Molecular approach to diagnose BCR/ABL negative chronic myeloproliferative neoplasms

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Chronic myeloproliferative neoplasms arise from clonal proliferation of hematopoietic stem cells. According to the World Health Organization myeloproliferative neoplasms are classified as: chronic myelogenous leukemia, polycythemia vera, essential thrombocythemia, primary myelofibrosis, chronic neutrophilic leukemia, chronic eosinophilic leukemia, hypereosinophilic syndrome, mast cell disease, and unclassifiable myeloproliferative neoplasms. In the revised 2008 WHO diagnostic criteria for myeloproliferative neoplasms, mutation screening for JAK2V617F is considered a major criterion for polycythemia vera diagnosis and also for essential thrombocythemia and primary myelofibrosis, the presence of this mutation represents a clonal marker. There are currently two hypotheses explaining the role of the JAK2V617F mutation in chronic myeloproliferative neoplasms. According to these theories, the mutation plays either a primary or secondary role in disease development. The discovery of the JAK2V617F mutation has been essential in understanding the genetic basis of chronic myeloproliferative neoplasms, providing some idea on how a single mutation can result in three different chronic myeloproliferative neoplasm phenotypes. But there are still some issues to be clarified. Thus, studies are still needed to determine specific molecular markers for each subtype of chronic myeloproliferative neoplasm.

Keywords: *Myelodysplastic-Myeloproliferative diseases; Hematologic neoplasm; Mutation; AK2V617F*

Introduction

Hematologic malignancies are broadly classified into myeloid and lymphoid disorders according to the morphological and immunological characteristics of the affected clonal cell population.[1] Among the myeloid disorders are myeloproliferative neoplasms (MPNs), which are caused by a clonal proliferation of a pluripotent hematopoietic progenitor. These neoplasms are characterized by an excessive production of mature blood cells of myeloid lineage, which are independent and/or hypersensitive to cytokines for cell survival, proliferation and differentiation.[2-5]

Recently, the World Health Organization (WHO) established new criteria for the diagnosis and classification of myeloproliferative neoplasms.[6] According to these criteria, the myeloid neoplasms were classified as chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia (CEL), hypereosinophilic syndrome (HES), mast cell disease (MCD) and myeloproliferative neoplasms, unclassifiable (MPN-u).[7]

In 1951, William Dameshek described a relationship between PV, ET and PMF, suggesting that these diseases, along with CML and erythroleukemia, were grouped into a general category of myeloproliferative disorders.[8,9] However, over the years, this classification was reviewed and currently PV, ET and PMF are called BCR/ABL-negative chronic MPNs (CMPNs).[7] These three neoplasms share some clinical features, such as the presence of hematopoietic stem cells independent of growth factors for proliferation, bone marrow hypercellularity, increased risk of thrombotic events and bleeding, spontaneous conversion to acute leukemia and bone marrow fibrosis.[10]

Evidence from the literature suggests that molecular features observed in CMPNs are caused due to disorders in the process of hematopoietic cell signaling. For instance, Gitler et al.[11] showed that overexpression of growth factors, which act as mediators of cell signaling, is able to induce MPN in mice. Roder et al.[12] demonstrated that hematopoietic cells from patients with CMPN express activated signaling molecules constitutively. Axelrad et al.[13] found that hematopoietic progenitors are hypersensitive to several growth factors in PV, PMF and ET. Based on this and other evidence, some groups of researchers have

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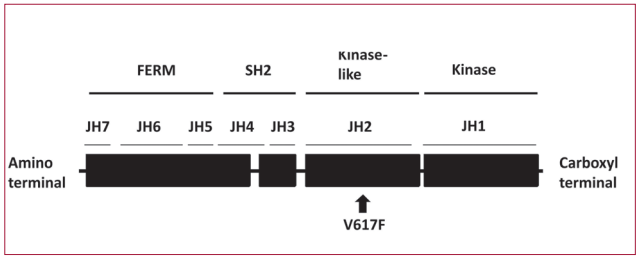
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hypothesized that abnormalities in the Janus kinase 2 protein (JAK2) may be related to CMPNs as this protein is responsible for the activation of several molecules involved in cell signaling processes.[14] This hypothesis was confirmed in 2005, when four different research groups identified a mutation in exon 14 of the JAK2 gene which causes the substitution of valine for phenylalanine at codon 617 in the JAK2 protein (JAK2V617F). This mutation was described in more than 95 percent of PV cases and in approximately 50 percent of ET and PMF cases.[3,15,16] The incidence of JAK2V617F in PV, ET and PMF was confirmed by several subsequent studies, which also reported its absence in normal cells and rare appearances in other hematological disorders.[17]

JAK2 gene structure and regulatory mechanisms

JAK2 is one member of the Janus kinase (JAK) family, along with: JAK1, JAK3 and Tyk2.[1,18] This family has seven homologous domains, termed the JAK homology (JH) domains 1 through 7, and a portion of the initial carboxyl group and an amino-terminal portion. Starting from the carboxyl domain toward the amino-terminal, the JH1 domain represents the kinase domain and JH2, the pseudokinase; the JH3-JH4 domain of JAKs shares homology with Src-homology type 2 (SH2) domains, while the JH5-JH7 has homology to the band 4.1, ezrin/radixin/moesin (FERM) domain.[16] Structurally, the JAKs can be divided into two parts. The amino-terminal portion is important for receptor binding and has surface stabilizing functions; the kinase domains (carboxyl-terminal portion) are crucial for the regulation of the cellular signaling process.[16] Research has shown significant involvement of the FERM domain in the interactions of the JAKs with transmembrane cytokine receptors and in the regulation of kinase activity.[19] The role of the SH2-domain in JAKs is unclear, although in other



Adapted from Radtcke et al., 2005

Figure 1 – The JAK2 gene has seven homologous domains, termed Jak homology (JH) domains 1 through 7, and a portion of initial carboxyl and an amino-terminal portion. Starting from the carboxyl domain toward the amino-terminal, the JH1 domain represents the kinase domain and JH2, the pseudokinase; the JH3-JH4 domain of JAKs shares homology with Src-homology type 2 (SH2) domains, while the JH5-JH7 has homology to the band 4.1, ezrin/radixin/moesin (FERM) domain. The arrow shows that the JAK2V617F mutation occurs in the domain kinase-like

protein kinases it plays a key role in molecule affinity to phosphotyrosine residues.[19] The pseudokinase domain precedes the kinase and due to the differences in residues required for catalytic activity, it cannot transfer phosphate; therefore, it is called catalytically inactive.[16,20] However, the pseudokinase domain is structurally necessary for the JAK response to cytokine receptor activation and inhibition of basal activity of the kinase domain.[1,16] The JAK2V617F mutation occurs in the pseudokinase domain resulting in a constitutive activation of the kinase domain (Figure 1).[16] Because of this, it was concluded that the pseudokinase domain is responsible for keeping the kinase domain in an inactive state at baseline.[21] JAK2 gene molecular models suggest that a mutation in the JH2 domain destabilizes the structural conformation of the protein,[17] which prevents its inhibitory effect on the JH1 domain, leading to the constitutive kinase activity characteristic of CMPNs.[22]

JAK2 protein kinase activity and the JAK2V617F mutation

JAK2 is a member of the JAK family of protein tyrosine kinases, enzymes that are capable of catalyzing the transfer of phosphate from the ATP molecule to tyrosine residues present in its own cytoplasmic domain (autophosphorylation) and tyrosine residues of other intracellular proteins.[9,23] These proteins are vital components in signaling mechanisms related to essential cellular functions, such as differentiation, proliferation and survival.[24]

In healthy individuals, JAK2 works in association with receptors that lack intrinsic kinase activity. The binding of cytokines, hormones and growth factors to their specific receptors results in multimerization, with cytoplasmic domains that are associated with JAK2.[1,25] This conformational change results in autophosphorylation and activation of the JAK2 protein, which consequently acts on receptor phosphorylation, and recruitment and phosphorylation of several proteins that act in cell signaling pathways. Thus, the function of the JAK2 protein is to act as a mediator between the membrane receptor and the molecules of cell signaling.[20] Some studies show that JAK2 is the sole Janus kinase responsible for the signaling of erythropoietin (EPO) receptors, since the deletion of this gene results in embryonic lethality due to the lack of erythropoietin.[26] Additionally, JAK2-deficient hematopoietic progenitors fail to respond to stimulation of EPO.[27]

The JAK2V617F mutation confers constitutive kinase activity to the protein. Thus, it remains constantly phosphorylated[28] which leads to uncontrolled proliferation of hematopoietic cells independently of cytokines. This event is observed in hematopoietic colonies from patients with PV.[29] This transformation mediated by the JAK2V617F mutation is more efficient in hematopoietic cells that coexpress the erythropoietin receptor, thrombopoietin receptor or granulocyte colony-stimulating factor receptor.

Unlike most cytokine receptors, the erythropoietin receptor, thrombopoietin receptor and granulocyte colony-stimulating factor receptor are homodimeric type I receptors, which are expressed in cells of the erythrocytic, megakaryocytic and granulocytic lineages, respectively. Thus, the propensity of the JAK2V617F mutation for proliferating neoplasms involving these three lineages can be explained partly by the differential expression of this type of cytokine receptor during hematopoietic differentiation.[30]

In vitro studies have shown that expression of the JAK2V617F mutation activates multiple signaling pathways that contribute to the neoplastic transformation process with increased proliferation and inhibition of apoptosis. Among the proteins involved in signaling pathways are the transcription activating proteins and signal transducers (STATs), especially STAT5, which, among other functions, positively regulate the production of the anti-apoptotic protein Bcl-xL.[31] Dimerization of this protein and translocation to the cell nucleus occur upon activation of STATs, where they interact with specific DNA domains to induce the transcription of the target gene.[25] Considerable evidence suggests that the constitutive activation of STAT5 is the primary cause for the malignant transformation process, leading to the development of CMPNs.[32] However, the key role of STATs in this transformation process has not been completely elucidated yet.[9] Other pathways may be involved, for example, phosphatidylinositol 3-kinase (PI3K), mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK) and protein kinase B (PKB/Akt), which have already been well characterized in leukemia models.[33]

The exaggerated activation of signaling pathways triggered by JAK2V617F may, in part, be explained by the fact that cells with such a mutation can escape from an important negative feedback mechanism that attenuates the signaling caused by the JAK2 protein.[34] The main mechanism for the regulation of Janus kinases is mediated by families of intracellular proteins, whose main function is to negatively regulate signal transduction by cytokines. Among these proteins are the suppressors of cytokine signaling (SOCS) and cytokine-inducible SH2 domain-containing protein (CIS).[35] The SOCS normally bind to JAK kinases resulting in their degradation. In particular, SOCS1 and SOCS3 proteins are responsible for binding to JAK2 and inhibiting its catalytic activity. Although the expression of SOCS1 results in JAK2 and JAK2V617F degradation which, in turn, leads to kinase activity inhibition, the expression of SOCS3, paradoxically, results in an increase in JAK2V617F protein stability and activity, i.e., the constitutively activated JAK2 protein may lead to hyperphosphorylation of the SOCS3 protein, which results in increased cell proliferation. In this case, the SOCS3 protein acts as a potentiator of JAK2-mediated signaling.[36]

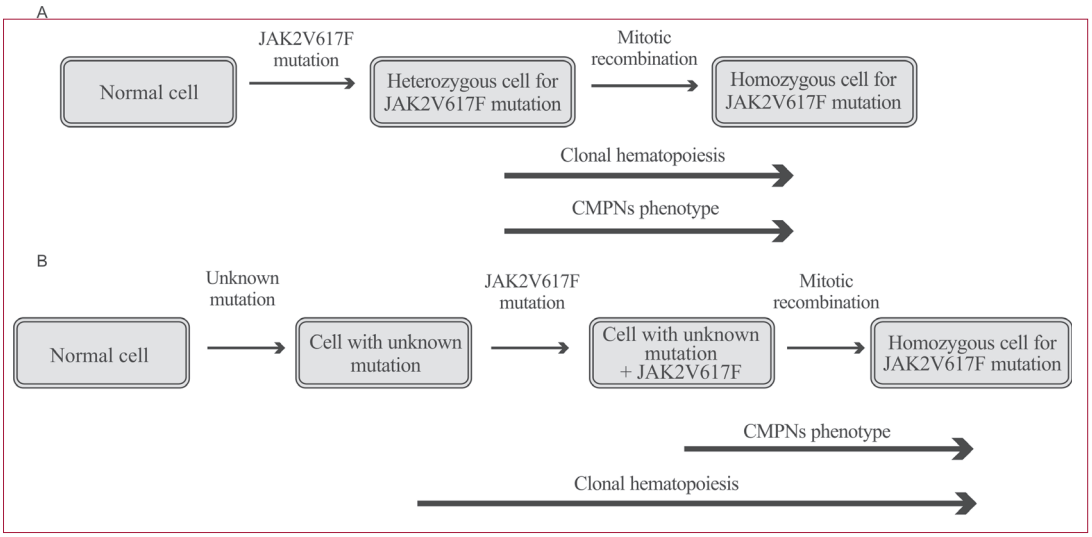
After the discovery of the JAK2V617F mutation, it became clear that this molecular abnormality could be used as a clonal marker for the diagnosis of CMPNs. Initially, the results indicate that this mutation would probably be specific to myeloid lineage precursors as it was not found in lymphocytes. However, with the development of more sensitive methods, the JAK2V617F mutation was observed in a small fraction of lymphocytes and natural killer cells of some patients.[15,37] These data suggest that cells mutate at an early stage of differentiation, which supports the hypothesis that CMPNs are disorders that originate in hematopoietic stem cells.[37]

Genetic complexity of MPN

There are still some issues on CMPNs to be clarified. The main one, from a pathogenic point of view, is to clarify how a single mutation may be associated with the pathogenesis of three distinct diseases: PV, ET and PMF. Some hypotheses are proposed to explain the phenotypic differences between them.[38]

There are currently two hypotheses explaining the role of the JAK2V617F mutation in CMPNs.[2,3,27,39-41] According to these hypotheses, the mutation plays a primary or secondary role in disease development. In the first hypothesis, JAK2V617F simultaneously induces clonal hematopoiesis and starts the myeloproliferative phenotype. The development of each subtype of CMPN is influenced by constitutive genetic factors of each patient. The second hypothesis argues that other mutations acquired prior to JAK2V617F are responsible for the development of the abnormal hematopoietic clone. These mutations, termed "pre-JAK2", besides promoting the acquisition of the JAK2V617F mutation, determine which CMPN the individual will develop.[42] Therefore, according to this hypothesis, mutation development is an event that occurs during the clonal evolution of CMPNs (Figure 2).[41] Data presented in some studies confirm a model that suggests the presence of mutations prior to JAK2V617F.[9,39] It is known that some chromosomal abnormalities, such as the chromosome 20q deletion, are frequently observed in patients with CMPN.[39] Based on this fact, some studies assessed the presence of cytogenetic abnormalities in patients with CMPN and found that all hematopoietic cells of these patients had a deletion of chromosome 20q, whereas only a portion of these cells was positive for the JAK2V617F mutation. This supports the hypothesis that a primary mutation establishes a clonal profile that predisposes patients to mutations in the JAK2 gene.[38] However, there are two main arguments against the hypothesis that the mutation is a secondary event in CMPNs. First, there are no reports of patients identified as being negative for JAK2V617 at diagnosis and who have acquired the mutation later, that is, during the course of the disease.[43] Second, in animal studies, the JAK2V617F mutation, by itself, is able to rapidly develop a disease similar to human PV.[32]

Another important fact in order to understand the pathogenesis of CMPNs was reported by Kralovics et al.[44] These investigators found that loss of heterozygosity (LOH)



Adapted from Kralovics, 2008

Figure 2 – In model A, the JAK2V617F mutation causes the onset of the CMPN phenotype and clonal hematopoiesis. In model B, the acquisition of an unknown mutation results in clonal hematopoiesis. The JAK2V617F mutation is acquired later, and at this point the CMPN phenotype appears. In some patients, mitotic recombination results in transition of cells heterozygous for JAK2-V617F to homozygosity

of chromosome 9p is a relatively common event in PV. Thus, it was shown that, unlike most tumors in which LOH is commonly a result of an unmutated gene copy deletion, in PV this event is a result of acquired uniparental disomy (aUPD),[3,27,44] in which mitotic recombination occurs between homologous regions of heterozygous chromosome-9. It is known that mitotic recombination results in the formation of homozygous chromosomes for the mutation, which have an additional proliferative advantage compared to heterozygous chromosomes (Figure 3).[3]

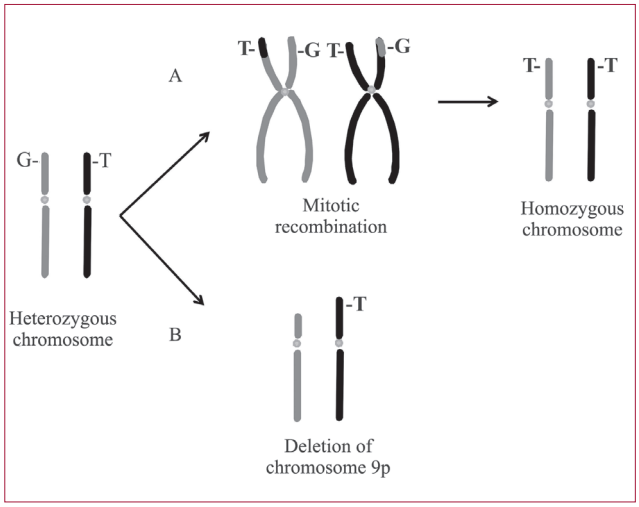


Figure 3 – In this figure two alternative models are presented, A and B. The chromosome 9 with the wild-type JAK2 sequence is depicted in gray (G), and chromosome 9 with the mutation is shown in black (T). In model A, mitotic recombination might result in 9pLOH. Alternately, deletion of the telomeric part of the wild-type chromosome 9p as a potential mechanism for 9pLOH is shown in model B

Some studies on the JAK2V617F mutation in cell lineages and in animal models demonstrate its oncogenic profile, as well as its potential to cause CMPNs.[4,32,45] These studies show that in models of bone marrow transplantation and in transgenic mice, the JAK2V617F mutation is capable of inducing a myeloproliferative phenotype, similar to what happens in human CMPNs. However, there was a difference in the intensity of erythrocytosis, leukocytosis and the induction of marrow fibrosis in mice with and without other gene alterations.[32,46] This observation shows that different types of genetic changes may alter the phenotype induced by JAK2V617F.[2,32,41,42,46]

In experiments with transgenic animals, Kralovics et al.[41] showed that the level of the JAK2V617F mutation expression also interferes with the phenotypes. For example, animals with low expression of the mutation developed thrombocythemia and those with high expression, developed polycythemia. As already mentioned, the homozygosity for the JAK2V617F mutation is the result of aUPD in chromosome 9p24. Some studies have shown that homozygosity is more common in PV than in ET.[3,7,15,38,47,48] Therefore, one can assume that aUPD of chromosome 9p24 and homozygosity for the JAK2V617F mutation are common in the development of PV, but not in ET.[3,4,15,46,47] *In vivo* studies are consistent with the hypothesis that overexpression of JAK2V617F in hematopoietic compartments causes polycythemia and leukocytosis without associated thrombocytosis[32,38,49] and that a low expression of this mutation is associated with thrombocytosis.[42,45] Some studies reveal the existence of a familial predisposition for the development of CMPNs, which reinforces the hypothesis of the presence of alleles that provide a selective advantage for the development of specific mutations.[50,51] Familial MPN is characterized by a dominant

autosomal inheritance with incomplete penetrance and variable presence of the three CMPNs with clinical and molecular characteristics indistinguishable from sporadic CMPNs.[50,52] In all cases examined, the JAK2V617F mutation is acquired, not inherited through the germinal lineage.[51] The phenotypic penetrance is said to be incomplete, because the mutations present in the inherited germinal lineage do not cause CMPNs by themselves; they rather depend on the occurrence of a somatic mutation of the JAK2 gene.[41]

Pardanani et al.[53] carried out research on the phenotypic influence of single nucleotide polymorphisms (SNPs) in the JAK2 gene. In that study, 32 SNPs were analyzed in 179 patients with different CMPNs. It was found that SNPs were associated with either PV or ET. Although this is just one isolated study, it provides evidence that genetic variations of each individual are relevant to the phenotype of CMPNs.[42]

Laboratory diagnosis

Until recently, there was no specific laboratory tests for the diagnosis of BCR/ABL-negative myeloproliferative neoplasms.[10] The criteria for the diagnosis of PV were extensively modified since standardization by the Polycythemia Vera Study Group (PVSG) over twenty years ago.[2,54] Tests were expensive and were not universally available. Moreover, the tests had low sensitivity and specificity. Those tests included the determination of red cell mass to distinguish between erythrocytosis vera and relative polycythemia, identification of erythroid colony growth independent of erythropoietin *in vitro*, cytogenetic analysis of bone marrow cells, checking of EPO levels, ultrasound of the spleen and analysis of the overexpression of polycythemia rubra vera-1 (PRV-1).[55]

Currently, according to a review of the criteria used in the diagnosis of CMPNs produced by WHO in 2008, the presence of mutations in the JAK2 gene is considered the most important criterion for the diagnosis of PV,[4,27] and represents a clonal marker in ET and PMF.[6,18] Furthermore, the presence of this mutation distinguishes clonal myeloproliferation of the CMPNs from those observed in secondary polycythemia and reactive fibrosis or thrombocytosis. Different methods can be used to investigate the JAK2V617F mutation, such as allele-specific polymerase chain reaction (PCR),[15,56,57] real-time PCR,[58] pyrosequencing,[47] and PCR coupled with enzymatic digestion.[15,57]

In the clinical practice, investigation of the JAK2V617F mutation is performed as a triage in patients with increased hemoglobin levels, thrombocytosis, neutrophilia, splenomegaly of unknown origin and abdominal vein thrombosis. In such cases, the detection of this mutation confirms the presence of CMPN, while the absence is of limited value for the diagnosis.[56,59]

Although the methods to detect mutations in the JAK2 gene are not well standardized, they are widely available and sensitive enough to detect the presence of heterozygous mutations in 5 to 10 percent of cells. They also have low rates of false-positive results, which makes them great tools for diagnostic use.[2] However, the odds of having false-positives and false-negatives cannot be ignored, especially considering the highly sensitive allele-specific testing and the presence of patients with very low rates of mutated alleles (below 5 percent).[6,60]

These aspects were taken into consideration by the WHO during the reformulation of the parameters used to diagnose the disease, in which the histological analysis of bone marrow is considered a necessary criterion for the diagnosis of ET, PV and PMF in the absence of mutations in the JAK2 gene. In addition, other parameters were considered as criteria of greater or lesser importance for the differential diagnosis of CMPNs, as shown in Tables 1, 2 and 3.[56]

Table 1 - The 2008 World Health Organization diagnostic criteria for polycythemia vera

	Polycythemia vera
Major criteria	1. Hgb > 18.5 g/dL (men), Hgb > 16.5 g/dL (women) or Hgb > 17 g/dL (men), Hbg > 15 g/dL (women) if associated with an increase of ≥ 2 g/dL from baseline that cannot be attributed to correction of iron deficiency
	2. Hgb or Htc > 99th percentile of reference range for age, gender or altitude of residence or red cell mass elevated > 25% above mean normal
Minor criteria	1. Presence of JAK2V617F mutation bone marrow trilineage myeloproliferation
	2. Subnormal serum Epo level
	3. EEC growth.

Adapted from Tefferi & Vardiman, 2008[6]
EEC - endogenous erythroid colony; Epo - erythropoietin; Htc - hematocrit; Hgb - hemoglobin
Diagnosis of polycythemia vera requires either both major criteria and one minor criterion or the first major criterion and 2 minor criteria

Table 2 - The 2008 World Health Organization diagnostic criteria for essential thrombocythemia

	Essential thrombocythemia
Criteria	1. Platelet count ≥ 450,000 cells/mm^3
	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. Presence of the JAK2V617F mutation or other clonal marker or No evidence of reactive thrombocytosis

Adapted from Tefferi & Vardiman, 2008[6]
CML - chronic myelogenous leukemia; PMF - primary myelofibrosis
MDS - myelodysplastic syndrome
Diagnosis of essential thrombocythemia requires all four criteria

Table 3 - The 2008 World Health Organization diagnostic criteria for primary myelofibrosis

	Primary myelofibrosis
Major criteria	1. Atypia megakaryocyte proliferation and accompanied by either reticulin and collagen fibrosis or In the absence of reticulin fibrosis, the megakaryocyte changes must be accompanied by increased marrrow cellularity, granulocytic proliferation and often decreased erythropoiesis 2. Not meeting WHO criteria for CML, PV, MDS or other myeloid neoplasm 3. Presence of JAK2V617F mutation or other clonal marker or No evidence of reactive marrow fibrosis
Minor criteria	1. Leukoerythroblastosis 2. Increased serum LDH 3. Anemia 4. Palpable splenomegaly

Adapted from Tefferi & Vardiman, 2008 [6]
CML - chronic myelogenous leukemia; PV - polycythemia vera;
MDS - myelodysplastic syndrome
Diagnosis of primary myelofibrosis requires all three major criteria and two minor criteria

Final considerations

The discovery of the JAK2V617F mutation was essential to establish ideas crucial to understanding the genetic basis of CMPNs, but many questions still remain unanswered. Although literature-based studies provide an idea of how a single mutation can result in three different phenotypes, further studies are needed to determine specific molecular markers for each CMPN subtype.

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