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MYELOPROLIFERATIVE NEOPLASMS (B STEIN, SECTION EDITOR)

# Novel Pathophysiological Mechanisms of Thrombosis in Myeloproliferative Neoplasms

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## Abstract

**Purpose of Review** Thrombosis remains a leading cause of morbidity and mortality in BCR/ABL negative myeloproliferative neoplasms (MPN). Circulating blood cells are both increased in quantity and qualitatively abnormal in MPN, resulting in an increased thrombotic risk. Herein, we review recently elucidated mechanisms of MPN thrombosis and discuss implications of drugs currently under investigation for MPN.

**Recent Findings** Recent studies highlight that in *JAK2*$^{V617F}$ granulocytes and platelets, thrombo-inflammatory genes are upregulated. Furthermore, in *JAK2*$^{V617F}$ granulocytes, protein expression of integrin CD11b, tissue factor, and leukocyte alkaline phosphatase are all increased. Overall, myeloid cells, namely neutrophils, may contribute in several ways, such as through increased adhesion via β1 integrin binding to VCAM1, increased infiltration, and enhanced inducibility to extrude neutrophil extracellular traps. Non-myeloid inflammatory cells may also contribute via secretion of cytokines. With regard to red blood cells, number, rigidity, adhesion, and generation of microvesicles may lead to increased vascular resistance as well as increased cell-cell interactions that promote rolling and adhesion. Platelets may also contribute in a similar fashion. Lastly, the vasculature is also increasingly appreciated, as several studies have demonstrated increased endothelial expression of pro-coagulant and pro-adhesive proteins, such as von Willebrand factor or P-selectin in *JAK2*$^{V617F}$ endothelial cells.

**Summary** With the advent of molecular diagnostics, MPN therapeutics are advancing beyond cytoreduction. Our increased understanding of pro-inflammatory and thrombotic pathophysiology in MPN provides a rational basis for evaluation of in-development MPN therapeutics to reduce thrombosis.

**Keywords** BCR/ABL negative myeloproliferative neoplasms · Thrombosis · Tissue factor · P-selectin · Neutrophils · Vascular inflammation

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## Introduction

BCR/ABL-negative myeloproliferative neoplasms (MPN) are composed of three phenotypically distinct clonal disorders, all with JAK-STAT activation central to their pathogenesis: polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF). Thrombosis is a leading cause of morbidity and mortality in MPN. Arterial thrombosis, particularly ischemic stroke, is most common and is increased 3-fold in MPN relative to the general population [1•]. The risk of venous thromboembolism (VTE) is 10-fold increased in MPN versus the general population, with a peculiar propensity for splanchnic vein thrombosis [1•, 2]. Microvascular disturbance is also common in MPN, resulting in migraine-type headaches, erythromelalgia, transient ischemic attacks, and coronary artery dysfunction.

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Somatic mutations in *JAK2*, *MPL*, and *CALR* genes lead to development of the various MPN phenotypes. *JAK2* mutations present as a pan-myelosis, and *CALR* and *MPL* often present with predominant thrombocytosis. Of these, the $JAK2^{V617F}$ mutation occurs in 95% of patients with PV and 60% of patients with ET or MF [3] and confers the highest risk of thrombosis [4••, 5] (Fig. 1a). Risk factors for MPN thrombosis include age > 60, prior thrombosis, cardiovascular risk factors, hematocrit > 45%, and presence of the $JAK2^{V617F}$ mutation. In PV and ET, treatment is based largely upon the risk of thrombosis, with lower risk patients managed with aspirin and therapeutic phlebotomy and higher risk patients with the addition of the cytoreductive agent hydroxyurea and more recently interferon. Cytoreduction predominantly aims to lower the number of blood cells to normalize blood rheology. Although this strategy has reduced the thrombotic risk in MPN, 16–25% of MPN patients still experience thrombotic events [6, 7]. Therefore, there remains a critical need to elucidate the pathogenesis of MPN-related thrombosis and to develop therapies to prevent it.

Why $JAK2^{V617F}$ mutations result in higher thrombotic risk than *MPL* and *CALR* mutations, when both lead to activation of the JAK-STAT pathway, is not yet clear. One possibility is that the mutations differentially affect the JAK-STAT pathway [8, 9]. Variant allele fraction (VAF) is the ratio of mutated to unmutated alleles within a sample. For $JAK2^{V617F}$, higher VAF is associated with increased myeloproliferation as well as arterial and venous thrombosis [10]. This association is further demonstrated by a recent study of $JAK2^{V617F}$ clonal hematopoiesis within the Danish General Suburban Population Study, wherein individuals with $^{+}$ VAF of > 1% but without an MPN diagnosis had an increased risk of thrombosis compared to those without the mutation [4••]. Curiously, in the past 5 years, basic and translational studies have advanced our understanding and revealed new potential mechanisms driving $JAK2^{V617F+}$ MPN pro-inflammatory and thrombotic pathophysiology (Fig. 1). Furthermore, several new, targeted drugs are under investigation which may interfere with pro-thrombotic milieu. Herein, we review these cell-specific mechanisms and how current and in-development therapies may prevent thrombosis.

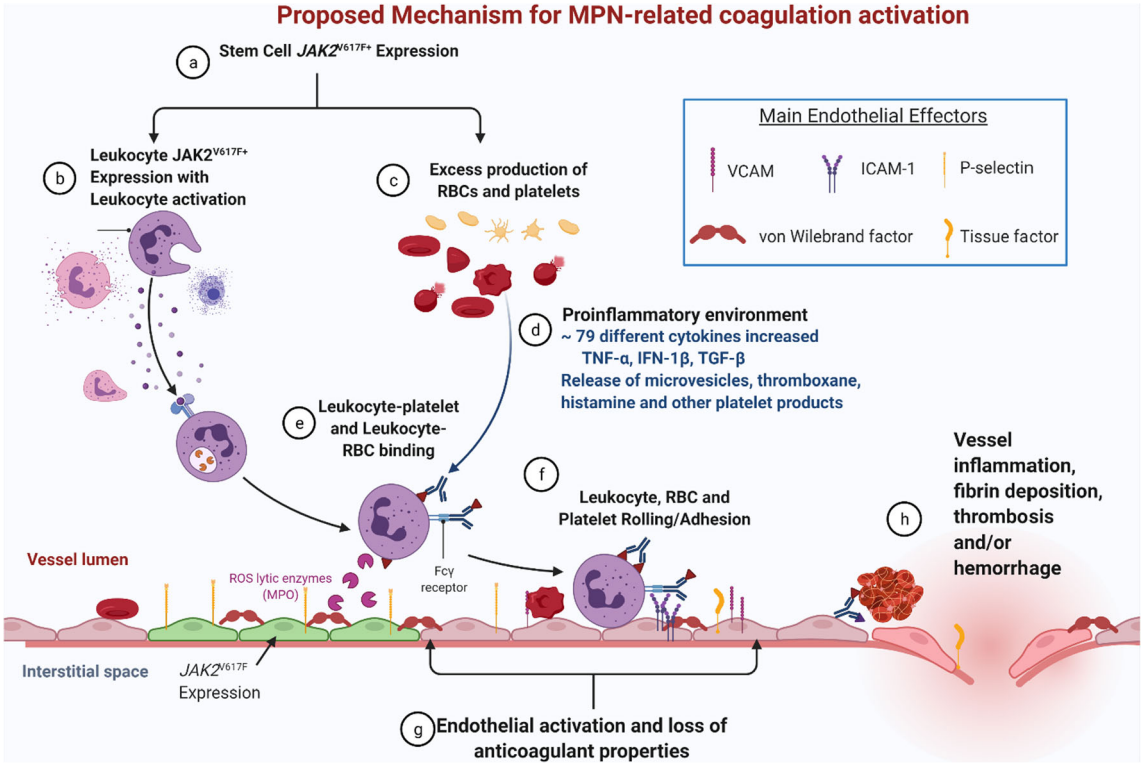


**Fig. 1** Proposed mechanisms for MPN-related thrombosis. **a** Somatically acquired mutations in stem cells lead to the development of myeloproliferative neoplasm. **b** Hematopoietically derived granulocytes and non-myeloid inflammatory cells are produced and activated. **c** Excess production of RBCs and platelets. Collectively, B and C lead to **d** a pro-inflammatory environment and **e** increased leukocyte-platelet and leukocyte-RBC binding as well as **f** leukocyte, RBC, and platelet rolling and adhesion. **g** Endogenous JAK expression and pro-inflammatory response lead to increased endothelial activation. Collectively, this develops into **h** an inflamed environment prone to fibrin deposition and thrombosis. Image created with Biorender.com

## Role of Leukocytes, Red Cells, and Platelets

### Leukocytes

Neutrophils are the most abundant leukocyte and are often increased in *JAK2*$^{V617F}$ MPN. Whether leukocytosis is a risk factor for thrombosis in MPN has remained a matter of debate, confounded by the retrospective nature of analyses performed. A recent meta-analysis linked leukocytosis with arterial thrombosis in ET, but not in PV [11]. MPN were not molecularly annotated in the analysis, however, and it is possible that an excess risk is due to the *JAK2*$^{V617F}$ mutation, which is associated with increased leukocyte counts and independently shown to increase thrombotic risk in ET. Prospective clinical trials are needed to determine whether leukocytosis is a bona fide risk factor for thrombosis.

Irrespective of quantity, MPN neutrophils exhibit increased JAK-STAT pathway activation as compared to healthy controls [8, 9]. *JAK2*$^{V617F}$ and *CALR* driver mutations differentially affect the pathway, perhaps due to paracrine versus direct activation (Fig. 1b) [8, 9]. Importantly, thrombo-inflammatory genes are upregulated in MPN granulocytes and correlate with thrombosis history [12••]. Upregulation of these thrombo-inflammatory genes is further influenced by iron deficiency, which would suggest reconsideration of the long-standing practice of therapeutic phlebotomy for MPN treatment [13, 14].

Downstream of gene regulation, protein expression of integrin CD11b, tissue factor (TF), and leukocyte alkaline phosphatase are all increased in *JAK2*$^{V617F}$ as compared to *CALR* granulocytes [15–18]. Increased neutrophil adhesion via β1 integrin binding to VCAM1 has been demonstrated in *JAK2*$^{V617F}$ MPN (Fig. 1e, f). Mouse models corroborate these human observations, with increased β1/β2 integrin implicated in venous thrombosis and increased neutrophil infiltration associated with atherosclerotic plaque formation [19••, 20].

MPN neutrophils also have enhanced inducibility to extrude neutrophil extracellular traps (NETs), which was shown in a *JAK2*$^{V617F}$ mouse model to result in spontaneous pulmonary emboli and increased provoked venous thrombosis [21••]. Peptidylarginine deaminase 4 (PAD4), an enzyme critical to NET formation, was increased in neutrophils in this *JAK2*$^{V617F}$ mouse model and deletion of *PAD4* abrogated thrombosis [21••].

### Non-Myeloid Inflammatory Cells

*JAK2*$^{V617F+}$ hematopoietic stem cells develop into multiple cell lineages—including myeloid and non-myeloid inflammatory cells, such as T-cells and natural killer (NK) cells [22, 23]. These non-myeloid inflammatory cells are responsible for cytokine secretion. Both *JAK2*$^{V617F+}$ T-cells and *JAK2*$^{V617F+}$ monocytes in mice demonstrate increased expression of pro-inflammatory cytokines [19••, 23]. Likewise, at least 79 different cytokines have been reported to have perturbations in individuals with MPN [24–27]. Of these, ~ 12 (IL-1α, IL-1β, IL-2Ra, IL-6, IL-8, IL-11, IFN, TNF-α, TGF-β, VEGF, PDGF, and MIP-1) have been correlated to leukemic progression and survival in MPN [24]. However, less work has been done to correlate cytokines to thrombosis. Studies with T-cell restricted *JAK2*$^{V617F+}$ mouse models demonstrate elevated secretion of tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ) [23]. This is notable, as TNF-α, IFN-γ, and other pro-inflammatory cytokines also increase endothelial expression of pro-adhesive VCAM-1, a ligand for β1/β2 integrins on leukocytes [19••, 28, 29]. Collectively, these studies demonstrate that non-myeloid inflammatory cells participate in generation of the pro-thrombotic milieu (Fig. 1d). Furthermore, they suggest that strategies to reduce cytokine secretion and/or effects may inform MPN thrombotic risk reduction strategies.

### Red Cells

In PV, as well as others with erythrocytosis disorders (i.e., Chuvash polycythemia), elevated hematocrit is associated with increased risk of arterial and venous thrombosis [30–33] (Fig. 1c). As the most abundant cell in circulation, several RBC biophysical properties may contribute to pro-thrombotic physiology. For example, over time, repetitive cycles of deformation under shear force reduce RBC membrane stability leading to the generation of microvesicles and increased exposure of negatively charged pro-coagulant phospholipid phosphatidylserine (PS) [34]. Indeed, several studies have found that MPN patients have increased circulating microvesicles and PS exposure [35–38]. As demonstrated in a recent study, *JAK2*$^{V617F+}$ RBC-derived microvesicles carry myeloperoxidase, which increase endothelial oxidative stress and arterial vascular tone (Fig. 1d, e) [39•]. Other work suggests that RBC-derived microvesicles may increase thrombin generation and activate platelets [40–43].

Besides microvesicles, in response to MAPK signaling, the RBC surface itself expresses pro-adhesive molecules [28, 44]. In PV, the *JAK2*$^{V617F+}$ mutation activates the RAP1/Akt signaling pathway leading to Lutheran/basal cell adhesion molecule (Lu/BCAM) expression and increased adhesion to extracellular matrix laminin-α5 [45] (Fig. 1e). Combined, these studies suggest that in MPN, increased RBC number, rigidity, adhesion, and presence of microvesicles/PS contribute to increased vascular resistance as well as increased RBC interactions that promote rolling and adhesion [34, 46–48] (Fig. 1f). Increased hematocrit also marginates platelets, allowing for increased interaction with a growing thrombus [49]. Indeed, pivotal studies in PV have clearly shown that a hematocrit of < 45% results in fewer thrombotic events [50].

### Platelets

On balance, elevated platelet counts are not associated with thrombosis in MPN [51–55]. Extreme thrombocytosis in fact has been implicated in MPN-associated hemorrhage, in part related to a type 2 acquired von Willebrand syndrome [56]. Qualitative platelet changes have been associated with both increased thrombosis and increased bleeding in MPN. This dichotomous finding is not fully understood but is thought to be secondary to abnormal activation of platelets, leading to granule deficiency and impaired platelet function [57]. Individuals with MF in particular have higher rates of bleeding than observed in ET or PV (Fig. 1h) [58]. This has also been observed in mouse models of MPN, suggesting that the disease phenotype plays a strong role in the hemorrhagic diathesis [59, 60, 61•].

With respect to thrombosis, platelets from MPN patients have an increased expression of thrombo-inflammatory genes, which correlate with an increased thrombotic risk [12••]. P-selectin, which is integral in recruitment of leukocytes, is increased in MPN platelets, and there are more platelet-leukocyte aggregates in circulation [62–65]. Increased P-selectin expression associates with higher emperipolesis of neutrophils through megakaryocytes, during which platelets can acquire bits of neutrophil membrane [66]. Microvesicles derived from platelet membranes are also found in increased quantities in the circulation of MPN patients, many bearing TF [67]. As in the case of neutrophil activation, the presence of a *JAK2*$^{V617F}$ mutation was associated with increased quantities of microvesicles and increased TF expression on them, highlighting the significance of this pathway in the pathogenesis of thrombosis [68, 69].

### Endothelial Cells and Vascular Inflammation

A collection of recent publications strongly suggest that vascular endothelial cells are involved in MPN pro-thrombotic physiology (Fig. 1g, h). First, in PV patients with prior splanchnic thrombosis, at least three studies demonstrate endothelial *JAK2*$^{V617F}$ expression [70–72]. Likewise, two recent studies directly assessed *JAK2*$^{V617F+}$ endothelial cells and found an increased expression of von Willebrand factor (VWF) and P-selectin [73, 74••]. Additionally, in static adhesion assays, *JAK2*$^{V617F+}$ endothelial cells exhibit increased leukocyte adhesion [73], and under venous flow rates, neutrophils exhibit increased adhesion to *JAK2*$^{V617F}$ endothelial cells [74••]. Last, transcriptome analysis of *JAK2*$^{V617F+}$ endothelial cells demonstrates increased cell adhesion, coagulation, and ECM receptor gene expression profiles [73, 75]. Collectively, these data suggest that an increased endothelial expression of pro-coagulant and pro-adhesive proteins, such as VWF or P-selectin, may account for an increased *JAK2*$^{V617F}$ VTE risk [76, 77]. Therefore, strategies that incorporate evaluation of vascular activation may advance strategies to reduce MPN thrombosis risk.

## Moving Beyond Aspirin and Phlebotomy: Potential of MPN Treatments to Prevent VTE

### Hydroxyurea

Overall, many randomized and non-randomized studies conducted over the span of several decades have established hydroxyurea (HU), a ribonucleotide reductase inhibitor, as a common first-line chemotherapy agent in MPN [78]. However, the mechanisms by which HU may reduce thrombosis in MPN remain elusive. Recent studies in *JAK2*$^{V617F+}$ mice have demonstrated that HU treatment reduces P-selectin expression in microvascular endothelial cells [74••]. Furthermore, older studies from the sickle cell disease literature suggest that in endothelial cells, HU is associated with increased nitric oxide production and improved vascular tone and function [79]. Likewise, in MPN, HU is associated with lower circulating RBC microvesicle levels [37]. However, offsetting these studies is data from *JAK2*$^{V617F+}$ patients that demonstrate HU treatment increases RBC adhesion to laminin and has a minimal effect on pro-inflammatory cytokines [45, 80]. Thus, continued evaluation of how HU may reduce thrombotic risk in MPN patients remains necessary and informative towards identification of pathophysiologic mechanisms essential in MPN thrombosis.

### JAK-STAT Inhibitors

The discovery of the *JAK2*$^{V617F+}$ mutation led to the development of JAK-STAT inhibitors that target the JAK ATP-binding site with various selectivities for each JAK kinase [81]. Ruxolitinib, a JAK1/JAK2 kinase inhibitor, was the first approved agent for use in PV and MF patients refractory to, or intolerant of, hydroxyurea [82]. Fedratinib, a more selective JAK2 inhibitor, is the only other JAK-STAT inhibitor with approval for front-line use in MF [83]. Two other JAK-STAT inhibitors, momelotinib and pacratinib, are currently undergoing phase 3 trials for MF (NCT 04173494 and NCT 03165734). Furthermore, JAK-STAT inhibitors are now being used or in development for numerous other disorders, including graft-versus-host disease (GVHD) [84].

To date, thrombosis and cardiovascular outcomes have not been a primary or secondary outcome for JAK-STAT inhibitor trials. Compared to standard of care, two meta-analyses of the ruxolitinib trials demonstrate a modest reduction in thrombotic events [85, 86••]. Conversely, post-marketing analysis suggests ruxolitinib may increase pulmonary embolism rates. However, several studies highlight potential mechanisms by which JAK-STAT inhibitors may reduce thrombosis. First, a

limited flow cytometry pilot study comparing pro-thrombotic markers in *JAK2*$^{V617F+}$ patient samples found that after starting ruxolitinib therapy, individuals had a reduction in monocyte tissue factor (TF) expression and monocyte number [87]. Notably, a larger study, comparing *JAK2*$^{V617F+}$ patients with thrombosis history to those without, found that in subjects with thrombosis history, granulocyte TF expression was upregulated 13-fold. Combined, these studies suggest TF as a potential marker for thrombosis. This is notable because an unbiased biomarker study suggests that several JAK-STAT inhibitors reduce TF expression as well as other pro-adhesive and inflammatory targets [88•]. Second, recent work by Wolach and colleagues demonstrated that treatment of *JAK2*$^{V617F+}$ neutrophils with ruxolitinib reduces *JAK2*$^{V617F+}$ NET formation and thrombosis in vivo [89]. Combined, these studies provide a mechanistic rationale that JAK-STAT inhibitors may reduce thrombosis in MPN and add support for continued efforts to assess both post-marketing and pre-clinical studies.

## Anti-Integrin Monoclonal Antibodies

Leukocyte, red cell, and platelet interactions with the endothelium are mediated through integrin binding. In platelets, αIIbβ3 and α2β1 integrins participate in platelet aggregation and binding. On leukocytes, six integrins are expressed, with β1 and β2 chains being critical for the formation of pro-adhesive VLA-4 (very late antigen-4, α4β1). VLA-4 binds to vascular cell adhesion molecule 1 (VCAM1) and other endothelial adhesion molecules to mediate adhesion [90]. Recent work found that blocking *JAK2*$^{V617F+}$ leukocyte-mediated β1/β2 integrin interactions with VCAM-1 decreases thrombosis [19••]. This data suggests that adoption of anti-integrin antibodies may be a strategy to reduce thrombosis in MPN. Overall, oral αIIbβ3 inhibitors failed phase 3 trials in cardiovascular disease due to excess bleeding and mortality [90]. Therefore, these drugs are unlikely to be re-evaluated in the context of MPN. However, natalizumab, an anti-α4 monoclonal antibody, is approved for use in multiple sclerosis (MS) and inflammatory bowel disorders, with the caveat that stringent monitoring is required due to development of progressive multifocal leukoencephalopathy (PML) [90]. Nonetheless, trials of natalizumab for GVHD are underway (NCT02133924). Therefore, future studies evaluating the feasibility of anti-integrin therapy to prevent MPN thrombosis may be warranted.

## Bromodomain and Extraterminal Domain (BET) and Histone Deacytelase Inhibitors

In MPN, epigenetic changes in immune cells lead to increased inflammatory response [91]. Bromodomain and extraterminal domain (BET) and histone deacytelase (HDAC) proteins are critical regulators of epigenetic programming, and several therapeutic strategies to inhibit these proteins are under clinical development for MPN patients. Thrombosis and cardiovascular outcomes are not primary or secondary outcomes for most MPN-related trials. However, mechanistically, there are several pathways by which these drugs may interfere with *JAK2*$^{V617F+}$ MPN pro-thrombotic physiology. First, with regards to BET inhibitors, the bromodomain-containing (BRD) proteins recognize acetylated lysine residues and non-histone proteins to transcriptionally regulate gene expression. For example, BRD proteins are coactivators of NF-κB p65 (aka RELA), which leads to downstream activation of pro-inflammatory cytokines, such as IL-6 [92]. BET inhibitors reduce activator protein 1 (AP-1) transcription, which along with RELA, controls TF transcription [93, 94]. Therefore, beyond reducing *JAK2*$^{V617F+}$ hematopoietic cells, BET inhibitors may reduce inflammation and pro-thrombotic activation. At present, several BET inhibitors are undergoing evaluation in MPN, including two ongoing phase 2/3 trials, MANIFEST and MANIFEST-2 (NCT02158858 and NCT04603495), and a phase 1b study of PLX2853; thus far, none of these trials has reported thrombosis outcomes.

With regard to HDAC proteins, several studies have demonstrated increased levels of HDAC4-6, HDAC9, and HDAC 11 are associated with MPN evolution and progression [95–98]. The HDAC inhibitor givinostat, a zinc-chelating inhibitor of class I and II HDACs, downregulates heat shock protein 90 acetylation leading to reduced JAK2 stability and action [99–101]. Mechanistically, givinostat and other HDAC inhibitors appear to target *JAK2*$^{V617F}$ hematopoietic cells [95, 96, 102–104]. Furthermore, givinostat reduces pro-inflammatory tumor necrosis factor-α (TNF-α) and interleukin-1β [105–107]. Interestingly, preclinical data in animal models of sickle cell disease and rheumatoid arthritis demonstrate that several other HDAC inhibitors decrease IL-6, TF, and VCAM-1 [108, 109]. Last, compared to the historical annual rate of 3.4% per patient year, a cohort of *JAK2*$^{V617F}$ patients followed 4+ years on givinostat had a lower thrombosis rate of 2.3% per year [110, 111]. Overall, these data suggest givinostat and other HDAC inhibitors may prevent MPN-related thrombosis.

## Mini-Hepcidins

Hepcidin is a liver-secreted peptide hormone that is critical in iron homeostasis [112]. Hepcidin expression is regulated by iron availability, inflammatory cytokines (i.e., IL-6), and erythropoietic demand [113]. Recent analysis of the hepcidin levels in the Tromsø Study, a single-center prospective cohort study of inhabitants of Tromsø, Norway, has noted elevated hepcidin levels were associated with an increased risk of VTE [114]. However, in other studies, iron deficiency was also associated with an increased risk of thrombosis [115, 116].

Compared to individuals without *JAK2*$^{V617F}$ mutation, *JAK2*$^{V617F}$ individuals exhibit decreased hepcidin levels [112]. Additionally, in *JAK2*$^{V617F}$ mice, administration of mini-hepcidins reduced iron supply to erythrocytes, resulting in decreased erythrocytosis and splenomegaly [117]. Therefore, mechanistically, the importance of iron homeostasis and hepcidin in the setting of *JAK2*$^{V617F}$ mutation is unclear, and further studies are needed. Currently, PTG-300, a hepcidin-mimetic, is under phase 2 clinical trials (NCT04057040 and NCT04767802) for use in PV. Thus far, these trials have not reported thrombotic outcomes [118].

### Crizanlizumab and Selectin Inhibitors

P-selectin is a transmembrane protein expressed on platelets and endothelial cells that plays a critical role in inflammation and thrombosis by mediating platelet-leukocyte, platelet-endothelial, and endothelial-leukocyte rolling and adhesive interactions. During inflammation, P-selectin is released from within Weibel-Palade body storage granules [119, 120]. Recent work has demonstrated that endothelial cells isolated from mice with endothelial-restricted *JAK2*$^{V617F}$ expression also displayed increased P-selectin and VWF expression [121]. Crizanlizumab, a humanized monoclonal antibody that inhibits P-selectin, was FDA approved in 2019 for use in sickle cell disease based on studies demonstrating reduction in painful vaso-occlusive crisis [122]. Intriguingly, addition of P-selectin blocking antibodies to mice with endothelial-restricted *JAK2*$^{V617F}$ expression eliminated lung thrombus formation [74••], suggesting a potential role for crizanlizumab in MPN. At present, there is currently only one non-US-based international trial evaluating the use of crizanlizumab combined with ruxolitinib in patients with MF (NCT04097821); however, this study, like many others, does not include thrombosis outcomes or measurements. Furthermore, several other selectin inhibitors, including pan-selectin inhibitor rivipansel, have not been evaluated. Therefore, further studies evaluating the anti-thrombotic potential of crizanlizumab and other selectin inhibitors are warranted.

## Conclusions

As MPN therapeutics are quickly moving beyond aspirin and phlebotomy, there remains an unmet need to identify and define surrogate biomarkers for thrombosis. However, as illustrated in Fig. 1, recent advances in our understanding of pro-inflammatory and thrombotic pathophysiology in MPN offer some mechanistic understanding that may lead to identification of surrogate thrombosis biomarkers. Furthermore, these studies provide rationales for how some candidate therapies may serve to reduce thrombosis. Overall, strategies that target the *JAK2*$^{V617F}$ hematopoietic clone, as well as downstream effects on granulocytes, red cells, and vascular interactions may prove more effective in preclinical and clinical studies. Moving forward, continued basic/translational research will advance our understanding in the prevention and management of thrombosis in MPN patients.

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## Declarations

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