + TIMP-1 + CXCL12 selected a subset of MF-derived CD34+ cells with higher clonogenic potential as compared with CXCL12 alone ($p \leq 0.05$, respectively) (Figure 7B). Conversely, the clonogenic output of CB-derived CD34+ cells after

migration toward various combinations of pro-inflammatory factors was unaffected (Figure 7A). Notably, according to mutation status, the CFU-C post migration assay demonstrated once again that various combinations of pro-inflammatory factors significantly stimulate the clonogenic ability of migrated CD34+ cells from *JAK2*^{V617F}, but not *CALR,* mutated patients (Supplementary Figure 7).

When the number of granulocyte and erythroid colonies were analyzed individually, only BFU-E growth was significantly increased with respect to controls ($p \leq 0.05$) after cells were migrated toward IL-1β + TNF-α + CXCL12 ± TIMP-1. Of note, IL-1β + TNF-α + TIMP-1 + CXCL12 significantly stimulated also CFU- GM growth as compared with CXCL12 alone ($p \leq 0.05$) (data not shown).

Taken together these results demonstrate that, irrespective of mutation status, IL-1β + TNF-α + CXCL12 ± TIMP-1 selectively enhances the migratory ability of MF- derived CD34+ cells. Interestingly, IL-1β + TNF-α + CXCL12 ± TIMP-1 promotes and selects the circulating HSPCs of *JAK2*^{V617F} mutated patients with higher clonogenic potential.

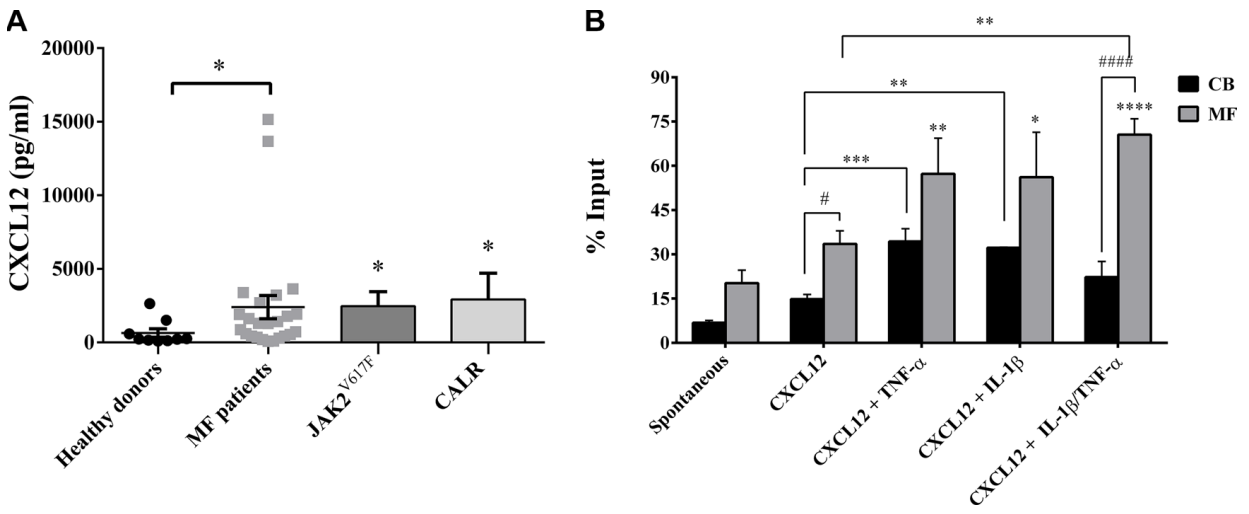

Figure 6: IL-1β and TNF-α significantly increases migration of MF-derived CD34+ cells. (A) CXCL12 plasma levels of MF patients (total *n* =24; *JAK2*^{V617F} mutated patients *n* = 16; *CALR* mutated patients *n* = 8) and controls (*n* = 10). Regardless mutation status, CXCL12 concentration was significantly higher in MF patients (**p ≤ 0.05 vs controls*). (B) When cells were migrated toward CXCL12 alone, an increased migration ability was observed in MF-derived (*n* = 15) CD34+ cells as compared with the CB-derived (*n* = 8) counterparts. The addition of inflammatory factors alone (IL-1β/TNF-α) plus CXCL12 significantly increased the migratory behaviour of MF-derived CD34+ cells as compared with CXCL12 alone. IL-1β + TNF-α synergistically enhanced the migratory behaviour of CD34+ cells as compared with spontaneous migration (*****p < 0.0001*), CXCL12 alone (***p <0.001*) and the CB-counterpart (*####p <0.0001).* Results are expressed as mean percentages ± SEM of input. *(**p ≤ 0.01; ***p ≤ 0.001 vs CXCL12 alone for CB-derived CD34+ cells*) (**p ≤ 0.05; **p ≤ 0.01; ****p < 0.0001 vs spontaneous migration for MF-derived CD34+ cells*) (*#p ≤ 0.05; ####p <0.0001 vs CB*).

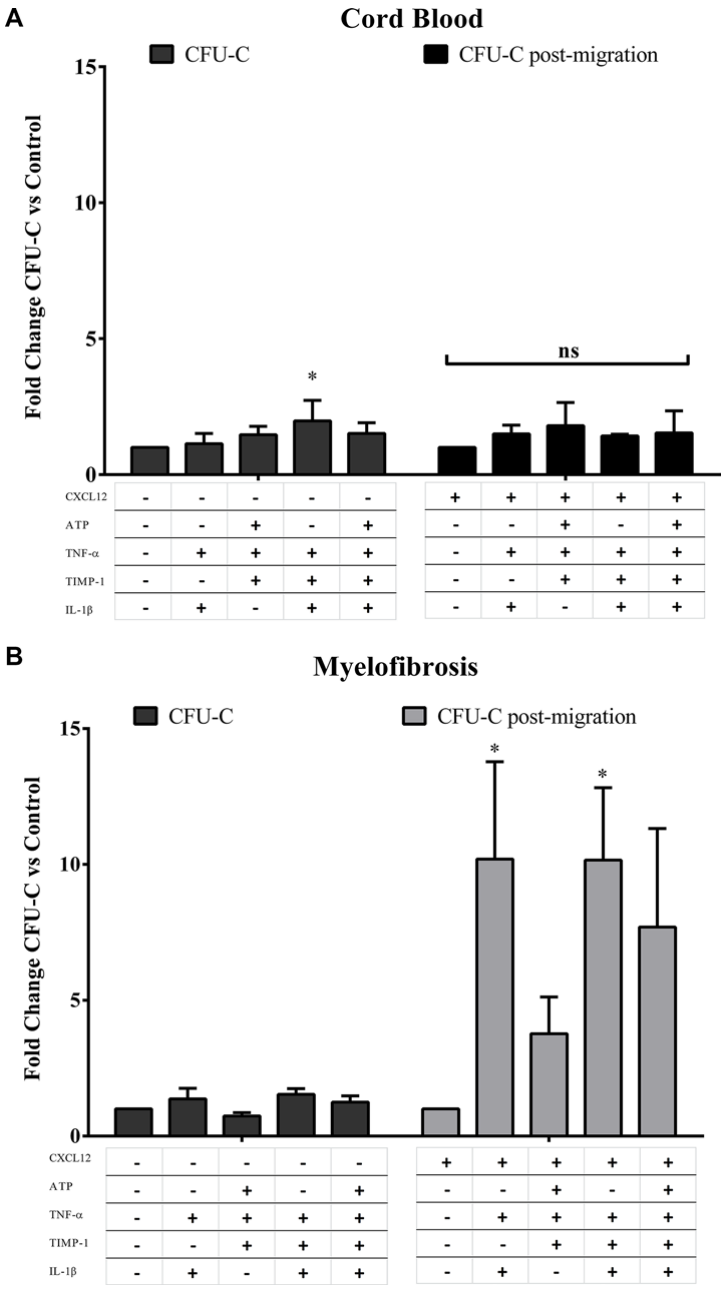

Figure 7: The clonogenic output of MF-derived CD34+ cells after migration toward IL-1β + TNF-α + CXCL12 ± TIMP- 1 is potently enhanced. Panels (**A** and **B**) show the clonogenic potential of CB-derived (A; *n* = 6) and MF-derived CD34+ cells (B; *n* = 14) at baseline with or without various combinations of pro-inflammatory factors (CFU-C) and after migration toward CXCL12 alone or various combinations of pro-inflammatory factors + CXCL12 (CFU-C post-migration). After migration toward IL-1β + TNF-α + CXCL12 ± TIMP-1, the MF-derived, but not CB-derived, CD34+ cells show significantly increased clonogenic potential. Results are expressed as mean fold change of CFU-C ± SEM. (**p ≤ 0.05 vs untreated cells (A) and CXCL12 alone (B)*).

DISCUSSION

Here, we evaluated the *in vitro* effects of four main crucial factors of the inflammatory microenvironment (IL-1β, TNF-α, TIMP-1 and ATP) on survival, clonogenic output and migration ability of MF HSPCs.

First, this study demonstrates that, regardless of mutation status, IL-1β, TNF-α and TIMP-1 are increased in the plasma of MF patients and the presence of IL-1β, TNF-α ± TIMP-1 confers a survival advantage of MF-derived HSPCs. Second, HSPCs from *JAK2*^{V617F} show *in vitro* enhanced proliferation over untreated cells and the CB counterparts in response to IL-1β, TNF-α ± TIMP-1 exposure (alone or, mostly, in combination). Accordingly, IL-1β + TNF-α stimulates cell cycle progression of MF-derived CD34+ cells to the S-phase. Third, IL-1β + TNF-α combination promotes the *in vitro* migration of MF-derived HSPCs. Interestingly, after migration toward IL-1β + TNF-α + CXCL12 ± TIMP-1, MF-derived CD34+ cells show increased clonogenic ability as compared with CXCL12 alone or the CB counterparts. This finding was mainly due to stimulation of the clonogenic growth of HSPCs from *JAK2*^{V617F} mutated patients.

TNF-α has already been shown to facilitate clonal expansion of *JAK2*^{V617F}-positive cells in MF [26]. The results of this study provide new evidences that, in addition to TNF-α, IL-1β and TIMP-1 promote the *in vitro* maintenance of the HSPCs.

Mutation status is associated with dysregulated hemopoietic function (clonogenic output and colony composition) of MF-derived CD34+ cells in presence of IL- 1β + TNF-α ± TIMP-1. Specifically, when pro-inflammatory factors were added in culture, CD34+ cells from *JAK2*^{V617F} mutated patients showed increased clonogenic potential and increased size of the erythroid progenitors compartment as compared with the *CALR*-mutated counterparts. Along with the CFU-C growth, IL-1β stimulates the *in vitro* growth of MK progenitors of *JAK2*^{V617F} mutated patients only. Consistent data on single cell assays suggested that HSC self-renewal capacity is negatively affected by *JAK2*^{V617F}, but progenitor cells have increased proliferation capacity [42]. In addition, these findings can be related to the fact that *JAK2*^{V617F} mutant, in contrast with the *CALR* counterpart, activates not only the MK cell line but also the erythroid and granulocytic lineages [43].

Despite increased frequency of MK progenitors (CD34+CD41+ cells) in the PB of *CALR* mutated patients, we clearly demonstrate that the hemopoietic function of CD34+ cells from *CALR* mutated patients is unmodified (megakaryocytic compartment) or significantly inhibited (erythroid compartment) by IL-1β or various combinations of inflammatory factors including IL-1β. It is therefore likely that these functional abnormalities may contribute to explain the lower hemoglobin concentration that is displayed by *CALR*-positive patients compared to *JAK2*^{V617F}-mutated patients [8]. Interestingly, at variance with *JAK2*^{V617F}, *CALR* mutants moderately activate the PI3K/AKT pathway, a critical determinant of erythropoiesis and megakaryocytopoiesis [43–46].

Of note, we also found increased number of circulating CD34+CD63+ cells in MF. However, despite TIMP-1 alone was ineffective, various combinations including TIMP-1 increased the proliferation/migration of MF-derived CD34+ cells. This finding was not due to upregulated expression of CD63 receptor on MF- derived CD34+ cells after exposure to IL-1β and/or TNF-α (data not shown). It is therefore likely that downstream intracellular signaling pathways are hyperactivated and stimulate clonogenic activity.

Overall, our data indicate that the *in vitro* behavior of the MF-derived HSPCs can be upregulated by regulatory signals provided by the microenvironment and, specifically, through the cooperation between various pro- inflammatory factors. Therefore, the increased number of HSPCs in the peripheral blood of MF may be due not only to the displacement of HSPCs from bone marrow into peripheral blood, but also to the proliferative/survival signals coming from the pro-inflammatory factors within the peripheral blood niche. As a consequence, the pro-inflammatory microenvironment emerges as central site for cell division and proliferation.

In conclusion, the *in vitro* interplay between identified pro-inflammatory cytokines, which are abnormally increased, promotes and selects the circulating MF-derived HSPCs with higher proliferative activity, clonogenic potential and migration ability. Thus, it is likely that the *in vivo* inflammatory niche plays a key role in the maintenance of the malignant hemopoietic clone. Targeting these inflammatory micro-environmental interactions may be a clinically relevant approach for MF.

MATERIALS AND METHODS

Patients and samples

Peripheral blood (PB) was obtained from 10 normal age-matched volunteers and 36 patients with MF in chronic phase. Patients characteristics according to mutational status are shown in Table 1. At the time of the study, patients were at diagnosis (19 cases) or untreated for at least two months. Previous therapies were: hydroxyurea (12 cases) and Ruxolitinib (3 cases). The diagnosis of MF was made according to WHO 2008 criteria [47]. Patients and controls provided written informed consent for the study. This study was approved by the medical Ethical Committee of the University Hospital of Bologna and was conducted in accordance with the Declaration of Helsinki. During the last trimester of pregnancy, an increased number of CD34+ HSPC are mobilized from the fetal liver and can be found in the circulating blood, including umbilical cord blood (CB). Therefore, since HSPC trafficking characterizes both the PB of MF patients and CB, we choose this physiological source for comparison. CB collections (14 cases) from normal full-term deliveries were provided by the Cord Blood Bank of the University Hospital of Bologna after written informed consent.

Table 1: Patients characteristics according to mutational status

Characteristics	*JAK2*^{V617F}-mutated patients (no. 23)	*CALR* mutated patients (no.13)	*P* value
Median age, years (range)	65 (40–82)	73 (70–84)	**0.01**
Male sex, n° (%)	12 (52%)	7 (54%)	1
Median allele burden, % (range)	89 (0,4–99)	56,5 (47–98)	0.07
Median WBC, $\times 10^9$/L (range)	8,6 (2,5–157,6)	6,6 (2,3–16)	0.48
Median hemoglobin, g/dL (range)	11,9 (8,6–15,1)	9,3 (7,7–12,7)	**0.04**
Median platelet count, $\times 10^9$/l (range)	270 (41–707)	198 (86–419)	0.44
High/intermediate 2 IPSS category, n° (%)	12 (52)	7 (54)	1
Unfavorable karyotype, n° (%)	9 (39)	3 (23)	0.46
PMF diagnosis, n° (%)	14 (61)	9 (69)r	0.7
BM fibrosis grade $\geq$ 2, n° (%)	14 (61)	13 (100)	**0.03**
Patients with splenomegaly, n° (%)	19 (83)	10 (77)	0.7
Median follow-up, months (range)	45 (1,9–114,9)	48 (10–136,3)	0.24

Cell isolation

PB, anticoagulated with ethylenediamine tetraacetic acid (EDTA), was obtained from patients/controls. Mononuclear cells (MNCs) were separated from MF and CB samples by stratification on Lympholyte-H 1.077 g/cm3 gradient (Gibco-Invitrogen, Milan, Italy), followed by red blood cell lysis for 15 min at 4°C. MNCs were then processed on magnetic columns for CD34+ cell isolation (mean purity 94% ± 5%) (MACS CD34 Isolation kit; Miltenyi Biotech, Bologna, Italy), as previously described [37].

Plasma levels measurement of selected circulating cytokines

We measured the cytokines plasma levels of patients/ controls by ELISA, according to the manufacturer's instructions. EDTA-anticoagulated PB was centrifuged for 15 minutes at 1000 g within 30 minutes of collection. The plasma was then collected and stored at -80°C until quantification. In particular, the TIMP-1 ELISA kit was provided from Boster Immunoleader (Boster Biological Technology Co., Pleasanton, CA, USA) and CXCL12 ELISA kit from Krishgen ByoSistems (Ashley CT, Whittier, CA, USA). The CiraplexTM immunoassay kit/ Human 9-Plex Array (Aushon BioSystems, Billerica, MA, USA) was used for the measurement of various cytokines including IL-1β and TNF-α.

Phenotype of circulating CD34+ cells

The phenotype of circulating CD34+ cells was evaluated in PB from MF patients and in CB samples by conventional immunofluorescence, as previously described [48]. Antibodies used to characterize the CD34+ cells are listed in Supplementary Table 1. A minimum of 1 × 104 CD34+ cells were acquired by ow cytometer BD Accuri C6 (Becton Dickinson). Analysis was performed excluding cellular debris in a SSC/FSC dot plot. The percentage of positive cells was calculated subtracting the value of the appropriate isotype controls. The absolute number of positive cells/mL was calculated as follows: percentage of positive cells × White Blood Cells count/100.

Apoptosis assay

Freshly isolated CD34+ HSPCs (2–5 × 105) from MF patients or CB units were maintained in RPMI 1640 with 10% FBS, with or without IL-1β (1,10 ng/mL), TNF-α (10,100 ng/mL), TIMP-1

(100,300 ng/mL), and ATP (100,1000 µM), alone or in different combinations. After 4 days, cells were stained for 15 min at RT with Annexin- V-FLUOS Staining Kit (Roche, Penzberg, Germany). Samples were then immediately analyzed by BD Accuri C6 (BD Bioscience). Results are expressed as percentage of live cells compared to the whole cells.

Erythroid and granulocytic progenitors assays

MF/CB-derived CD34+ cells were cultured *in vitro* to achieve hematopoietic cell differentiation and the formation of multi-lineage colony-forming units (CFU-Cs), including colony forming unit-granulocyte macrophage (CFU-GM) and BFU-E. Specifically, CD34+ cells were seeded in methylcellulose-based medium (human StemMACS HSC-CFU lite w/Epo, Miltenyi Biotech) at 5 × 102 cells/mL in 35-mm Petri dishes in the presence or absence of the selected pro-inflammatory factors: TIMP-1 (100 ng/mL; Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA), ATP (1000 µM; Sigma Aldrich, Milan, Italy), TNF-α (10 ng/mL; Thermo Scientific) and IL-1β (1 ng/mL; Thermo Scientific), alone or in combination. After 2 weeks of incubation at 37°C in 5% humidified CO2 atmosphere, CFU-C growth was evaluated by standard morphologic criteria using an inverted microscope (Axiovert 40, Zeiss).

Megakaryocytic progenitors assay

Megakaryocytic colonies (Colony Forming Unit-Megakaryocyte (CFU-MK)) were obtained using MegaCultTM-C assay (Stem Cell Technologies; Vancouver, BC, Canada), according to the manufacturer's protocols. Brie y, 5 × 103 MF/CB-derived CD34+ cells were seeded in a collagen-based medium in double chamber slides in the presence or absence of the inflammatory factors, alone or in combination. Cultures were incubated for 12 days and then dehydrated, fixed and stained with a primary antibody to the MK-specific antigen GPIIb/IIIa (CD41) linked to a secondary biotinylated antibody-alkaline phosphatase avidin conjugated detection system. CFU-MK were counted using a light microscope.

Cell cycle analysis

A total of 106 CD34+ cells was maintained in Roswell Park Memorial Institute (RPMI)-1640 (Lonza) supplemented with 10% fetal bovine serum (FBS Thermo Fisher Scientific, Waltham, MA

USA). Cells were resuspended in complete medium at a concentration of 1 × 106/mL, and primed for 24 hours with the pro- inflammatory cytokines (1 ng/mL IL-1β, 10 ng/mL TNF-α, 100 ng/mL TIMP-1, 1000 μM ATP), alone or in combination. Treated cells were first permeabilized with NP-40 (15 min at RT) and then labeled with propidium iodide (PI)/RNAse staining kit (BD Bioscience) for 15 min at RT, in the dark. The DNA content was assessed by BD Accuri C6 (BD Bioscience) and results were analyzed by FCS express 4 software.

Changes in the cell-cycle distribution were evaluated using PI. The percentage of cells in the G0/ G1, S, and G2/M phases was determined by measuring simultaneously the DNA and RNA total cellular content.

Migration assay

Migration of MF/CB purified CD34+ cells was assayed towards a CXCL12 gradient (150 ng/mL) in transwell chambers (diameter 6.5 mm, pore size 8 μm; Costar; Corning), as previously described [38]. Brie y, 50 μl of RPMI 1640 plus 10% FBS containing 0,5 × 105 cells were added to the upper chamber and 150 μl of medium with or without CXCL12 ± IL-1β (1 ng/mL), TNF-α (10 ng/mL), TIMP-1 (100 ng/mL), and ATP (1000 μM) (alone or in combination) were added to the bottom chamber. After overnight incubation at 37°C in 5% humidified CO2 atmosphere, inserts (upper chambers) were removed and cells transmigrated into lower chamber were recovered and counted by Trypan Blue exclusion test in a Neubauer chamber using an inverted microscope (Nikon) with a 10× magnification. The amount of migrated cells was expressed as a percentage of the input, applying the following formula: (number of migrated cells recovered from the lower compartment/total number of cells loaded in the upper compartment) × 100. In addition, migrated cells were assayed in methylcellulose-based medium for their ability to form hematopoietic colonies (as above described).

Mutation analysis

JAK2^{V617F} allele-burden was assessed in granulocyte DNA by quantitative polymerase chain reaction–based allelic discrimination assay (ipsogen *JAK2* MutaQuant Kit) on 7900 HT Fast Real Time PCR System (Applied Biosystem) [49]. *CALR* exon 9 *s*equencing was performed by Next Generation Sequencing (NGS) approach with GS Junior (Roche-454 platform); analysis was carried

out with AVA Software (GRCh38 as reference) [50]. Rare *CALR* mutations identified by NGS were confirmed by Sanger sequencing.

Individual colonies were harvested at day 12– 14 from 3 *JAK2*^{V617F} and 3 *CALR* mutated patients (20 individual colonies each condition). Molecular characterization of single colonies was performed on DNA extracted using REPLI-g Single Cell Kit (QIAGEN, Marseille, France), which provides accurate genome ampli cation from single cells or limited samples with high efficiency. Brie y, 4 µL of cell material (supplied with PBS) were firstly lysed. After denaturation, isothermal amplification reaction was performed and amplified DNA was used for *JAK2*^{V617F} and *CALR* mutations assessment, as above described.

Cytogenetic analysis

Chromosome banding analysis was performed on bone marrow cells by standard banding techniques according to the International System for Human Cytogenetic Nomenclature. At least 20 metaphases were required. Unfavourable karyotype included complex karyotype or single or two abnormalities including +8, -7/7q-, i(17q), -5%5q-, 12p-, inv(3) or 11q23 rearrangement [51, 52].

Statistical analysis

Numerical variables have been summarized by their median and range, and categorical variables by count and relative frequency (%) of each category. Comparisons of quantitative variables between groups of patients were carried out by the nonparametric Wilcoxon rank-sum test. All *p* values were considered statistically significant when ≤ 0.05 (2-tailed). Statistical analyses were performed using Graphpad (Graphpad Software Inc., La Jolla, CA) and SPSS software (PASW Statistics for Windows, Version 18.0. Chicago, IL).

CONFLICTS OF INTEREST

The Authors declare no conflicts of interest.

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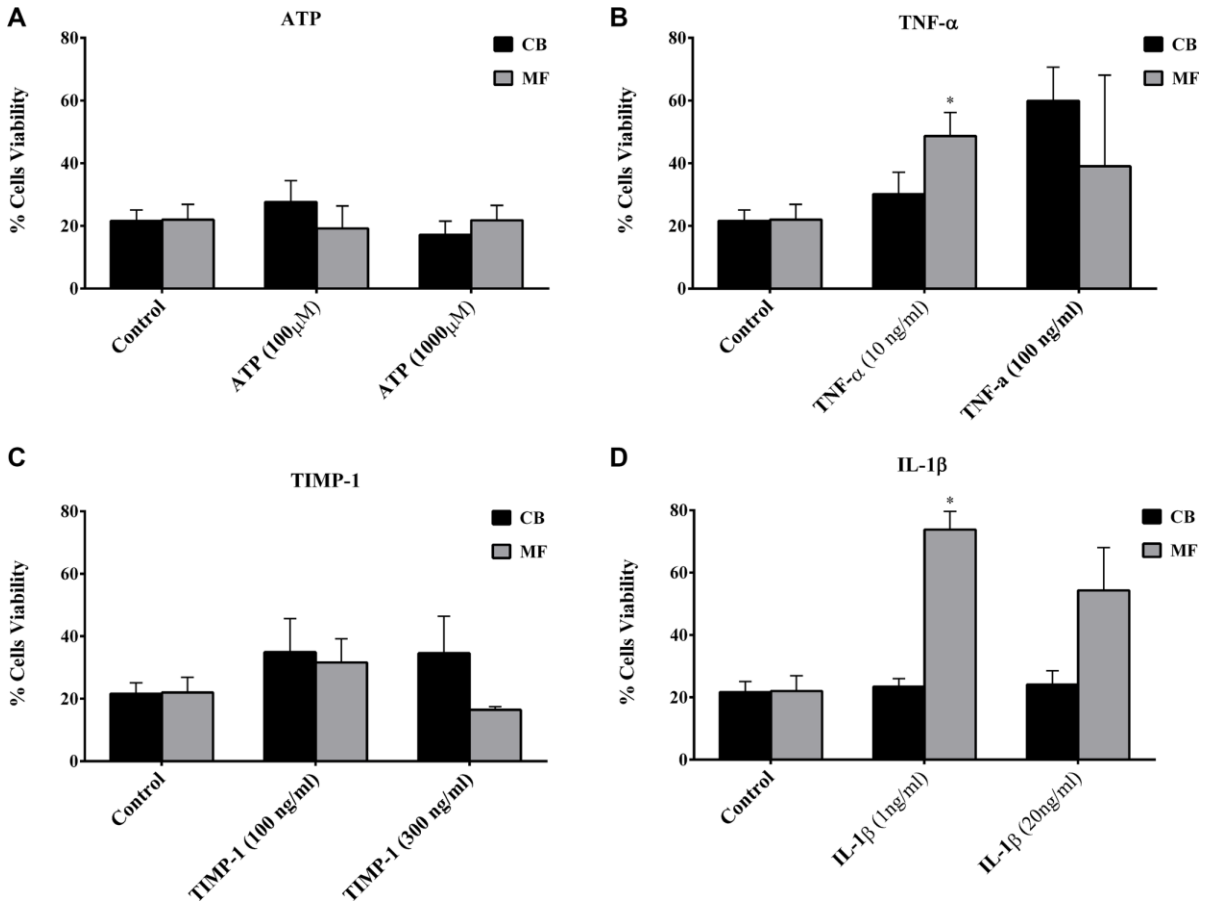
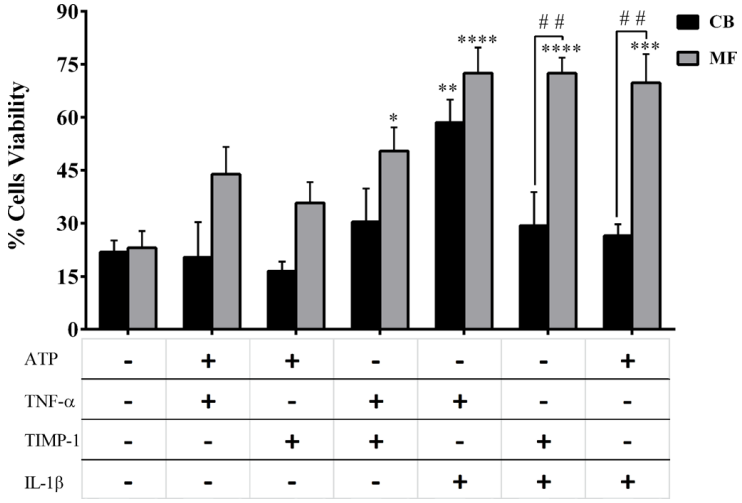
Supplementary Materials

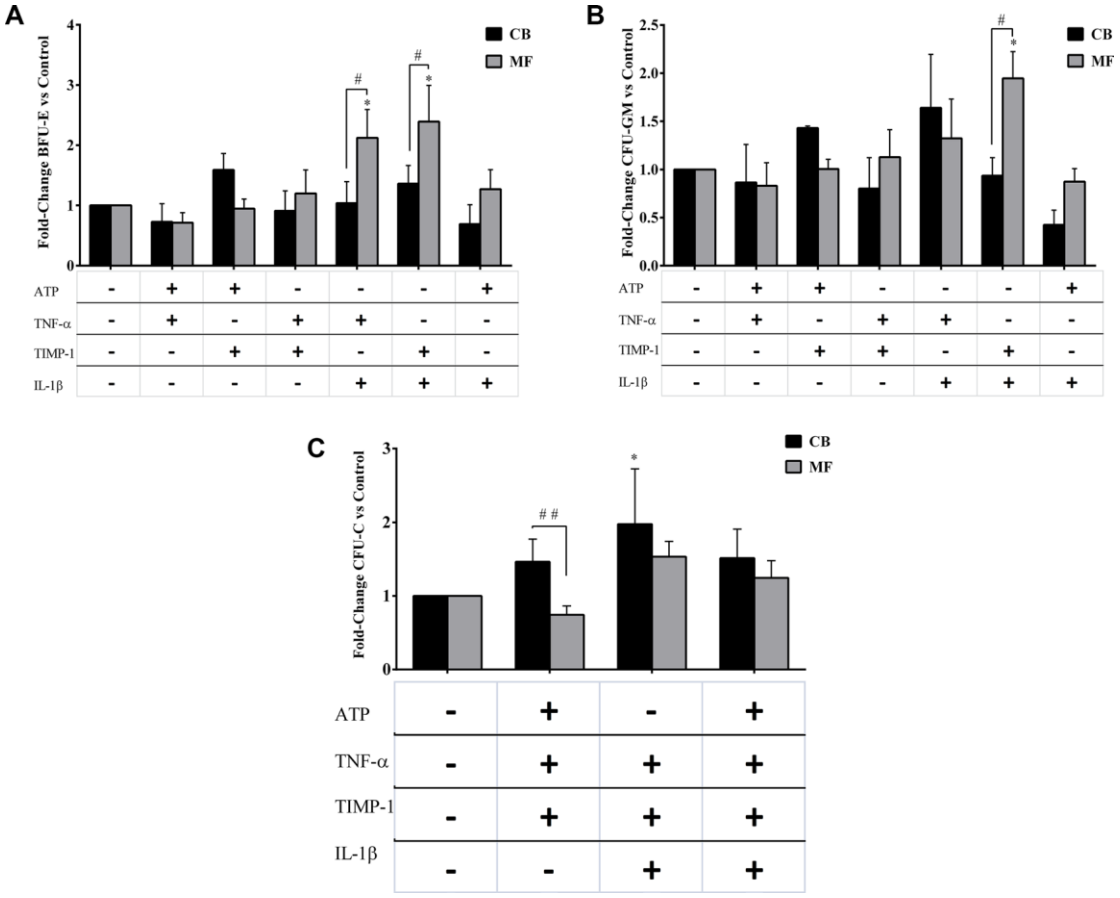
Crucial factors of the inflammatory microenvironment (IL-1β/ TNF-α/TIMP-1) promote the maintenance of the malignant hemopoietic clone of myelofibrosis: an *in vitro* study



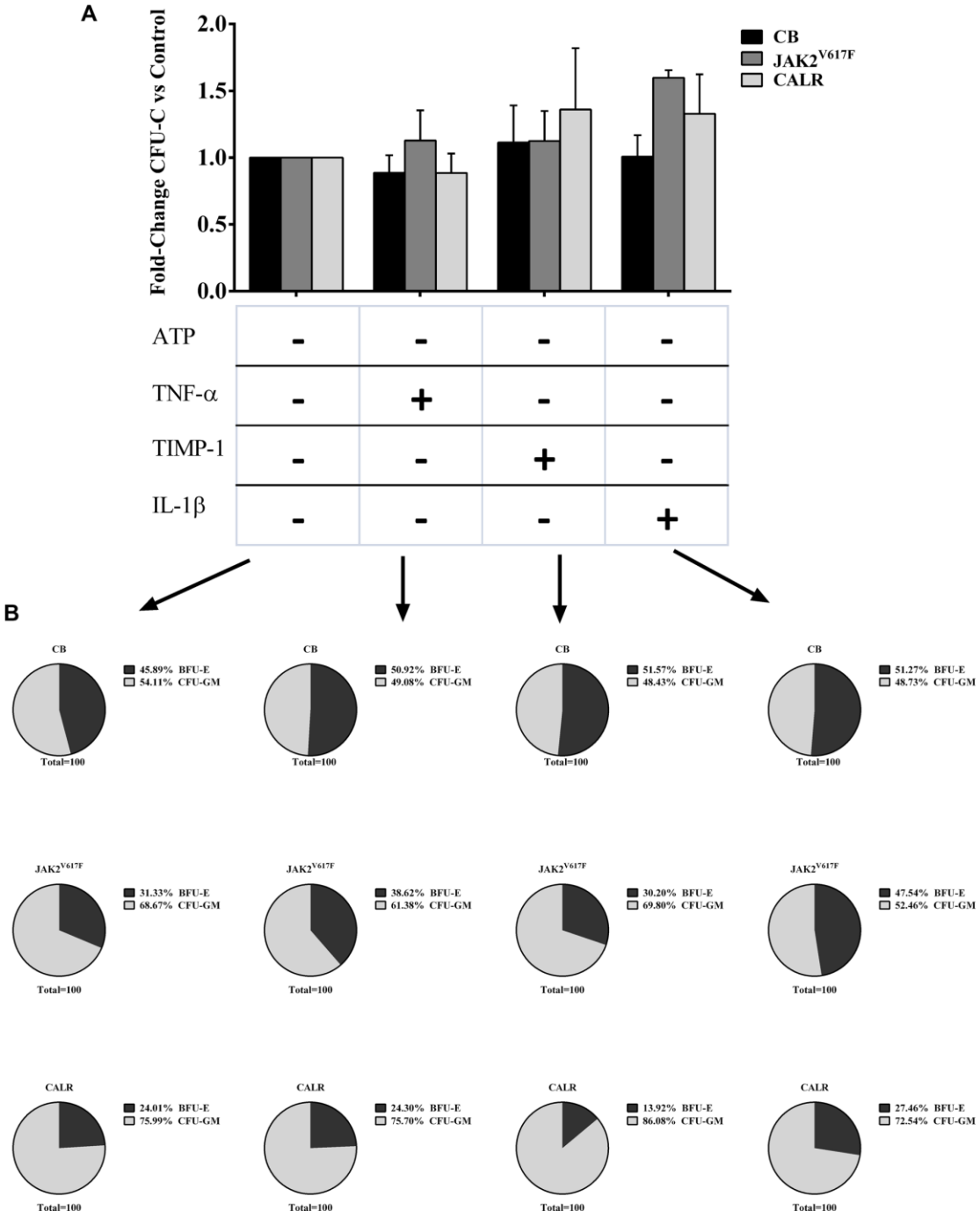
Supplementary Figure S1: Survival of MF- and CB-derived CD34+ cells after *in vitro* treatment with increasing doses of inflammatory factors. CD34+ cells from MF patients (*n* = 4) or CB (*n* = 3) were *in vitro* treated for 4 days with factors alone (ATP (100, 1000 μM), TNF-α (10,100 ng/mL), TIMP-1 (100,300 ng/mL) and IL-1β (1,20 ng/mL)) and the percentage of cell viability was assessed after AnnexinV/PI staining, as described in methods. (*$p \leq 0.05$ *vs untreated cells*).



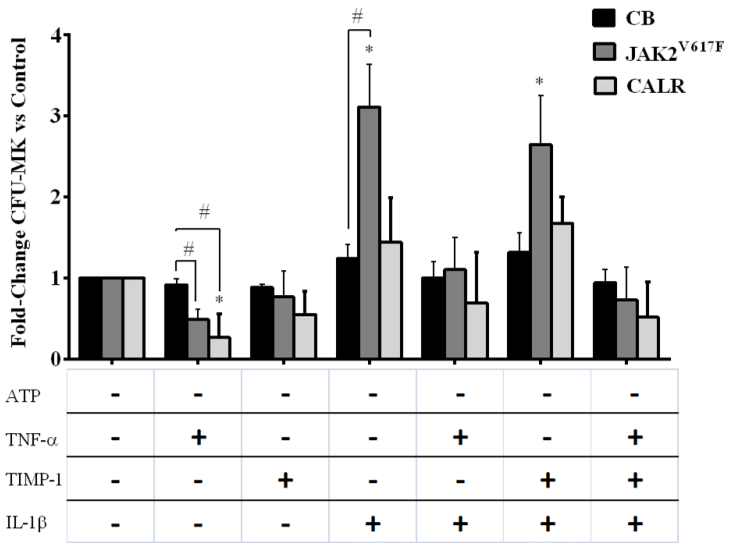
Supplementary Figure S2: Survival of CD34+ cells from MF patients is increased by various combinations of pro-inflammatory factors. CD34+ cells from MF patients (*n* = 20) or CB (*n* = 8) were *in vitro* treated for 4 days with factors two by two and the percentage of cell viability was assessed after AnnexinV/PI staining, as described in Methods. Cells viability of MF-derived CD34+ cells was significantly increased by IL-1β + TNF-α, IL-1β + TIMP-1, TNF-α + TIMP-1 and IL-1β + ATP as compared with untreated cells. Conversely, only the IL-1β + TNF-α combination was effective in stimulating the *in vitro* survival of the CB-derived CD34+ cells. Comparing MF vs CB, IL-1β + TIMP-1 and IL-1β + ATP significantly promoted the survival of the MF-derived cells. All data are presented as mean ± SEM. (**p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001 vs untreated cells (CTR)*) (*##p ≤ 0.01 vs CB*).



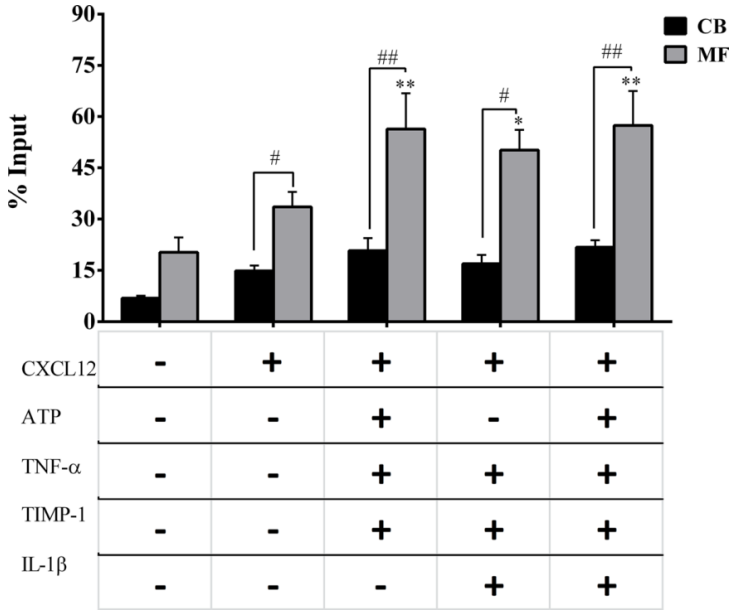
Supplementary Figure S3: Effects of inflammatory factors on BFU-E/CFU-GM growth from MF- and CB-derived CD34+ cells. Circulating CD34+ cells were isolated from MF patients (*n* = 20) and CB units (*n* = 8) and cultured in semisolid medium in the presence of the selected two-by-two or multiple pro-inflammatory factors. After 14 days, the BFU-E/CFU-GM (**A** and **B**) and the total CFU-C (**C**) output was assessed. (A) When IL-1β + TIMP-1 and IL-1β + TNF-α were added in culture, the BFU-E growth of MF-derived, but not CB-derived, CD34+ cells was significantly increased as compared with the untreated samples and the CB counterparts. None of the others combinations were effective. (B) The growth of CFU-GM from MF-derived CD34+ cells showed the same pattern displayed by BFU-E. (C) When various combinations of inflammatory factors were tested, MF-derived CD34+ cells showed a decreased number of CFU-C compared to CB in response to ATP, TNFα, and TIMP. All data are presented as mean ± SEM. (**p ≤ 0.05 vs untreated cells*) (*#p ≤ 0.05; ##p ≤ 0.01 vs CB*).



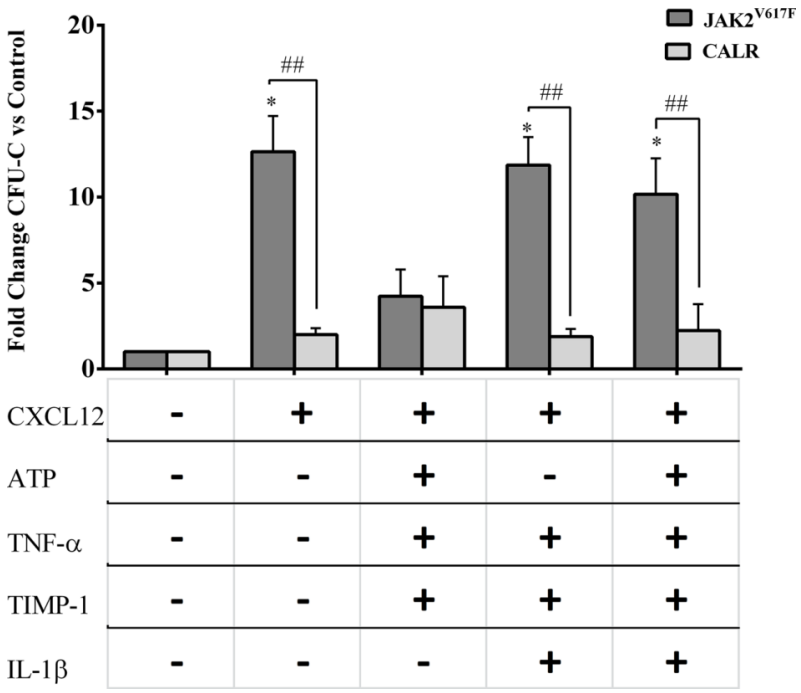
Supplementary Figure S4: Effects of pro-inflammatory factors alone on CFU-C growth of CD34+ cells from *JAK2*^{V617F} and *CALR* mutated patients. (**A**) When CFU-C growth of CD34+ cells from *JAK2*^{V617F} (*n* = 10) and *CALR* (*n* = 6) mutated patients was assessed in the presence of factors alone, no differences were observed between CB samples and between the two mutated groups. (**B**) Colony composition analysis demonstrated that only IL-1β enhanced the erythroid compartment of the *JAK2*^{V617F} mutated group.



Supplementary Figure S5: CFU-MK growth according to mutation status. CFU-MK growth from *JAK2*^{V617F} (*n* = 6) and *CALR* (*n* = 4) mutated patients was significantly inhibited by TNF-α as compared with CB counterparts. By contrast, IL-1β and IL1β + TNF-α stimulated the CFU-MK growth of *JAK2*^{V617F}mutated patients (**p ≤ 0.05 vs untreated cells*) (*#p ≤ 0.05; ##p ≤ 0.01 JAK2*^{V617F}*/CALR mutated patients vs CB*).



Supplementary Figure S6: Various combinations of pro-inflammatory factors significantly promote migration of MF- derived CD34+ cells. When migration toward multiple combinations of factors + CXCL12 was analyzed, the migration ability of MF- derived (*n* = 15), but not CB-derived (*n* = 8), CD34+ cells was signi cantly increased. (**p ≤ 0.05; **p ≤ 0.01 vs spontaneous migration*) (*#p ≤ 0.05;##p ≤ 0.01 vs CB*). Results are expressed as mean percentages ± SEM of input.



Supplementary Figure S7: CFU-C post-migration assay according to mutation status. The clonogenic potential of CD34+ cells from *JAK2*^{V617F} (*n = 8*)*/CALR* (*n = 6*) mutated patients after migration toward CXCL12 alone or various combinations of pro- inflammatory factors + CXCL12 (CFU-C post-migration) is shown. After migration toward various combinations of pro-inflammatory factors, only the *JAK2*^{V617F}-derived CD34+ cells show significantly increased clonogenic potential. Results are expressed as mean fold change of CFU-C ± SEM. (**p ≤ 0.05 vs spontaneous migration*) (*##p ≤ 0.01 JAK2*^{V617F} *vs CALR mutated patients*).

Supplementary Table S1: The following MoAbs were used to phenotypically characterize the MF- and CB-derived cells

• anti-CD34 (clone 8G12)
• anti-CD38 (clone HIT2)
• anti-CD47 (clone B6H12)
• anti-CD45 (clone HI30)
• anti-CD184 (CXCR4; clone 12G5)
• anti-CD49d (clone 9F10)
• anti-CD44 (clone G44-26)
• anti-CD41a (clone HIP8)

All from BD Biosciences (San Jose, CA USA)
• anti-CD63 (TIMP-1 receptor; clone H5C6) from eBioscience (San Diego, CA USA),
• anti-CD133 (clone AC133) (Miltenyi Biotech, Bologna, Italy).
Negative controls were isotype-matched irrelevant MoAbs (BD Biosciences and Miltenyi Biotech).

RESULT V

Ongoing study, preliminary results

INTRODUCTION

Molecular routine diagnostics for Ph-negative myeloproliferative neoplasms (MPN) is currently focused on mutations in *JAK2*, *CALR* and *MPL*. In addition to those three driver mutations, alterations in additional genes, i.e. *ASXL1*, *EZH2*, *IDH1*, *IDH2* and *SRSF2*, have been described, and have shown correlation with worse prognosis in PMF[1]. Interestingly, the presence of mutations in two or more of these five genes predicts the worst survival[2]. Furthermore, mutations in *TP53* and *TET2* also correlate with worse prognosis and an increased risk of leukemic transformation[3].

With an increasing number of molecular markers the need for clinical assays based on massively parallel next-generation sequencing (NGS) methods rises. These methods allow for a fast, sensitive, and cost-efficient high-throughput screening of genomic aberrations and their application has already fundamentally improved our understanding of how genetic alterations affect health and disease. Indeed, beyond additional diagnostic markers, NGS provides quantitative information on sequence abnormalities, which can be used to estimate the clone size and thus allows to identify potential driver mutations in a given patient. Also, NGS enables the detection of clonal evolution over the course of the disease or during treatment. Finally, by identifying multiple mutations and even low-level mutations, NGS has revealed potential prognostic impact of various mutations[4-9].

In a clinical molecular diagnostic laboratory, multigene screening with conventional platforms proves challenging due to the need for relatively large amounts of DNA to assess one gene at a time and the coordination of the several results into an integrated report. For this reason, NGS technologies are highly relevant for diagnostic purposes as alternatives to first-generation sequencing techniques. However, inclusion of NGS technologies into the diagnostic routine has been delayed due to the lack of adequate guidelines and rigorous validation. In particular, valid cutoff values are needed when describing infrequent mutations.

Today, the use gene panels is becoming more widespread, for a comprehensive characterization of patients at diagnosis. This could allow to better stratify patients into subgroups with different diagnostic and prognostic risk, in order to identify the best therapeutic strategy (personalized theraphy approach).

The aim of the present work was to identify a NGS assay that would provide accurate molecular characterization of myeloid malignancies, including MPN, for future personalized diagnosis and treatment approaches. Therefore, three different gene panels were tested and their technical performance was evaluated by various validation experiments.

MATERIALS AND METHODS

Primary cell samples

Samples from 113 patients with myeloid malignancies (76 AML, 13 secondary AML, 19 MPN, 2 CML and 3 atypical CML), were used for sequencing. Peripheral blood (PB) or bone marrow (BM) samples were taken from the patients at diagnosis (107 samples), at the time of relapse (14 samples) or during follow-up (4 samples) for a total of 125 samples. The PB from 4 healthy donors were also sequenced to detect germline variants. Blood cells were isolated by stratification on Ficoll-Hypaque gradient and were stored at -20°C until further use. The study was approved by the local ethics committee and written consent was obtained from all patients before sample analysis.

Sequencing gene panels

Three different gene panels were evaluated in this study: TruSight Myeloid Sequencing Panel (Illumina), Myeloid Solution Panel (Sophia Genetics) and AML NGS Diagnostic Custom Panel (Thermo Fisher). The TruSight Myeloid Sequencing Panel (Illumina) consists of 568 amplicons, covering 54 target genes which is associated with different haematological disorders (such as Acute Myeloid Leukemia (AML), Myelodysplastic Syndromes (MDS), Philadelphia- negative Chronic Myeloproliferative Neoplasms (MPN), Chronic Myelomonocytic Leukemia (CMML) and Juvenile Myelomonocytic Leukemia (JMML)). Of the 54 genes, 15 are fully sequenced while the remaining 39 are covered only at the "hotspot" regions (Table 1).

ABL1	**CEBPA**	HRAS	MYD88	SF3B1
ASXL1	CSF3R	IDH1	NOTCH1	SMC1A
ATRX	**CUX1**	IDH2	NPM1	SMC3
BCOR	**DNMT3A**	**IKZF1**	NRAS	SRSF2
BCORL1	**ETV6/TEL**	JAK2	PDGFRA	**STAG2**
BRAF	**EZH2**	JAK3	**PHF6**	TET2
CALR	FBXW7	**KDM6A**	PTEN	TP53
CBL	FLT3	KIT	PTPN11	U2AF1
CBLB	GATA1	KRAS	**RAD21**	WT1
CBLC	GATA2	MLL	**RUNX1**	**ZRSR2**
CDKN2A	GNAS	MPL	SETBP1	

Table 1. List of the 54 genes covered by TruSight Myeloid Sequencing Panel (Illumina). In red are signed the fully sequenced genes.

The Myeloid Solution Panel (Sophia Genetics) covers the coding regions of 30 genes associated to Myelodysplastic Syndromes (MDS), Philadelphia- negative Chronic Myeloproliferative Neoplasms (MPN) and Acute Myeloid Leukemia. Only 10, out of 30, genes are fully covered (Table 2).

ABL1	ASXL1	BRAF	CALR	CBL	**CEBPa**
CSF3R	**DNMT3A**	**ETV6**	**EZH2**	FLT3	HRAS
IDH1	IDH2	**JAK2**	KIT	KRAS	MPL
NPM1	NRAS	PTPN11	**RUNX1**	SETBP1	SF3B1
SRSF2	**TET2**	**TP53**	U2AF1	WT1	**ZRSR2**

Table 2. List of the 30 genes covered by Myeloid Solution Panel (Sophia Genetics). In red are signed the fully sequenced genes.

Finally, the AML NGS Diagnostic Custom Panel (Thermo Fisher) The panel consists of 22 genes, of which only 6 (*CEBPA*, *DNMT3A*, *EZH2*, *KDM6A*, *RUNX1* and *SETD2*) fully sequenced (Table 3). Unlike the previous two panels, this one allows to analyze simultaneously 2 pools: one of DNA and one of RNA (the latter for the research of fusion genes).

CBL	**EZH2**	HRAS	NRAS	**RUNX1**	WT1
CEBPa	FLT3	IDH1	KRAS	**SETD2**	PPM1D/WIP1
C-KIT	GATA1	IDH2	MLL	TET2	
DNMT3A	GATA2	**KDM6A**	NPM1	TP53	

Table 3. List of the 22 genes covered by AML NGS Diagnostic Custom Panel (Thermo Fisher). In red are signed the fully sequenced genes.

TruSight Myeloid Sequencing Panel (Illumina) and Myeloid Solution Panel (Sophia Genetics) were run on the Illumina platform (MiSeq) whereas AML NGS Diagnostic Custom Panel (Thermo Fisher) was run on Ion Torrent S5 platform (ThermoFisher).

Variant calling and data analysis

Human genome build 19 (hg19) was used as reference for all the three panels. Alignment to the hg19 genome and variant calling was performed by Variant Studio 2.0, Sophia DDM and Torrent Variant Caller, respectively. Run parameters and data output from each run were obtained and compared against specifications outlined by the manufacturer. Panel performance characteristics were assessed and genomic regions requiring additional analysis or wet-bench approaches identified. A sequencing coverage of 500X and a minimum variant frequency of 5% in the background of wild type were established as cut-offs for clinical reporting. As consequence, the identified variants were filtered for the minimum recommended coverage (> 500X) and for their frequency in the European population (<1%, according to the definition of non-polymorphic variant).

Subsequently, variants were further filtered for a Variant Frequency (VF, %), i.e. the ratio between the number of mutated reads and the number of wild-type reads, of 5 (cut-off for clinical reporting, as already mentioned above), 10 and 25 (which is the sensitivity of Sanger sequencing) %. Finally, a filter for all the sinonimous, intronic, intergenic or UTR regions-mapped variants (thus potentially non-pathogenic) has been apply. Analytical validation was performed by comparison with pre-derived diagnostic data.

RESULTS

With regard to the TruSight Myeloid Sequencing Panel (Illumina), more than 95% of target regions were covered with depth greater than 500X, as guaranteed by manufcturer. The medium number of nucleotide variants (nVNT) per patient, filtered only for 500X minimum coverage and for non-polymorfic variants, was 21.94, 6.86 and 3.89 with a VF of 5, 10 and 25%, respectively. By adding the filter for all non-pathogenic variants, nVNT decreas to 9.66, 3.74 and 2.16. These results show that for almost all covered genes, the majority of variants are present with a VF <25%. This means that in the diagnostic routine, we are not able to identify a good percentage of most alterations with traditional methods. This concept is clearly visible in Figure 1. Moreover, we invastigated on which genes were the most mutated in our population, also distinguishing by type of disease. The results of this analysis are shown in Figure 2.

Notably, some genes, such as *TET2*, *MPL* and *ASXL1*, have been found mutated with a frequency higher than described in literature. On the contrary, *JAK2* resulted mutated in a lower percentage of patients and this is maybe due to the low number of MPN samples in our cohort. More interenstingly, no mutations have been found in 8 out of 54 genes of the Illumina Panel (i.e. *CALR, CBLB, GATA1, HRAS, FBXW7, CDKN2A, GNAS* and *CBLC*). Therefore, we decided to perform a coverage analysis for each probe of the panel, to define if the absence of mutations in these genes could be ascribed to a low (< 500X) depth of sequencing or to a systematic error in variant calling process. The resulting data demonstarted that only *CALR, CBLB, GATA1* and *HRAS* have a poor coverage (Figure 3). Similarly, also *CEBPA* showed a poor coverage: indeed, a large proportion (5 of 6 amplicons) covering the clinically actionable CEBPA gene did not meet a minimum depth of coverage of 500X.

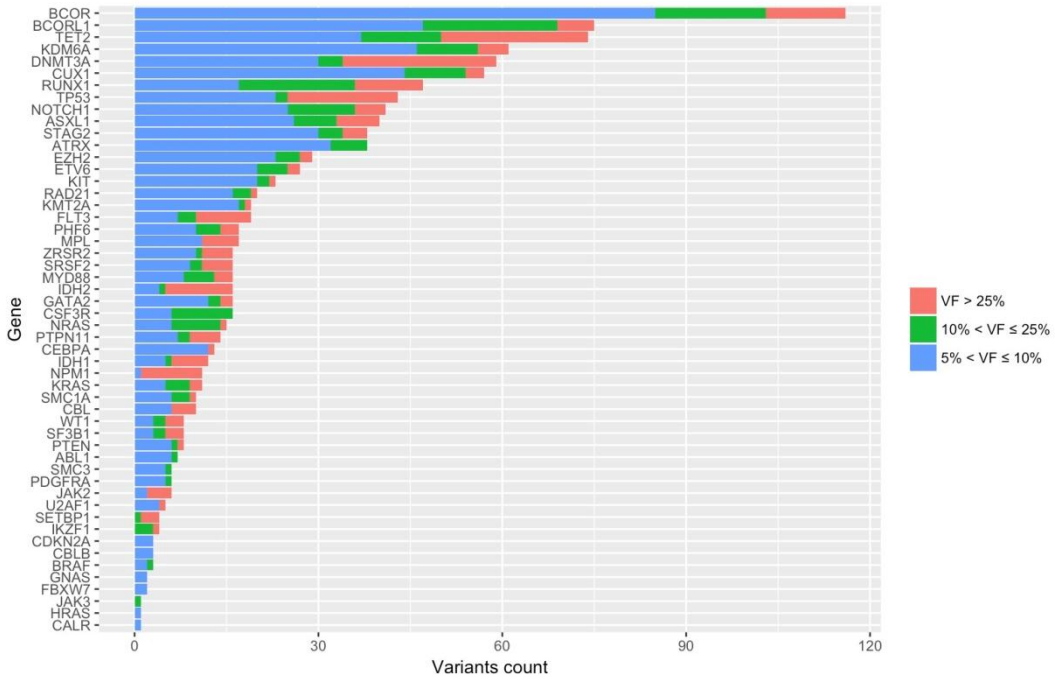


Figure 1. Schematic representation of number of variants per gene of the TruSight Myeloid Sequencing Panel (Illumina). For each gene, variants have been distributed for variant frequency (VF, %) at 5, 10 and 25%.

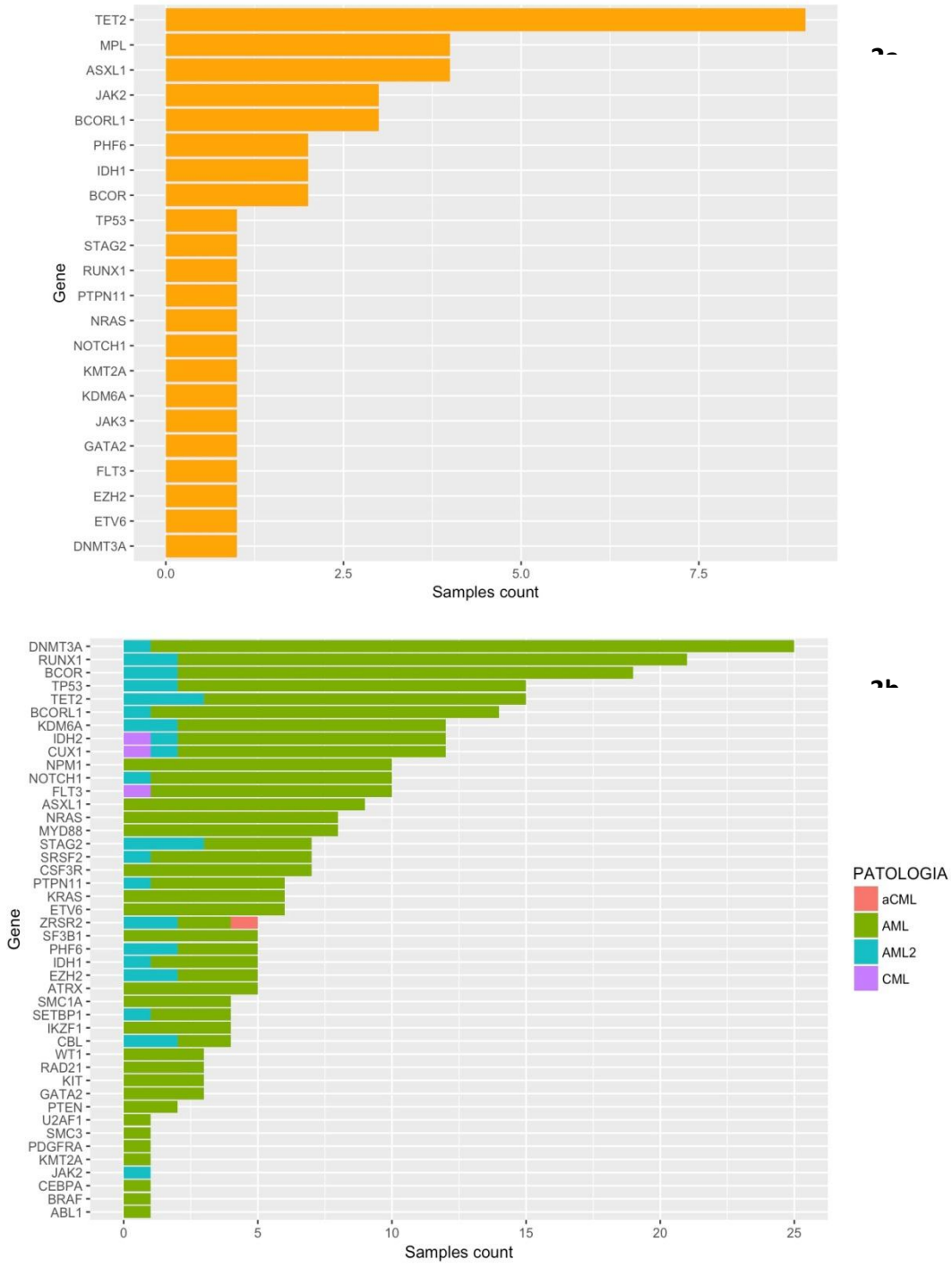


Figure 2. Graphical representation of the genes most frequently mutated in patients diagnosed with **(2a)** Ph-negative chronic myeloproliferative neoplasms (MPN) and **(2b)** with other myeloproliferative disorders.

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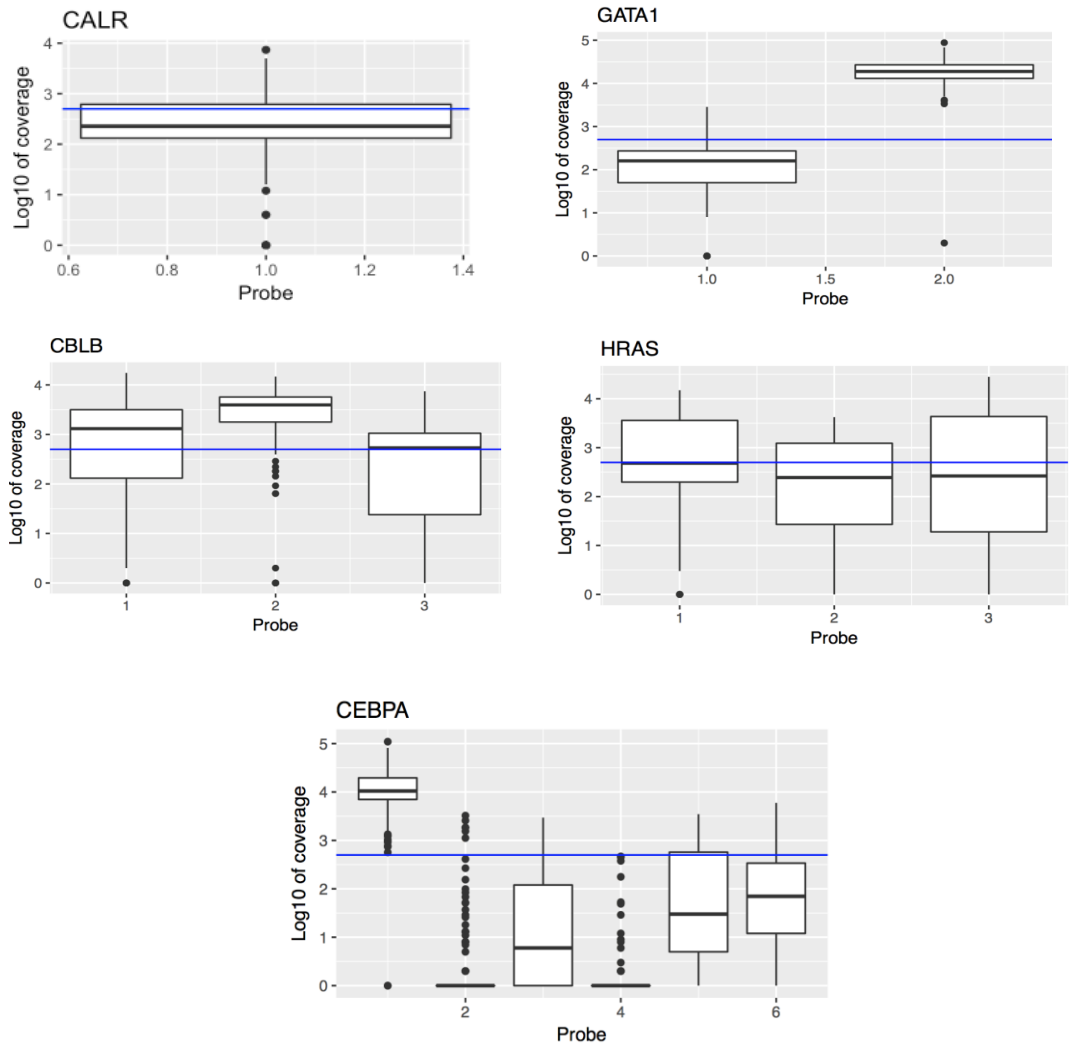

Figure 3. Boxplot of coverage data for each probe, grouped by gene. Coverage analysis has highlighted a low depth of sequencing of *CALR*, *GATA1*, *CBLB*, *HRAS* and *CEBPA* genes. Blu line represent the cut-off value of minimum recommended coverage (500X).

Based on these results, we decided to test the Genetics Myeloid Solution Panel (Sophia) in order to identify a gene panel with a better performance. A total of 16 samples (5 at diagnosis, 7 relapses and 4 follow-up) of patients with myeloid disease have been sequenced. Panel performance characteristic were similar to Illumina panel. In this case, the medium number of nucleotide variants (nVNT) per patient, filtered only for 500X minimum coverage and for non-polymorfic variants, was 11.62, 8.5, 7.2 with a VF of 5, 10 and 25%, respectively. As already described for Illumina gene panel, nVNT decreas to 3.43, 3.17 and 2.45 by adding the filter for all non-pathogenic variants. Also in this case, the variants of each gene have been subdivided according to their frequency, resulting in a different profile from the previous one: indeed, the majority of variants have been detected with a VF >25% (Figure 4). Finally, data of mean coverage per target region, extraxted from "Sophia QA" report file, has been plotted (Figure 5) keeping into account only genes with a coverage lower than guaranted from manufacturer (1000X). Overall, the Genetics Myeloid Solution Panel (Sophia) showed a better coverage than TruSight Myeloid Sequencing Panel (Illumina) except for CEPBA.

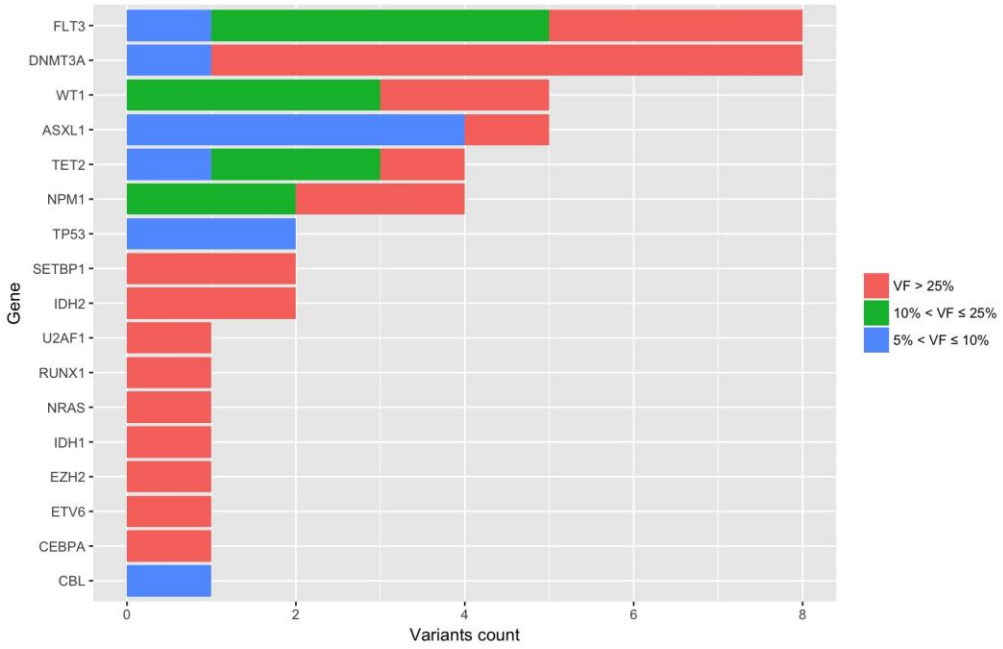

Figure 4. Schematic representation of number of variants per gene of the Genetics Myeloid Solution Panel (Sophia). For each gene, variants have been distributed for variant frequency (VF, %) at 5, 10 and 25%.

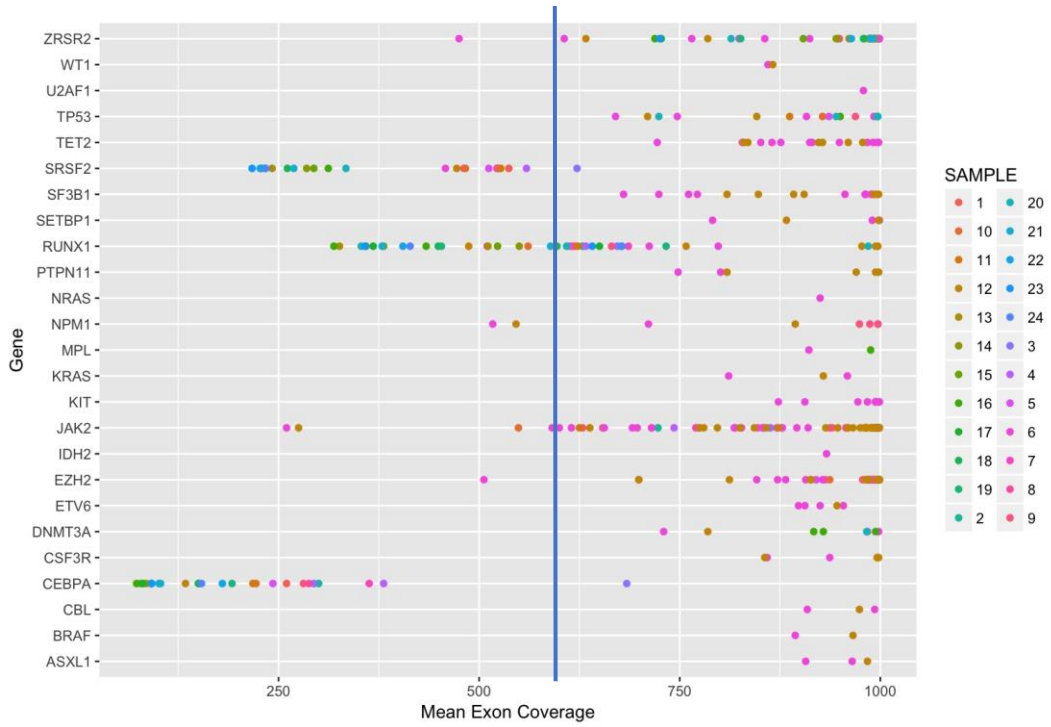

Figure 5. Representation of coverage data for each probe, grouped by gene. Coverage analysis has highlighted a low depth of sequencing of *CEBPA* gene. Blu line represent the cut-off value of minimum recommended coverage (500X).

However, these are preliminary data that need to be validated in a larger cohort, as well as the ones derived from AML NGS Diagnostic Custom Panel (Thermo Fisher). This latter gene panel has been recently acquired and, therefore, very few samples have been sequenced by using it. However, from an initial analysis, some genes (CEBPA above all) resulted to be poor covered.

CONCLUSIONS

Others have previously reported on the validation of custom NGS panels for hematologic malignancies[10-17]. These studies have shown the range of application of NGS in the context of molecular diagnostics for hematologic malignancies.

Here we report on preliminary results of the evaluation of the analytical performances of three different gene panels: TruSight Myeloid Sequencing Panel (Illumina), Myeloid Solution Panel (Sophia Genetics) and AML NGS Diagnostic Custom Panel (Thermo Fisher).

Our study demonstrated that both the TruSight Myeloid Sequencing Panel (Illumina) and Genetics Myeloid Solution Panel (Sophia) performed well across different myeloid malignancies,

covering more than 95% of target regions with depth greater than 500X. Moreover, they showed a high sensitivity, allowing to identify gene variants with very low mutation burden (<1%) in all tested patients. However, it remains to clarify how discriminate between relevant and irrelevant mutations as well as the precise pathogenetic and phenotypic role of the identified alterations.

Notably, a comprehensive validation approach needs to establish both test performance characteristics and test limitations. This approach allows for the appropriate application of the test to routine diagnostic conditions. Although both the TruSight Myeloid Sequencing Panel (Illumina) and Genetics Myeloid Solution Panel (Sophia) showed an overall high analytical performance, covering analysis revealed a low (< 500X) depth of sequencing in some recurrent genes, i.e. *CALR*, *GATA1*, *CBLCB*, *HRAS* and *CEBPA*. Evaluating the ability of gene panels to detect large insertions or deletions (such as in *CALR*) or variations in regions with high GC content (such as in *CEBPA*) may enable an assessment of whether alternative approaches to improve sequence coverage are needed. More interestingly, on the other hands, the use of multiple bioinformatics approaches may allow for maximizing variant calling. Indeed, given the large amount of data generated by a NGS approach, the improvement of informatics tools is mandatory.

In conclusion, the identification of factors contributing to disease initiation and progression, as well as the respective influence on the clinical disease course is needed to optimize the diagnosis, prognostic scoring and therapeutic approaches. Therefore, with improved turnaround time, decreasing costs, and an expanding knowledge of the therapeutic and prognostic significance of the detected variants, NGS-based panel testing is likely to play a major role in the management of patients with myeloid malignancies. However, some efforts are necessary to identify the most suitable approach in order to translate it into diagnostic process. Interestingly, setting of bioinformatics tools may simply the interpretatiosn of results.

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CONCLUSIONS

The overall aim of this study was the molecular characterization of MPN patients, and the study of interactions between malignant hematopoietic stem/progenitor cell (HSC/PC) and the inflammatory cell micro-environment.

- ✓ Mutational status and outcome of young (≤ 40 years old) ET and early-PMF patients have never been investigated. The comparison between the two groups of patients showed that mutation frequency were 61% for *JAK2*^{V617F}, 25% for *CALR* and 1% for *MPL*W515K, and were comparable across WHO diagnosis; however, *JAK2*^{V617F} allele burden was higher in the early-PMF group. Compared with *JAK2*^{V617F}-positive patients, *CALR*-mutated patients presented lower thrombotic risk (9.1% versus 21.7%, P = 0.04), longer survival (100% *vs* 96%, P = 0.05) and better combined-EFS (86% *vs* 71%, P = 0.02). Interestingly, non-type 1/type 2 *CALR* mutations ('minor' mutations) and abnormal karyotype were found to correlate with increased risk of disease evolution. The assessment of the molecular status is of utmost importance in these cohorts of patients, as both thrombosis and survival were significantly influenced by mutation type and burden.

- ✓ The determination of the mutation load is becoming a standard diagnostic procedure in most molecular laboratories, though WHO criteria do not specify a cut-off value for the diagnosis of a MPN. Our results highlight that the detection of a *JAK2*^{V617F} mutation at low levels (0.1-3%) is difficult to be interpreted in everyday clinical practice, since not all positive patients received a hematological diagnosis. However, all patients with an AB ≥0.8% finally received a diagnosis of MPN; therefore, a mutation load above this cut-off may be considered very indicative for the presence of a myeloproliferative disease. Additionally, the study identified the prospective evaluation of *JAK2*^{V617F} mutation load as a convenient and non-invasive method to evaluate patients with small mutated clones in order to timely detect the expansion of clonal hematopoiesis and diagnose a full blown disease.

- ✓ In the context of highly sensitive allele-specific assays and low mutant AB in the peripheral blood, the standardization of molecular techniques is urgently needed. We demonstrated that a qualitative approach is not sensitive enough to detect *JAK2*^{V617F} mutation at low mutation burden (i.e. < 1%). Conversely, the quantitative approach proved to be highly efficient and sensitive, although a modest variability was observed between all participating centers. Interestingly, between all tested *JAK2*^{V617F} mutation detection assays, only the qualitative ARMS-PCR methods (both "in-house" and ipsogen *JAK2* MutaSearch kit) and the quantitative approach (ipsogen *JAK2* MutaQuant kit) were able to detect the positivity of samples with an AB of 1%, with an acceptable variability observed at this AB. With regard to samples with AB >1%, both qualitative and quantitative methods allow to detect mutation burden with a lower inter-laboratory variability. Overall, the quantitative ipsogen *JAK2* MutaQuant kit assay performed consistently across different platforms, affirming itself as a robust method to obtain comparable results. This study sets the basis for the standardization of molecular techniques.

- ✓ The *in vitro* interplay between various pro-inflammatory cytokines, which are abnormally increased, promotes and selects the circulating MF-derived HSPCs. Interestingly, we found that IL-1β and TIMP1 (in addition to TNF-α) confer a survival advantage of MF-derived HSPCs, enhancing their proliferation and *in vitro* migration. Additionally, after migration toward IL-1β + TNF-α (± TIMP-1), MF-derived CD34+ cells show increased clonogenic ability. Therefore, the increased number of HSPCs in the peripheral blood of MF may be due not only to the displacement of HSPCs from bone marrow into peripheral blood, but also to the proliferative/survival signals coming from the pro-inflammatory factors within the peripheral blood niche. As a consequence, the pro-inflammatory microenvironment emerges as central site for cell division and proliferation. Thus, it is likely that the *in vivo* inflammatory niche plays a key role in the maintenance of the malignant hemopoietic clone. Targeting these inflammatory micro-environmental interactions may be a clinically relevant approach for MF.

- ✓ Althought NGS technologies proved to be highly efficient for diagnostic purposes, no adequate guidelines and rigorous validation have been done. Our preliminary date suggested that both the TruSight Myeloid Sequencing Panel (Illumina) and Genetics Myeloid Solution Panel (Sophia) performed well across different myeloid malignancies, covering more than 95% of target regions with depth greater than 500X. Moreover, they showed a high sensitivity, allowing to identify gene variants with very low mutation burden (<1%) in all tested patients. However, it remains to clarify how discriminate between relevant and irrelevant mutations as well as the precise pathogenetic and phenotypic role of the identified alterations. Interestingly, some recurrent genes (i.e. CALR, GATA1, CBLCB, HRAS and CEBPA) showed a lower depth of sequencing, requiring an alternative molecular approach. Alternatively, the optimization of bioinformatics tools could be utmost important to facilitate variant calling process and data interpretation.

In conclusion, our study allows to better characterize MPN patients on a molecular level and from the point of view of the inflammation. Of note, we set the basis for the standardization of molecular techniques and for the validation of a robust NGS approach to be transferred into a diagnostic setting. An investigation on a larger cohort of patients might be useful to strengthen our data and to better stratify patients at diagnosis, in order to improve clinical outcome as well as to identify new potential molecular therapeutic targets for personalized therapies.

On the other hand, we identify IL-1β and TIMP-1 as novel promoting factors of the *in vitro* maintenance of MF-derived HSPC. These results elucidate the mechanism of maintenance of the malignant hemopoietic clone and identified two molecular biomarkers that may be exploited as potential targets of therapy. The investigation on patients treated with ruxolitinib will allow to shed light into the effect of the therapy on the mutational status and the inflammatory microenvironment.