JAK2 Mutation-Related Disease and Thrombosis

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Abstract

A recurrent *JAK2*V617F mutation is typically associated with chronic myeloproliferative neoplasms (MPNs) that include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis. This mutation results in a gain of function that is credited to underlie most of the pathogenesis and phenotypic characteristics of these disorders; it serves as a key diagnostic marker and represents a suitable target for JAK2 inhibitors. Because cardiovascular events represent the main cause of morbidity and mortality in PV and ET, current patients' risk stratification is based on variables predicting individual thrombotic risk (age and previous thrombotic history). However, evidence is accumulating that supports a role of *JAK2*V617F mutation as a novel risk factor for thrombosis, although prospective validation has not been provided yet. In this review, we discuss about potential mechanisms that link mutated JAK2 with the thrombotic propensity of MPN and the clinical correlates; hopefully, novel information could result in better patient management.

Keywords

- *JAK2* mutation
- myeloproliferative neoplasms
- thrombosis
- risk factors
- leukocytosis
- endothelial cells

Polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) were initially grouped together in a family of "myeloproliferative syndromes" by William Dameshek in 1951,[1] who speculated about their overlapping clinical phenotypes and formulated the hypothesis that they all derived from a similar, unknown pathogenetic event leading to a disordered global myeloproliferation.[2] This intuition remained valid over the years and has been further reinforced by novel molecular discoveries that inform the revised 2008 classification of the World Health Organization (WHO), where the name of these disorders was modified to "myeloproliferative neoplasms" (or MPNs) (►**Table 1**).[3,4]

An overproduction of mature blood elements, with predominance of erythroid and megakaryocytic lineage in PV and ET, respectively; a disordered myeloproliferation eventually resulting in decreased production of mature blood cells and/or associated with variable degree of bone marrow fibrosis in PMF; the progressive accumulation of bone marrow fibers during the transition of PV and ET to post-PV and post-ET myelofibrosis (PPV/PET-MF)[5]; the development of extramedullary hematopoiesis, particularly in the spleen and the liver, typical of PMF and more advanced phases of PV and ET; an exceedingly high rate of vascular complications, including thrombosis in atypical sites, and common, disturbing microvessel manifestations[6]; and the propensity to evolve to acute myelogenous leukemia, and the lack of a curative approach, a part for allogeneic stem cell transplantation in some patients with PMF, all represent distinctive features of the MPN.

Some fundamental experiments and clinical achievements produced over the last few years have contributed to an improvement in understanding and management of these disorders.[2] The most relevant discoveries include the demonstration of the clonal origin of PV and ET in a common myeloid stem cell, based on enzymatic or genetic markers,[7] and the knowledge of an hypersensitivity of hematopoietic progenitors to several cytokines, including erythropoietin (Epo), a property that is at the basis of the "endogenous erythroid colonies" generated in vitro by progenitor cells capable of proliferating in the absence of Epo.[8] Among the clinical achievements, it is worthwhile mentioning that the basis for the classification and management of MPN was developed in the 1980s along with studies of the Polycythemia Vera Study Group (PVSG),[9,10] the "Bergamo" trial on the

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Table 1 Criteria for the diagnosis of chronic myeloproliferative neoplasms according to the 2008 WHO classification

	Polycythemia vera	Essential thrombocythemia	Primary myelofibrosis
Major criteria	1. Hb $>$ 18.5 g/dL (men) or $>$ 16.5 g/dL (women) *or* Hb or Hct $>$ 99th percentile of reference range for age, sex, or altitude of residence *or* Hb $>$ 17 g/dL (men) or $>$ 15 g/dL (women) if associated with a sustained increase of $\geq$ 2 g/dL from baseline that cannot be attributed to correction of iron deficiency *or* elevated red cell mass $>$ 25% above mean normal predicted value 2. Presence of *JAK2*V617F or similar mutation	1. Platelet count $\geq 450 \times 10^9$/L 2. Megakaryocyte proliferation with large and mature morphology. No or little granulocyte or erythroid proliferation. 3. Not meeting WHO criteria for CML, PV, PMF, MDS, or other myeloid neoplasms 4. Demonstration of *JAK2*V617F or other clonal marker *or* no evidence of reactive thrombocytosis	1. Megakaryocyte proliferation and atypia accompanied by either reticulin and/or collagen fibrosis *or* in the absence of reticulin fibrosis, the megakaryocyte changes must be accompanied by increased marrow cellularity, granulocytic proliferation, and often decreased erythropoiesis (i.e., prefibrotic PMF) 2. Not meeting WHO criteria for CML, PV, MDS, or other myeloid neoplasms 3. Demonstration of *JAK2*V617F or other clonal marker *or* no evidence of reactive marrow fibrosis
Minor criteria	1. BM trilineage myeloproliferation 2. Subnormal serum Epo level 3. EEC growth		1. Leukoerythroblastosis 2. Increased serum LDH 3. Anemia 4. Palpable splenomegaly
Diagnostic combinations	Both major criteria + one minor criterion *or* first major criterion + two minor criteria	All four criteria must be met	All three major criteria + two minor criteria

Abbreviations: BM, bone marrow; CML, chronic myelogenous leukemia; EEC, endogenous erythroid colonies; Epo, erythropoietin; Hb, hemoglobin; Hct, hematocrit; LDH, lactate dehydrogenase; MDS, myelodysplastic syndrome; PMF, primary myelofibrosis; PV, polycythemia vera; WHO, World Health Organization.

use of hydroxyurea in ET,[11] the European Collaboration on Low-Aspirin in Polycythemia vera (ECLAP),[12] and the Primary Thrombocythemia-1 (PT-1)[13] trials. However, the molecular pathogenesis of MPN remained substantially unknown until 2005, when the first recurrent molecular abnormality, a V617F point mutation in *JAK2*, was described,[14–17] which allowed to reconcile several of the original speculations of Dameshek as well as the consistent body of knowledge collected over the years. As a matter of fact, following the description of the *JAK2*V617F mutation, there has been a renewed interest in this field that resulted in an incredible amount of novel discoveries concerning molecular and cellular abnormalities and the development of large clinical studies that are paving the pathway for refined diagnosis and more effective management. This review will focus on recent insights into the pathogenesis of thrombosis in PV and ET and the emerging role of *JAK2*V617F mutation.

*JAK2*V617F (and Other) Mutation(s) in the Pathobiology of MPN

The MPN-associated *JAK2* mutation, a valine-to-phenylalanine substitution at position 617 (V617F), was described almost concurrently by four different research groups.[14–17] The mutation is located in the JH2 (JAK homolog 2) autoinhibitory domain, which does not possess enzymatic activity on target substrates but negatively regulates the function of the catalytic JH1 domain. This initial model of JAK function positing that the JH2 domain prevents activation of the JH1 domain[18,19] has been challenged in part by demonstrating that the JH2 domain is required for physiologic cytokine-dependent JAK activation[20] and that it actually functions as a dual specific kinase, capable of auto-phosphorylation at S523 and Y570.[21] However, the V617F mutation finally results in a gain of function of JAK2 which autonomously—that is, in the absence of a cytokine bound to the cognate receptor—activates downstream pathways, including JAK-STAT, PI3K/Akt, and ERK1/2 MAPK signaling.[22] The central role of this mutation in MPN pathogenesis is supported by the growth-factor independence acquired by factor-dependent cell lines that had been transduced with the V617F allele and by modeling the disease in animals; in fact, retroviral, transgenic, and conditional knock-in mouse models have shown that expression of *JAK2*V617F is sufficient to recapitulate a myeloproliferative disease,[23] usually with the characteristics of PV eventually followed by changes suggestive of myelofibrotic transformation.[22] Of interest, transgenic mice that express varying ratios of the V617F and wild-type *JAK2* alleles suggested that the phenotype may be at least in part dependent on the burden of mutated allele; in fact, when the V617F allele was less expressed than the wild-type one, mice presented thrombocytosis but minimal erythrocytosis or leukocytosis, while in the presence of a relative preponderance of the V617F allele, a more pronounced erythrocytosis and leukocytosis developed.[24] Finally, most of the Epo-independent erythroid colonies in PV patients were found to harbor the *JAK2*V617F mutation.[25,26]

The *JAK2*V617F mutation is detected in over 95% of PV and 60% of ET or PMF patients. In most PV patients, as opposite to a

minority of ET, only the mutated allele is found in hematopoietic cells (homozygosity) due to a process of mitotic recombination.[14,16,17] Recent observations indicate that homozygous progenitors can be found in both PV and ET, but only in PV, an expansion of an homozygous clone that becomes prevalent over the heterozygous clones takes place.[27] Therefore, acquisition of a dominant homozygous clone may be associated closely with the development of a polycythemic phenotype.[28] In 40 to 50% of patients with a diagnosis of V617F-negative PV, additional genetic defects including mutations, deletions, or insertions were described in *JAK2* exon 12, upstream to V617F.[29] These abnormalities induce a constitutive activation of JAK-STAT pathway at even greater level than the V617F mutation, and produced an overt polycythemic phenotype when expressed in mice. In a study involving 106 patients with 17 different exon 12 mutations, an isolated erythrocytosis was detected in two-thirds, while the remaining subjects also presented leukocytosis and/or thrombocytosis; collectively, at diagnosis, the hemoglobin level was higher and the platelet and leukocyte counts were lower compared with *JAK2*V617F-positive PV patients.[30] However, the rate of thrombosis, myelofibrosis, leukemia, and death were similar in *JAK2*V617F and *JAK2* exon-12 mutated patients.

An additional recurrent molecular abnormality discovered in 5 and 10% of patients with ET and PMF is mutations involving codon 515 of *MPL*,[31,32] the gene encoding the receptor for the cytokine thrombopoietin. These point mutations cause a transition of W to L (most commonly), K or A residue. The 515 codon is located in a RWFQP motif in the transmembrane–juxtamembrane junction of *MPL*, and residue substitutions at this position were previously shown to affect the stability of *MPL* resulting in its ligand-independent activation.[33] Mice with retroviral expression of the W515L allele develop an acute aggressive disorder with extreme thrombocytosis and leukocytosis, bone marrow reticulin fibrosis, extramedullary hematopoiesis, and have significant shortening of life span.[32] *MPL* mutations in ET patients were associated with significantly higher platelet count and lower hemoglobin levels,[34,35] as observed also in PMF patients.[36] In some patients, *MPL* and *JAK2*V617F mutations coexist.[34,36]

It is still debated how a single mutation in *JAK2* or *MPL* might associate with different clinical phenotypes.[37] Several not mutually exclusive explanations have been considered, such as the varying burden of hematopoietic cells bearing the V617F allele and/or the relative contribution of V617F homozygous progenitors; the concomitant presence of additional mutations, either preceding or accompanying the *JAK2* or *MPL* mutations[38]; and the contribution of individual characteristics, genetic modifiers, or epigenetic modulators.[39,40] In addition, in the last few years, an unexpected molecular complexity of MPN emerged because of the concomitance of several additional mutations that can coexist with or without *JAK2*V617F or *MPL* mutations; most commonly affected are genes involved in epigenetic gene regulation (*TET2, DNMT3A, EZH2, ASXL1, IDH1,* and *IDH2*) or RNA splicing (*SRSF2*)[41–44] or are preferentially acquired at the time of leukemic transformation (*TP53, NRAS, IKZF*).[45–47]

Characteristics of Thrombosis in MPN and Risk Stratification Criteria

The mortality rate is increased in PV patients in an age-dependent manner,[48] while life expectancy may be normal in the majority of patients with ET.[49,50] However, a recent study from the Swedish Cancer Registry challenged, in part, this belief by showing considerably lower survival compared with general population. The relative survival rates were 0.64 (95% confidence interval [CI], 0.62 to 0.67) for PV and 0.68 (95% CI, 0.64 to 0.71) for ET. There was evidence of significant improvement in survival in patients diagnosed after 1993 as compared with previous years.[51]

The events that impact on survival in PV and ET patients are represented by fatal thrombosis, evolution to myelofibrosis, transformation to leukemia, and hemorrhages. Among these, cardiovascular events are the most impacting on the length and quality of life, although there is a trend to declining rates in recent years possibly as the result of better management and earlier diagnosis. Arterial thromboses represent 60 to 70% of all cardiovascular events in patients with PV and ET; they include transient ischemic attack, stroke, acute myocardial infarction, and peripheral arterial occlusion.[52] Venous thromboses are more common in PV than in ET patients, and they occur as deep vein thrombosis of the extremities, pulmonary embolism, and splanchnic vein thromboses (SVTs), such as portal vein thrombosis, mesenteric thrombosis, thrombosis of the hepatic veins causing Budd–Chiari syndrome,[53,54] and cerebral sinus thrombosis. In addition to large vessel occlusions, ET and PV patients suffer from microcirculatory symptoms, such as headache, dizziness, visual disturbances, distal paresthesia, and acrocyanosis. Erythromelalgia, the most typical although relatively uncommon of the microvascular disturbances, consists of congestion, redness, and burning pain involving the extremities and is usually highly responsive to aspirin, supporting the pathogenetic role of platelet aggregates.[55] Thrombosis occurred in 1.75% patient-year in a study involving 707 patients with myelofibrosis, with an adjusted rate of 2.2% patient-year, thus comparable with that observed in patients with ET.[56]

The information that is currently available on cardiovascular events derive from several retrospective series and a few seminal prospective trials. They include the PVSG studies that explored the use of phlebotomy, radioactive phosphorus, hydroxyurea, and chlorambucil in PV[9,10]; the "Bergamo trial" that compared hydroxyurea versus no treatment in 114 ET patients[11]; the experimental ($n = 518$)[12] and observational ($n = 1{,}638$)[57] arm of the ECLAP trial; the PT-1[13] study in 809 patients with high-risk ET comparing hydroxyurea plus aspirin *versus* anagrelide plus aspirin; and the most recent CYTO-PV study that randomly assigned 365 PV patients already under treatment with phlebotomies, hydroxyurea, or both, to a more intensive (target hematocrit, $< 45\%$) or less intensive (target hematocrit, 45 to 50%) treatment.[58]

The first thrombotic event may occur as the manifestation leading to the diagnosis of MPN or during the follow-up of an already known disease. In a survey conducted by the GIMEMA group in 235 patients with PV and 259 with ET with previous

arterial or venous event (67.6 and 31%, respectively; 1.4% for both districts), the recurrence rate was 7.6% patient-year.[59] In another series of 143 *JAK2*V617F-positive patients with ET, the cumulative probability of a second event at 10 years was 42.2%.[60] In patients with PV included in the ECLAP study,[12] cardiovascular mortality accounted for 1.5 deaths per 100 persons per year and the cumulative rate of nonfatal thrombosis was 3.8 events per 100 persons per year. Age older than 60 years and previous thrombosis have been identified as major predictors of vascular complications.[16,17] By incorporating these variables in a clinically oriented scheme (►**Table 2**), useful for therapeutic indications,[61] patients with PV or ET can be stratified in a "high-risk" or "low-risk" category according to their age and previous history of thrombosis; an "intermediate-risk" category, which include younger patients with coexisting generic cardiovascular risk factors such as hypertension, diabetes, hyperlipidemia, smoking, or genetic alterations of hemostatic factors in the absence of previous thrombosis, is also considered by some investigators, but formal proof of its relevance for therapeutic decisions is still lacking.[62] Guidelines of the British Committee for Standards in Haematology for ET also include a platelet count $> 1{,}500 \times 10^9$/L among thrombotic risk factors, although such extreme thrombocytosis has been associated with increased hemorrhagic rather than thrombotic risk; furthermore, low-risk patients are subclassified according to age lower than 40 years and between 40 and 60 years.[63] However, the concept of "younger age" as equivalent to "low risk" is challenged by recent observations in a retrospective series of 120 PV patients younger than 45 years showing that, despite they presented a lower leukocyte count and V617F allele burden, the rate of vascular complications was similar to a group of 84 patients older than 65 years (27 vs. 31%, respectively); of note, there was a striking prevalence of SVT in the younger subjects (13 vs. 2%, $p = 0.005$), particularly in females.[64]

Pathogenesis of Thrombosis in MPN: The Role of Abnormal Blood Cell Count

Different factors may concur to the multifactorial and complex pathogenesis of thrombosis in patients with PV and ET. They include rheological abnormalities due to increased red cell mass in PV, abnormalities in platelet function, activation of leukocytes, abnormalities of endothelial cells, and a hypercoagulable state.[65]

In the large majority of studies, thrombocytosis has not been identified as risk factor for thrombosis. A post hoc analysis of the ECLAP trial showed that platelet count greater than 500×10^9/L did not impact on thrombosis,[66] confirming observations of the PVSG-01 trial; even in the presence of extreme thrombocytosis, thrombotic events were not directly correlated with platelet count.[67] On the contrary, a platelet count in excess of $1{,}500 \times 10^9$/L is usually considered as risk factor for bleeding due to an acquired von Willebrand disease, thus suggesting caution in the use of anti-aggregating agents.[68] It is worthwhile mentioning that even extremely elevated platelet count in the setting of reactive thrombocytosis is not credited to favor thrombosis.

On the contrary, there has been much debate about the contributing role of increased hematocrit to thrombosis in PV,[66] notwithstanding the known negative effects of erythrocytosis on blood flow stasis, hypercoagulability, and endothelial injury.[69] In a seminal study, yet conducted in a small population of patients with PV, a clear correlation between raised hematocrit and thrombosis was demonstrated;[70] it was based mainly on these information that target hematocrit levels for treatment were conventionally set at 45 and 42% for men and women, respectively.[71] However, a time-dependent multivariate analysis of the ECLAP patient population failed to confirm a correlation between increased hematocrit up to 52% and major cardiovascular events.[66] Findings from a large, multicenter, randomized, and controlled trial, the CYTO-PV trial, have been reported recently; the aim of the study was to assess the benefit/risk profile of cytoreductive therapy with phlebotomy or hydroxyurea, or both, on the top of low-dose aspirin, to maintain hematocrit below 45 versus 45% to 50%.[58] In that study, 365 patients with PV were randomly divided to a more intensive treatment to a target hematocrit of 45% or a less intensive treatment to hematocrit in the range 45 to 50%. After a median follow-up of 31 months, there were more patients fulfilling the primate composite endpoint of death from cardiovascular causes or major thrombotic events in the higher hematocrit arm (9.8%) compared with lower hematocrit (2.7%), accounting for a hazard ratio of 3.91 (95% CI, 1.45 to 10.53; $p = 0.007$). Results of this study clearly established the contributing pathogenetic role of a raised hematocrit and blood viscosity to the pathogenesis of thrombosis in PV, and definitely set the optimal hematocrit level for treatment. Uncertainties still remain, based also on blood volume physiology reasonings, whether women should be maintained to a more physiologic hematocrit level of less than 42%.[72]

The role of leukocytosis as an independent risk factor for thrombosis has been investigated more recently.[73] In a time-dependent analysis of PV patients in the ECLAP observational arm, a leukocyte count greater than 15×10^9/L was associated with a significantly greater risk of thrombosis (hazard ratio, 1.71; 95% CI, 1.10 to 2.65), mainly due to myocardial

Table 2 Criteria for risk stratification of patients with polycythemia vera and essential thrombocythemia

Risk category	Age > 60 years or history of thrombosis	Generic cardiovascular risk factors
Low	No	No
Intermediate	No	Yes
High	Yes	–

infarction (hazard ratio, 2.84; 95% CI, 1.25 to 6.46), after adjustment for potential confounders including cytoreductive and antithrombotic treatment.[74] Also in ET, the presence of a baseline leukocyte count greater than 11×10^9/L was associated with a higher risk of thrombosis.[75,76] In another cohort of 194 low-risk patients with ET, progressive leukocytosis in the 2 years after diagnosis, rather than leukocytosis at diagnosis, was found to associate with a higher risk of vascular complications during the follow-up.[77] However, a retrospective analysis of 407 low-risk patients with ET from the Mayo Clinic could not confirm the association of leukocytosis with thrombotic risk.[78] Leukocytosis may have an impact on recurrent arterial thrombosis especially in younger patients.[79] The still pending issue is whether leukocytosis is simply a marker for vascular disease or rather it has a causative direct or indirect role in the pathogenesis of vascular events, and as such also the target of treatment; only a prospective study could help resolve these aspects.[73]

Pathogenesis of Thrombosis in MPN: The Role of *JAK2*V617F Mutation

A *JAK2*V617F-mutated status in ET[80–82] and a high V617F allelic burden in both ET[81,83] and PV[84] have been variably associated with increased risk of thrombosis.[37,85] In the first of three independent meta-analyses, 2,905 patients with ET were considered[86]; of these, 778 referred a thrombotic event. Results indicated that the presence of the *JAK2*V617F mutation was associated with a significantly higher risk of venous thrombosis (odds ratio [OR], 2.09; 95% CI, 1.44 to 3.05), arterial thrombosis (OR, 1.96; 95% CI, 1.43 to 2.67), and thrombosis at presentation (OR, 1.88; 95% CI, 1.38 to 2.56) compared with *JAK2* wild-type patients. In the second analysis by Dahabreh et al,[87] 2,436 patients were analyzed; the overall incidence of thrombosis of 26.4% and the risk of arterial (OR, 1.68; 95% CI, 1.31 to 2.15) and venous (OR, 2.5; 95% CI, 1.71 to 3.66) thromboses resulted significantly increased in mutated patients. Similar conclusions were obtained in the third meta-analysis of Lussana et al[88] who analyzed 3,150 patients with ET. This study also concluded for significantly higher thrombotic events in *JAK2*V617F-mutated patients (32 vs. 20% in wild type). Among mutated patients, the overall risk of thrombosis was 1.92 (95% CI, 1.4 to 2.53), 1.77 (95% CI, 1.29 to 2.43) for arterial thrombosis and 2.49 (95% CI, 1.71 to 3.61) for venous thrombosis. Information about the occurrence of microvessel disturbances are limited to a few series, but in any case the risk of these manifestations also resulted significantly increased in *JAK2*V617F-mutated patients (OR, 2.1; 95% CI, 1.18 to 3.63). Studies in PV are fewer, and results are more heterogeneous; since almost all PV patients harbor the *JAK2*V617F mutation, the influence of allelic burden only on the rate of thrombotic events can be evaluated. In a series of 173 PV patients who were prospectively followed since diagnosis, those who presented a mutated allele burden greater than 75% suffered from a 3.6-fold higher relative risk (RR) (95% CI, 1.47 to 7.1) of total thrombosis, which was largely accounted for by thromboses occurred during the follow-up (RR, 7.1; 95% CI, 1.6 to 10.1).[84] Other studies failed to described similar correlations[89,90] or find weaker, yet suggestive, associations.[91] However, the frequency of thrombosis was found to increase progressively according to the presence and/or the V617F allele mutation burden in a retrospective study of 867 ET (57% of whom were *JAK2*V617F mutated) and 415 PV (all mutated) patients; the rate of thrombosis was 1.4, 2.1, and 2.7% patient-year in the categories of *JAK2* wild-type ET, *JAK2*V617F-mutated ET, and *JAK2*V617F-mutated PV patients, respectively.[92] Only 2 to 4% of ET patients present the mutation in an homozygous status; in this subgroup of patients, the risk of total thrombosis, after multivariate adjustment for potential confounders, resulted almost fourfold greater compared with *JAK2*V617F heterozygous and wild-type patients.[83] Finally, in patients with PMF, a *JAK2*V617F-mutated status and age older than 60 years were significantly associated with thrombosis; the highest incidence of thrombosis was observed when the mutation was present along with leukocytosis (3.9% patient-year; HR, 3.13; 95% CI, 1.26 to 7.81).[56] The presence of the *JAK2*V617F mutation has been associated with more frequent occurrence of thrombosis also in the settings of familial MPN.[93] In summary, there is evidence that the *JAK2*V617F mutation status and/or its allelic burden are associated with thrombotic propensity in MPN.

The association between a relatively uncommon thrombosis in the splanchnic vein district (SVT) and an MPN is known since time,[53,94] and has been reinforced by the discovery that up to 45% of patients with Budd–Chiari syndromes and 34% of portal vein thromboses harbor the *JAK2*V617F mutation.[54] The calculated risk of having a SVT if harboring a *JAK2*V617F mutation was 53.98 (95% CI, 13.10 to 222.45) compared with *JAK2* wild-type subjects in a large study[95]; this fully justifies the routine use of *JAK2*V617F genotyping in all patients with idiopathic Budd–Chiari or SVT.[54,95] Of interest, unknown population-related genetic variants might underlie the otherwise unexplained low prevalence of *JAK2*V617F mutation ($< 5\%$) among Chinese patients with idiopathic Budd–Chiari syndrome, while the prevalence among subjects with portal vein thrombosis was similar (27%) to Caucasians.[96] In subjects with Budd–Chiari syndrome, the *JAK2*V617F mutation was associated with a higher risk of extrahepatic thrombotic complications after liver transplantation.[97] Finally, it has been reported that the *JAK2* 46/1 haplotype predisposes to SVT in the settings of an MPN.[98] On the contrary, there is no evidence of significant associations of a *JAK2*V617F mutation and retinal vein thrombosis[99] or recurrent miscarriages,[100] while the incidence of the mutation may be increased in subjects with cerebral sinus vein thrombosis.[94]

Therefore, *JAK2*V617F mutational status might represent a novel disease-associated risk factor that would deserve to be incorporated in the current risk stratification; however, a more rigorous prospective validation is definitely necessary. Of further importance are the relationships between *JAK2*V617F mutation and leukocytes, owing to the increasing experimental evidences that support a pathogenetic role of neutrophils in thrombosis of MPN patients.[73] Activated neutrophils and platelets can be detected in the circulation

of MPN patients, particularly in those who are *JAK2*V617F mutated. Activated neutrophils display overexpression of membrane adhesion molecules such as the β_2 integrin CD11b, express leukocyte alkaline phosphatase in a JAK2V617F-dependent manner,[84,101] and have a characteristic gene expression profile; plasma levels of neutrophil-derived enzymes, such as myeloperoxidase, CD14, CD11b, and elastase, are typically increased.[102] Platelet–leukocyte aggregates are increased in the circulation of ET and PV patients, are dependent on leukocyte CD11b expression, and were reduced by aspirin treatment; these abnormalities were accompanied by an elevated expression of tissue factor in the platelets of ET patients, particularly in those *JAK2*V617F mutated. The gene expression profile of neutrophils from PV patients was found to express a set of genes similar to granulocytes from patients with sepsis,[103] consistent with the activation of JAK/STAT signaling initiated by granulocyte colony-stimulating factor receptor. More recent data, however, suggest that the activation profile of MPN granulocytes is largely independent of JAK2V617F allele in patients with ET, suggesting that similar mechanisms of cell activation occur through other pathways in patients lacking the *JAK2*V617F mutation.[104] Finally, a direct positive correlation between the amount of circulating immature platelets, hemostatically more competent, and the *JAK2*V617F mutation has been observed in patients with PV and ET,[105] and might contribute to the prothrombotic phenotype. The platelet thrombin generation potential was significantly higher in MPN patients and progressively increased by *JAK2*V617F allele burden increment. Of interest, thrombin generation potential was significantly lower in hydroxyurea-treated compared with nontreated patients and was lowest in hydroxyurea-treated *JAK2*V617F-mutated patients.[106] A similar behavior has been reported for the immature platelet numbers.[105]

These findings support the existence of a platelet-dependent form of hypercoagulability in MPN patients, particularly in those harboring the *JAK2*V617F mutation. It is therapeutically relevant that cytoreductive therapy with hydroxyurea significantly affected this prothrombotic phenotype.

Activated neutrophils and platelets may also facilitate a thrombophilic condition in MPN patients, as supported by elevated plasma markers of coagulation[104] and contribute to the acquired activated protein C (APC) resistance associated with low free protein-S levels.[107] Such abnormalities can also interact with a *JAK2*V617F-mutated status by further raising the risk of thrombosis in younger ET patients.[108] In fact, *JAK2*V617F-mutated patients, particularly if homozygous for the mutation, resulted more APC resistant than the *JAK2* wild-type counterpart; furthermore, the levels of prothrombin, factor V, free protein S, and tissue factor pathway inhibitor were all significantly reduced in *JAK2*V617F-mutated patients. Decreased levels of free protein S, higher levels of tissue factor, von Willebrand factor, and soluble thrombomodulin were also reported in a study including 59 ET patients and correlated with the burden of *JAK2*V617F allele[109]; these abnormalities were more typically associated with a thrombotic history.

A further mechanism contributing to thrombosis in PV patients might be represented by the abnormal interactions occurring between circulating cells other than leukocytes and endothelial cells. A recent study demonstrated that an increased adhesion of red cells in PV patients is mediated by an Epo receptor-independent, *JAK2*V617F-dependent, increased phosphorylation of the adhesion protein Lu/BCAM, a unique erythroid receptor of laminin α5 chain expressed on red cell membrane.[110] The involvement of endothelial cells in the process of thrombosis associated with MPN, possibly contributing also to disease mechanisms in myelofibrosis, has received experimental support in the last few years. Endothelial cell progenitors, marked by CD34/CD133/VEGFR2-positivity, circulate in increased numbers in the peripheral blood of patients with myelofibrosis: their frequency (median, 0.26%) was significantly higher than in healthy subjects (0%) and the other MPN (0.1%), and marked preferentially subjects with early stage disease.[111] In a subsequent study, increased circulating endothelial colony–forming cells (ECFCs) were associated with history of SVT, with an OR of 6.6 (95% CI, 2.54 to 17.2); also, these patients had features of a nonactive myeloproliferative disease.[112] Patients with myelofibrosis showed the highest number of circulating ECFC among all MPN patients in the study by Teofili et al[113]; in 5 of 22 evaluable patients, ECFCs were *JAK2*V617F mutated and presented enhanced STAT5 and STAT3 phosphorylation as well as a more pronounced tendency to adhere to mononuclear cells. Of interest, ECFCs were detected only in patients suffering from thrombotic events. The presence of the *JAK2*V617F mutation was detected also in the liver endothelial cells of patients with Budd–Chiari syndrome,[114] and Yoder et al reported that a minority of ECFC from patients with a diagnosis of PV and history of thrombosis expressed the *JAK2*V617F mutation.[115] In a recent study, Barosi et al[5] used a very complex and controlled set of approaches to demonstrate that 12 of 18 patients with myelofibrosis had *JAK2*V617F-mutated endothelial cells obtained by splenic vessels as well as 6 of 10 subjects in whom endothelial cells were cultured ex vivo.[116] In addition to microvessels, a large vessel such as the splenic vein also contained *JAK2*V617F-mutated endothelial cells. Although no correlation could be found with thrombotic events in these patients, the study reinforces the possibility that at least a subset of *JAK2*V617F-mutated endothelial cells in the splanchnic district may display thrombogenic properties.

Altogether, these results are supportive of an endothelial cell dysfunction, possibly associated with the expression of mutated *JAK2* in a subset of endothelial cells, which contributes to determine and/or sustains the thrombophilic condition of MPNs, especially in the splanchnic district. However, several additional experimental steps should be accomplished to reconcile this hypothesis with the current knowledge concerning the existence and/or identity of a hemangioblast cell and/or common hematopoietic and endothelial cell lineage progenitor harboring the *JAK2*V617F mutation rather than angiogenic monocytes derived from the mutated myeloid clone. This notwithstanding, unraveling the contribution of mutated endothelium in the pathogenesis of

thrombosis could also highlight novel therapeutic targets and/or clarify the mechanisms of action of current therapies.

The role of inflammation in the pathogenesis of thrombotic events in MPN has raised much interest in the last few years.[117] Accelerated/premature atherosclerosis has been observed in different clinical conditions characterized by chronic inflammation. The MPNs are characterized by a chronic inflammatory status, as documented by raised levels of several plasma cytokines and chemokines, which are also credited to be responsible for systemic manifestations of the disease, particularly in myelofibrosis, and to serve as biomarkers associated with prognosis.[118–120] Two inflammatory biomarkers, the short pentraxin C-reactive protein (CRP) and the long pentraxin-3 (PTX3), both belonging to the superfamily of pentraxins, previously shown to represent markers of thrombosis and atherogenesis in the general population, have been studied in relation to thrombosis in PV and ET.[121] In patients presenting higher high-sensitive CRP levels (hsCRP; $>$ 3 mg/L), significant correlation with age, disease type (PV vs. ET), the presence of at least one CV risk factor, and *JAK2*V617F allele burden $>$ 50% were found. The number of patients with major cardiovascular events increased progressively according to increments of hsCRP values ($p = 0.01$). Also the levels of PTX3 were significantly correlated with the burden of *JAK2*V617F mutation; however, opposite to hsCRP, thrombotic episodes and CV risk factors were less frequent in patients with higher PTX3 levels.[121] As a matter of fact, the most potent and significant correlation with thrombosis was in the category of patients presenting high hsCRP and low PTX3 values (OR, 2.66; $p = 0.045$).[121] Since blood values of CRP and PTX3 were correlated with *JAK2*V617F allele burden, it is very likely that increased production of these inflammatory proteins may result from a direct effect on gene transcription by activated JAK-dependent signaling and to increased stimulation by inflammatory cytokines, including IL6, whose production is both directly and indirectly regulated by JAK signaling. Of outmost interest, several inhibitors of activated JAK2, including Ruxolitinib, approved for the treatment of myelofibrosis,[122,123] have shown strong efficacy in reducing the abnormal cytokine levels in patients with MF.[124] Recently, it has been reported in a phase II study that Ruxolitinib also significantly reduced hsCRP levels in patients with PV (Verstovsek et al,[125] presented at ASH2012). Whether beneficial effects of drugs on downregulation of inflammatory milieu could translate into a benefit in terms of reduced thrombosis remains to be evaluated.

To summarize, although a unifying mechanistic interpretation of the events that link the expression of *JAK2*V617F mutation with propensity to thrombosis in patients with MPN is still lacking, there are evidences that abnormal cellular signaling mediated by mutated *JAK2* in different cellular systems, as summarized in ►**Fig. 1**, has the potential to strongly contribute to the pathogenesis of thrombosis in

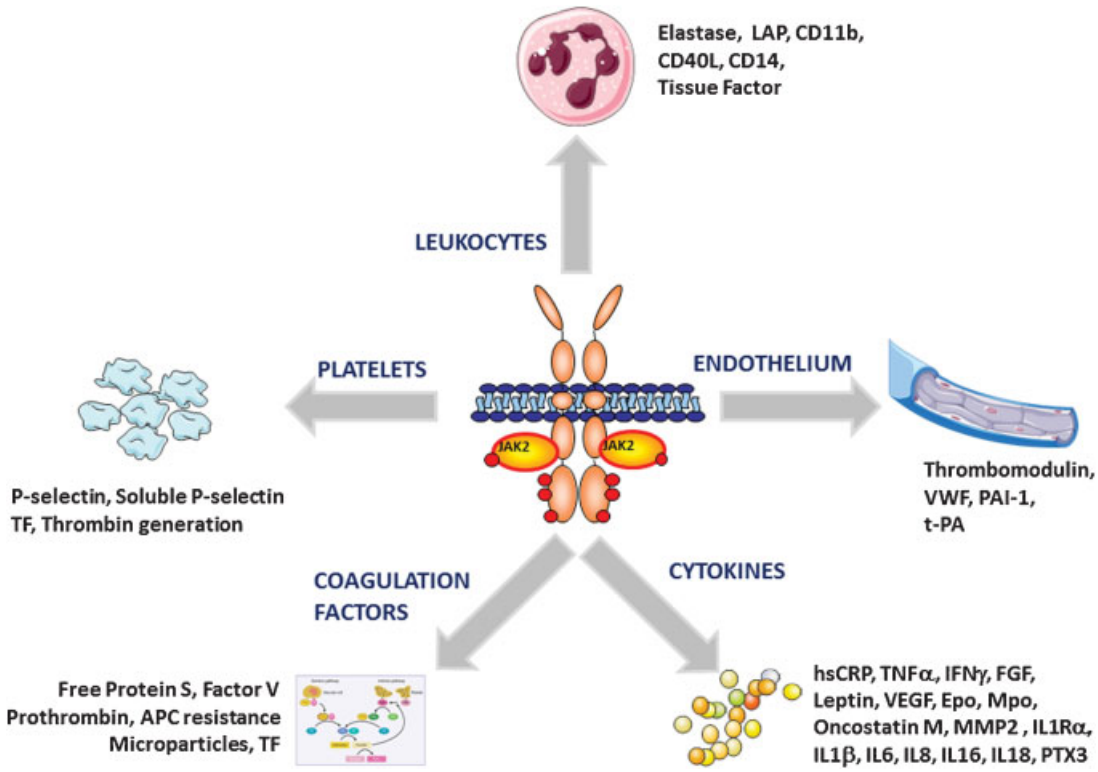

Fig. 1 A schematic representation of the several targets that can be affected by a V617F-mutated *JAK2* (here depicted as associated with the cytoplasmic domains of a cytokine receptor in its autonomously phosphorylated status, that is, in the absence of a cytokine bound to the extracellular portion of the receptor itself), and can overall contribute to the thrombotic propensity associated with myeloproliferative neoplasms. APC, activated protein C; CD, cluster of differentiation; Epo, erythropoietin; FGF, fibroblast growth factor; hs-CRP, high-sensitivity C-reactive protein; IFN, interferon; IL, interleukin; IL1R, interleukin-1 receptor; LAP, leukocyte alkaline phosphatase; MMP2, matrix metallopeptidase 2; Mpo, myeloperoxidase; PAI-1, plasminogen activator inhibitor-1; PTX3, pentraxin 3; TF, tissue factor; TNF, tumor necrosis factor; t-PA, tissue plasminogen activator; VEGF, vascular endothelial growth factor; VWF, von Willebrand factor.

MPN. These recent discoveries have improved our understanding of thrombosis mechanisms in MPN and will certainly help in improving therapeutic management in the near future.

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