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Chapter

Non-receptor Tyrosine Kinases Role and Significance in Hematological Malignancies

Ana Azevedo, Susana Silva and José Rueff

Abstract

This chapter presents a review about non-receptor tyrosine kinases, their structure, mechanisms of action and physiopathology, and how they are regulated and interact with other molecules and other signaling pathways, contributing to the regulation of fundamental cellular functions such as cell division and differentiation, stress responses, apoptosis, survival, and proliferation, gene expression, immune response, inter alia. Special emphasis will be assigned to the JAK family, the processes whereby it can be mutated/regulated and aberrantly activated, clinical significance and association with hematological disease progression and malignancy, mainly in myeloproliferative neoplasms. Consideration of these mechanisms may have important implications for selection of anti-cancer targeted therapies.

Keywords: tyrosine kinase, non-receptor, JAK, mutation, driver mutations, myeloproliferative, malignancy, drug resistance

1. Introduction

The existence and homeostasis of all living multicellular organisms depend on the existence of critical links established by several complex signaling pathways forming a circuitry of regulation.

The development of the Human Genome Project was crucial for the knowledge of the protein kinase, responsible for phosphorylation of other molecules, mostly proteins which can be grouped in two main classes, tyrosine kinases and serine-threonine kinases [1].

Tyrosine kinases (TKs) are a family of more than 90 enzymes that act as fundamental mediators of all signal transduction processes, contributing to a variety of biological mechanisms in response to internal and external triggers, modulating cellular growth, differentiation, migration, metabolism, apoptosis, and survival [2, 3]. Though their activity is very well regulated in normal cells, recent studies have implicated TKs in human neoplastic disorder development and progression, including hematological malignancies [4], assuming a dominant oncoprotein status, either by acquiring transforming functions due to mutations by enhanced expression or by autocrine paracrine stimulation [2, 3]. These mechanisms of abnormal activation of TKs led to important efforts in the development of newly target-directed molecules for cancer therapy as selective TK inhibitors [2–6].

Tyrosine kinases are responsible for the selective phosphorylation of tyrosine residues in specific target protein substrates, using ATP, thus allowing transmission of signals from the cellular surface to cytoplasmic proteins and the nucleus, to regulate physiological circuits [2, 3, 5]. They can be further subdivided into two groups, receptor proteins and non-receptor proteins (which will be discussed below).

Briefly, receptor tyrosine kinases (RTKs) include several families, namely, epidermal growth factor receptor (EGFR), insulin receptor (IR), fibroblast growth factor receptor (FGFR), and platelet-derived growth factor receptors (PDGFR). They function as transducers of extracellular signals to cytoplasm and contain several domains, multiple extracellular ligand binding (e.g., EGF, PDGF, etc.) sites, a cytoplasmic portion with catalytic and regulation features, and a single transmembrane hydrophobic disulfide bond that links the two other regions [1, 5]. RTKs function as cell surface receptors, being activated by ligand binding to the extracellular domain, with subsequent dimerization of receptors and transphosphorylation in the cytoplasmic domain [5]. They constitute also enzymes with kinase activity, which are associated with altered gene expression, interfering with cellular division, migration, and survival functions [3].

Non-receptor tyrosine kinases (NRTKs) are organized into nine subfamilies based on sequence similarities, primarily within the kinase domains, and are able to regulate several cellular processes, such as cellular division, proliferation and survival, gene expression, and immune response, among others [3]. The role of their deregulation, genetic alterations, and abnormal activation in the development of hematological malignancies will be covered in this review.

Novel therapeutic compounds able to target kinases have been developed for the treatment of patients with this kind of disorders.

2. Non-receptor tyrosine kinase families

Non-receptor tyrosine kinases (NRTKs) are a subgroup of tyrosine kinases, intracellular cytoplasmic proteins, or anchored to the cell membrane, which can trigger intracellular signals derived from extracellular receptor [3]. They can be classified into nine subfamilies according to sequence similarities, primarily within the kinase domains. These include ABL, FES, JAK, ACK, SYK, TEC, FAK, SRC, and CSK family of kinases, which will be presented below in this section.

Unlike RTKs, NRTKs lack receptor-like features, such as an extracellular ligand-binding domain and a transmembrane-spanning domain, exhibiting considerable structural variability (**Figure 1**). They comprise a shared kinase domain, which spans approximately 300 residues and consists of an N-terminal portion (five stranded β-sheet and one α-helix), and a large cytoplasmic C-terminal domain (mainly α-helical). Moreover, they often possess several additional signaling or protein-protein interacting domains, such as SH2, SH3, and PH domains. The ATP molecule binds between the two domains, and the tyrosine sequence of the protein substrate links with the residues of the C terminal domain [5].

The activation of NRTKs involves several complex mechanisms of heterologous protein-protein interaction to enable cellular tyrosine kinase phosphorylation, highly regulated by antagonist effects of tyrosine kinase versus phosphatases, which results in the successive activation of specific signaling pathways and messenger proteins that regulate cellular functions, such as growth, division, and apoptosis [5].

In the last few years, it has been substantiated that NRTKs can suffer two types of oncogenic mutations, namely, intragenic point mutations, duplications, or deletions and insertions, or in addition chromosomal rearrangements may occur, resulting in the fusion of genes (e.g., most famously BCR-ABL), associated with
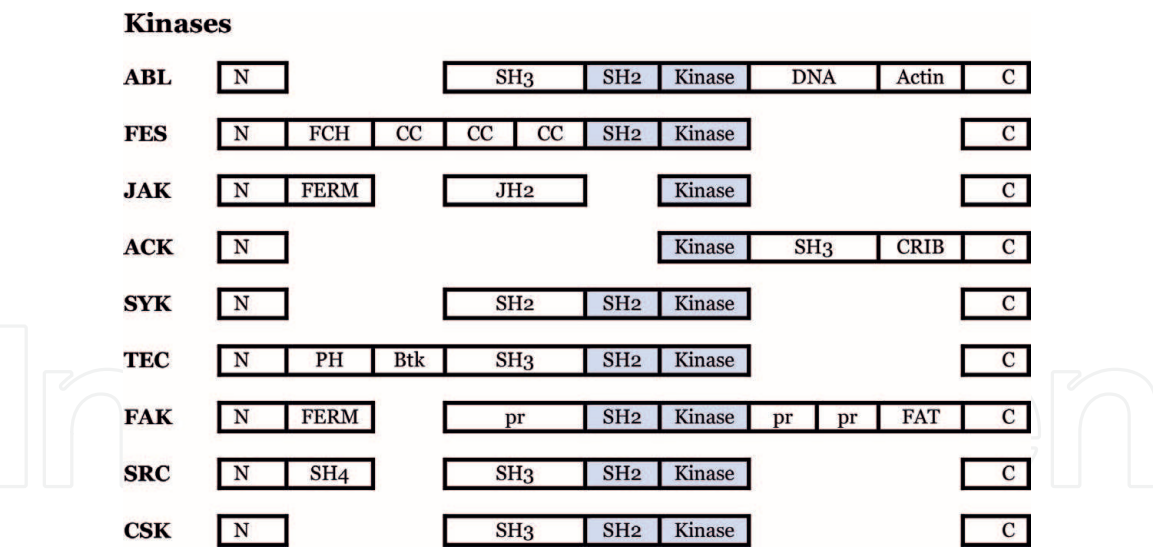
Figure 1.
Domain organization of the major non-receptor tyrosine kinase families (adapted from Siveen et al. [3]). Actin, actin-binding domain; Btk, Btk-type zinc finger motif; C, carboxy-terminus; CC, coiled coil motif; CRIB, Cdc42/Rac-interactive domain; DNA, DNA-binding domain; FAT, focal adhesion targeting domain; FCH, FES/Fer/Cdc-42 interactive protein homology domain; FERM, four-point-one, ezrin, radixin, moesin domain; JH2, Janus homology domain 2 (or pseudokinase domain); kinase, catalytic kinase domain (or SH1 domain); N, amino terminus; PH, pleckstrin homology domain; pr, proline-rich region; SH2, SRC homology 2 domain; SH3, SRC homology 3 domain; SH4, SRC homology 4 domain.

the development of hematological malignancies, either leukemia, lymphoma, or myeloma [3]. These mutations lead to aberrant kinase activation and signaling or a constitutive kinase activity, associated with the formation of oncogenes (or "driver mutations"), such as ABL, FES, SRC, and others, implicated in the process of hematopoiesis, contributing to cellular prolonged viability and survival [3]. Although some NRTK oncogenes exhibit structural, functional, and cellular localization differences, many of them share the same molecular pathways for cellular proliferation and viability regulation [3]. Later in this revision, we will focus the role of some NRTK families, mainly JAK, involved in the development of specific hematological malignancies, covering their associated genetic alterations and mutations, deregulation, and abnormal activation.

Recent advances have also been made in the development of specific kinase inhibitors and new therapies in order to target mutated kinases and inhibit their activity, showing to be very effective and remarkably well tolerated [3].

NRTKs play a crucial role in several cellular mechanisms. Some examples are the involvement of JAK family in cell signaling, through activation of signal transducers and activators of transcription (STAT); the role in cellular growth of nuclear TKs (e.g., ABL), through activation of transcription factor Rb, and of ACKs via the induction of JAK and SRC; the regulation of cell adhesion and proliferation mediated by FAK; the association of Fyn and ACKs with signal transduction pathways and of TEC families with intracellular signaling processes; and the intervention of SYK in immune response [3].

While BCR-ABL occurs exclusively in leukemia, many of the subsequently discovered tyrosine kinase fusions occur in multiple tumor types, including both liquid and solid malignancies [5].

2.1 ABL kinases

The Abelson (ABL) kinase family includes ABL1 and ABL2 (ABL-related gene, ARG) proteins, which are ubiquitously expressed and necessary for normal cellular function, encoded by ABL1 and ABL2 genes.

ABL family is involved in the regulation of several cellular mechanisms, namely, proliferation, migration, invasion and adhesion, reaction to DNA lesion and stress, and survival, through the interaction of distinct extracellular stimuli with specific signaling pathways [7]. Several growth factors, such as PDGF, EGFR, transforming growth factor β, and angiotensin subtype 1 receptors, are responsible for the activation of cytoplasmic c-ABL [8].

The identification of the fusion oncoprotein BCR-ABL1, which results from the translocation leading to the Philadelphia chromosome (Ph), by the American geneticist Janet Rowley (1925–2013) in 1972, formed by the reciprocal translocation between chromosomes 9 and 22 (t(9;22)(q34.1;q11.2)), and in 1985–1986, the knowledge of the *BCR-ABL1* transcript and its P210 fusion protein product, reinforced the role of ABL family in malignant disorders, especially hematological, such as acute myeloid leukemia (AML), chronic myeloid leukemia (CML), and acute lymphoblastic leukemia (ALL). The translocation of the breakpoint cluster region (BCR) sequences of chromosome 22 with the c-ABL tyrosine kinase of chromosome 9 gives origin to a fusion gene, responsible for the production of three oncoproteins. The BCR-ABL chimeric gene product has an enhanced tyrosine kinase activity, contributing to disease phenotype [2].

In 1996, in the era of the Human Genome Project development, these discoveries led Nicholas Lydon (b.1957), a British scientist, and Brian Druker (b. 1955), an American physician scientist, to the elaboration and therapeutic use of imatinib (a tyrosine kinase inhibitor) in CML [9].

The several products of malignant ABL fusion gene result in constitutively activated ABL kinases that can lead to cellular transformation and cancer. Activation of ABL kinases due to chromosome translocation is very rare in solid neoplasms, but usually there is overexpression, upstream oncogenic TKs or other chemokine receptors, inactivation of negative regulatory proteins, and/or oxidative stress [3].

There is a large number of signaling pathways that are activated by BCR-ABL, but those critical for BCR-ABL-dependent transformation include Gab2, Myc, CrkL, and STAT5 [3].

The first human malignancy to be associated to a specific genetic abnormality was chronic myelogenous leukemia, a clonal bone marrow stem cell malignancy, which accounts for 15–20% of adult leukemia's with a frequency of 1–2 cases per 100,000 individuals. It is more common in men and is rarely seen in children.

The formation of constitutively active chimeric BCR-ABL1 fusion oncoproteins leads to the creation of three distinct BCR-ABL variants, namely, p185, p210, and p230. The most common variant in CML is p210, in which the first exon of c-ABL has been replaced by BCR sequences, encoding either 927 or 902 amino acid, observed in hematopoietic cells of CML-stabilized patients, and in ALL and AML [3]. The p230 form is associated with acute leukemias, neutrophilic-CML, and rare cases of CML. The p185 form, containing BCR sequences from exon 1 fused to exons 2–11 of c-ABL, is found in about 20–30% of adults and about 3–5% of children with B-cell ALL [3].

BCR-ABL is the most common chromosomal translocation, but several other chromosomal abnormalities result in the expression of various fusion proteins, yet there are no activating point mutations identified in the ABL1/ABL2 genes [3].

BCR-ABL oncoprotein is the most frequent genetic defect found in adult ALL patients. Nearly 3–5% childhood and 25–40% adult cases of ALL have Philadelphia chromosome, associated with an aggressive phenotype and a worst prognosis [3].

The identification of BCR-ABL expression as the determinant leukemogenic event in CML and the use of BCR-ABL tyrosine kinase inhibitors (TKIs) since 2001 have changed the course of the disease and the management of patients, leading to a reduction in mortality rates and a consequent increase in the estimated prevalence of this disorder [10].

Imatinib mesylate, also known as STI571, was initially the standard of care for the first-line treatment of CML patients in chronic phase, due to its high long-term response rates and favorable tolerability profile compared with previous standard therapies [10]. The majority of kinase inhibitors are currently in clinical use to target BCR-ABL [11]. Imatinib is an ATP-competitive inhibitor that works by stabilizing the inactive ABL kinase domain conformation. Combining imatinib mesylate with standard chemotherapy also increases the overall long-term disease-free survival in both adults and children [3].

Approximately 15–30% (2–4% annually) of patients treated with imatinib discontinues treatment after 6 years due to resistance or intolerance, particularly in the accelerated and blast phase [10]. Nilotinib, dasatinib, bosutinib, and ponatinib are second-generation TKIs used for imatinib mesylate-resistant cases.

A literature review shows that pre-existing mutations at baseline confer a more aggressive disease phenotype and patients with advanced stages of the disease often do not respond to therapy or relapse [10].

The role played by efflux ABC transporters in resistance to TKI in CML has deserved studies indicating its possible major role in drug resistance, besides the acquisition of mutations in the fusion leading to inefficacity of the TKI [12–14].

2.2 Feline sarcoma (FES) kinases

Feline sarcoma (FES) and FES-related (FER) proteins are proteins included in another group of NRTKs, called FES kinase family. These kinases are homologous to viral oncogenes responsible for cancerous transformation, namely, feline v-FES (Feline sarcoma) and avian v-fps (Fujinami poultry sarcoma).

Fer is ubiquitously expressed, while FES is a proto-oncogene expressed mostly in myeloid hematopoietic, neuronal, epithelial, and vascular endothelial cells.

There is recent evidence that both kinases are activated in AML blasts and regulate vital functions related with internal tandem duplication containing FLT3. FES is associated with phosphorylation/activation of STAT family, with signaling proteins such as phosphatidylinositol-4,5-bisphosphate 3-kinase, mitogen-activated protein kinases, and extracellular signal-regulated kinases and with signaling of the mutated oncogenic KIT receptor [15]. It is involved in several cellular mechanisms such as migration, survival and immune response, myeloid differentiation, and angiogenesis, through interaction with multiple cell surface growth factors and cytokine receptors (e.g., IL3, IL4, and GM-CSF receptors) [3]. Fer kinase participates in cell cycle progression.

FES kinases consist of a unique amino-terminal FCH (FES/FER/CDC-42-interacting protein homology) domain, three coiled coil motifs that promote oligomerization, a central SH2 domain for protein interactions, and a kinase domain in the carboxy-terminal region. FCH domain together with the first coiled coil motif corresponds to FCH-Bin-Amphiphysin-Rvs (F-BAR) domain (**Figure 1**) [16]. Although there is no negative regulatory SH3 domain, the catalytically repressed state of FES is strongly regulated through a tight interaction between SH2 and kinase domain.

Activation of FES kinase requires active phosphorylation of Tyr713 located inside the activation loop and of Tyr 811. Hyperactivation of FES kinase is necessary for deregulated proliferation in human lymphoid malignancies, but aberrant activation is not associated with human tumors [17].

Four somatic mutations within the kinase domain of FES were identified in colorectal cancers, and Fer mutations have been associated to small-cell lung cancer [3].

2.3 JAK kinases

This family comprises four members, JAK1, JAK2, JAK3, and TYK2, originally named "just another kinase." They owe their name due to the similarity of kinase (JH1) and pseudokinase (JH2) symmetrical domains with Janus, the Roman god of two faces [18, 19]. TYK2 was the first family member to be identified by Krolewski in 1990, through libraries of complementary DNA from human T lymphocytes, while JAK1, JAK2, and JAK3 were identified using conserved motif clonation of the catalytic domain [18]. They comprise seven homologous JH domains organized into four regions: kinase (JH1), pseudokinase (JH2), FERM (four-point-one, ezrin, radixin, moesin, including the N-terminal JH7, JH6, JH5, and part of JH4), and SH2-like (JH3 and part of JH4) (**Figure 1**) [20]. The carboxy-terminal portion of these molecules includes the distinctive kinase domain (JH1) which is catalytically active and the catalytically inactive pseudokinase domain (JH2) which is felt to regulate the activity of JH1. The other amino-terminal JH domains, JH3–JH7, mediate association with receptors. FERM domain regulates the binding to the membrane-proximal part of the cytokine receptors [21].

In humans, JAK1 gene is located on chromosome 1p31.3, JAK2 gene on 9p24, JAK3 gene on 19p13.1, and TYK2 gene on 19p13.2 [9].

JAK proteins interact with different intracellular domains of cytokine receptors (discussed below) and are present in a variety of cellular subtypes. Expression is ubiquitous for JAK1, JAK2, and TYK2 but restricted to hematopoietic cells for JAK3 [9].

Many malignancies, including hematological neoplasms, are associated with deregulated activation of JAK family members, through aberrant cytokine production via autocrine/paracrine processes, point mutations within JAKs, or any other oncogene upstream of signaling cascade (discussed below).

Several studies reported various JAK mutations, mostly point mutations, occurring in all members [22–24]. *JAK2* V617F is one of the most studied mutations affecting JAK family, strongly associated with myeloproliferative neoplasms, which will be discussed in the next section of this chapter, and Hodgkin lymphoma and primary mediastinal B-cell lymphoma [3]. Other mutations have been described, such as 1) JAK1 A634D, localized in the pseudokinase domain, affecting signaling functions (STAT5), in AML, and T-cell and B-cell ALL; 2) JAK3 point mutations associated with various T-cell leukemia/lymphomas, poor prognosis and clinical outcome in juvenile myelomonocytic leukemia, and acute megakaryoblastic leukemia; 3) TYK2 kinase mutations have been reported in T-cell ALL and promote cell survival via activation of STAT1 as well BCL2 upregulation [3].

2.4 ACK kinases

ACKs also known as activated Cdc42 kinases are the fundamental components of signal transduction pathways linked to non-receptor tyrosine kinases. There are seven different types of ACKs, namely, ACK1/TNK2, ACK2, DACK, TNK1, ARK1, DPR2, and KOS1 [25].

The majority of these kinases include both N-terminal and C-terminal domains followed by a SH3 domain along with CRIB, which makes them unique NTRKs, and finally a kinase domain (**Figure 1**) [25].

ACK1 (ACK, TNK2, or activated Cdc42 kinase) is one of the most studied and well-known members of the ACKs. It is a ubiquitous 140-kDa protein located on the chromosome 3q, with the presence of multiple structural domains for its functional diversity, including cell survival, migration, growth, and proliferation, via acting as an integral cytosolic signal transducer for the array of receptor tyrosine

kinases (MERTK, EGFR, PDGFR, IR, etc.) to different intracellular effectors which includes both cytosolic and nuclear, and for epigenetic negative regulation on tumor suppressors [26]. It has been linked to several forms of human cancers, including gastric, breast, ovarian, pancreatic, colorectal, head, and neck squamous cell carcinomas, osteosarcoma, hepatocellular carcinoma, and prostate cancers [26].

Mutations in ACK1/TNK2 gene are the main oncogenic cause for AML, atypical CML, and chronic myelomonocytic leukemia. TNK1 has both tumor-suppressing and oncogenic potential as it can mitigate the growth of tumor cells by downregulating Ras-Raf1-MAPK pathway, induce apoptosis through NF-κB inhibition, and activate cellular transformation and growth of neoplastic cells. TNK1 has oncogenic potential implicated in hematological carcinogenesis such as in AML and Hodgkin's lymphoma, which may open new targets for therapy [3].

2.5 SYK kinases

Spleen tyrosine kinase (SYK) is one of the important classes of soluble cytosolic NRPKs and was first cloned in porcine spleen cells, with high expression hematopoietic cells [3]. It is a 72-kDa protein, encoded by SYK gene located on chromosome 9q22 and is highest homologous to ZAP-70, formed by two highly conserved SH2 domains with N-terminal and one tyrosine kinase domain at C-terminal (**Figure 1**) [3]. Activation of SYK occurs with the intervention of C-type lectins and integrins and the downstream signaling cascade, including VAV family members, phospholipase Cγ isoforms, the regulatory subunits of phosphoinositide 3-kinases, and the SH2 domain-containing leukocyte protein family members (SLP76 and SLP65) [27].

The SYK family is important in immune response between cell receptors and intracellular signaling mechanisms, through phosphorylation of cytosolic domain of the immunoreceptor tyrosine-based activation motifs (ITAMs), resulting in the conformational changes and further activation of SYK and signal transduction to other downstream target/effector proteins [27]. Its stimulatory effect on various survival pathways/signaling molecules supports the crucial role that SYK family has in many forms of hematological malignancies [28]. On the other hand, they also have a tumor-suppressive effect in the disorders of nonimmune origin [29]. Progress can be made in the development of targeted effective therapy.

2.6 TEC kinases

TEC kinase family is the second largest subclass of the NRTKs. It includes five members, namely, Bruton's tyrosine kinase (BTK), interleukin 2-inducible T-cell kinase (ITK/EMT/TSK), tyrosine-protein kinase (RLK/TXK), bone marrow tyrosine kinase on chromosome (BMX/ETK), and tyrosine kinase expressed in hepatocellular carcinoma (TEC) [30]. Their structure is characterized by the presence of an amino-terminal (PH) that can bind phosphoinositides, enabling the interaction between phosphotyrosine-mediated and phospholipid-mediated signaling pathways, and Btk-type zinc finger (BTK) motif followed by two domains, SH3 and SH2, and a carboxy-terminal kinase domain (**Figure 1**).

TEC proteins are expressed in hematopoietic cells and involved in cellular signaling pathways of cytokine receptors, RTKs, lymphocyte surface antigens, G-protein-coupled receptors, and integrins, contributing to cellular growth and maturation of blood cells [3]. For example, it has been shown that BTK mutations are associated with B lymphocytes and other relevant cells contributing to the tumor microenvironment (e.g., dendritic cells, macrophages, myeloid-derived suppressor cells, and

endothelial cells) development impairment [31, 32], increasing the need of innovative immunochemotherapies, such as BTK inhibitors (e.g., ibrutinib), which have improved disease control rates but, unfortunately, not survival [33].

BTK, ITK, and TXK are predominately expressed in bone marrow cells, whereas BMX and TEC even extend to normal somatic cells (e.g., cardiac endothelium) [3, 30]. BMX is expressed in myeloid lineage hematopoietic cells (e.g., granulocytes and monocytes), endothelial cells, and numerous types of oncologic disorders, having a preponderant role in cellular survival, differentiation and motility, and playing a key role in inflammation and cancer [30]. Furthermore, TEC is expressed in hematopoietic cells, namely, myeloid and lymphoid, B and T, lineages; is involved in the stabilization, signaling, and activation of lymphocytes [34]; and acts as a regulator of pluripotent stem cells, through the regulation of fibroblast growth factor-2 secretion, associated with tumorigenesis and hepatocellular carcinoma progression [3].

2.7 Focal adhesion kinases

FAK family includes two members, namely, the ubiquitously expressed focal adhesion kinase and the associated adhesion focal tyrosine kinase (Pyk2), which is expressed in the central nervous system and in hematopoietic cells.

FAK and Pyk2 share a domain structure that includes an N-terminal FERM domain, followed by a residue linker region, a central kinase domain, a residue proline-rich low complexity region, and a C-terminal focal adhesion targeting domain (**Figure 1**) [35].

FAKs are involved in cell propagation and adhesion and in cell to microenvironment communications [36]. They are associated with B-lymphoblastic leukemia and lymphoma cells but are usually absent in leukemias/lymphomas of T-cell origin and in myeloma [3]. These kinases are involved in regulation of cellular proliferation and migration, via response to extracellular stimuli. Interaction with growth factor leads to phosphorylation/activation of SRC kinase, which in turn is associated with various signaling pathways, and modulates proliferation and survival of tumor cells in AML and MDS patients [37]. FAK overexpression has been associated with leukemic cell migration from the marrow to the circulating compartment, drug resistance, and poor survival outcome [3].

2.8 SRC kinases

The SRC family of tyrosine kinases (SFKs) is membrane-associated NRTKs, acting as key mediators of signal transduction pathways and modulators of RTK activation, promoting mitogenesis. This class includes 11 related kinases: BLK, FGR, FYN, HCK, LCK, LYN, c-SRC, c-YES, YRK, FRK (also known as RAK) and Srm [38].

Their structure includes in the amino-terminal region a membrane-targeting myristoylated or palmitoylated SH4 domain; a specific domain of 50–70 residues different for each member of the family, trailed by SH3, SH2, and kinase domains; and a short carboxy-terminal tail with an auto-inhibitory phosphorylation site (**Figure 1**) [39, 40].

BLK, FGR, HCK, LCK, and LYN expression predominates in hematopoietic cells, whereas c-SRC, c-YES, YRK, and FYN are highly expressed ubiquitously in platelets, neurons, and some epithelial tissues; Srm is found in keratinocytes; and Frk is present primarily in the bladder, breast, brain, colon, and lymphoid cells [38, 39].

SFKs are involved in a wealth of cellular mechanisms, such as cell survival regulation, DNA synthesis and division, actin cytoskeleton rearrangements, and

motility, through a major role in a variety of cellular signaling pathways activated by several RTKs (PDGF-R, EGF-R, FGF-R, IGF1-R, and CSF-R) and G-protein-coupled receptors [3]. Catalytic activity is exercised upon phosphorylation of a critical residue (Tyr419) within the activation loop and of the auto-inhibitory site Tyr530 within the carboxy-terminal tail, forming a closed auto-inhibited inactive conformation via the association of the SH2, SH3, and kinase domains by intramolecular interactions. However, these interactions could be broken by mutations or specific cellular triggers that are able to disrupt the inactive conformation of SFKs [3].

There is evidence that SFKs are involved in cancer development, by several different mechanisms. They are implicated in the regulation of cell-cell adhesion, involving different molecules, such as p120-catenin protein, a substrate of SRC; on the other hand, particularly SRC might be involved in the activation of STAT (STAT3 and STAT5) transcription factors which regulate cytokine signaling in hematopoietic cells and regulation of RAS/RAF/MEK/ERK MAPK and VEGF pathways and apoptosis molecules, having a role in the progression of CML, AML, CLL, and ALL. SFKs such as focal adhesion kinase, paxillin, and p130CAS have been implicated in monitoring of signaling pathways mediated by integrin, whose functional alterations are associated with several tumor types [3, 41]. SFKs are also associated with the development and signaling of T and B cells, particularly LCK, LYN, and FYN [39, 42–44].

Activation of SFKs due to mutation or binding to activating partners such as growth factor receptors (HER2/NWU, PDGF, EGFR, and c-kit), adaptor proteins, and other NRTKs (focal adhesion kinase and Bcr-ABL) can be detected in several cancers [45]. However, oncogenic mutations are rarely observed in the progression of hematopoietic malignancies such as leukemia and lymphomas (AML, ALL, CML, Burkitt's lymphoma, etc.), which are especially the result of constitutive activation of SFKs and amplification of anti-apoptotic and oncogenic downstream signaling pathways [41]. Moreover, there is evidence that SFKs promote cancer cell resistance to chemotherapy, radiation, and targeted RTK therapies. For example, Lyn and Hck have demonstrated upregulation and interaction with the oncogenic BCR-ABL fusion protein in specimens from patients with advanced CML and ALL who showed relapse after imatinib mesylate treatment [46, 47].

Due to the importance of SFKs in cancer development, it has been considered that inhibition of these proteins in combination with standard therapies may represent a great clinical potential in disease control [48].

2.9 C-terminal SRC kinases

C-terminal SRC kinases (CSK) and CSK-homologous kinase (CHK) are the two members included in this family of NRTKs. CSK is a 50-kDa protein ubiquitously expressed in all cells, primarily present in cytosol, with an amino-terminal SH3 domain followed by a SH2 domain and a carboxy-terminal kinase domain (**Figure 1**). CSK protein has no site for the activation loop for autophosphorylation nor a transmembrane domain or any fatty acyl modifications. However, the mobility of CSK to the membrane is a critical step in the regulation of its own activity, so that it is achieved by means of numerous scaffolding proteins (caveolin-1, paxillin, Dab2, VE-cadherin, IGF-1R, IR, LIME, and SIT1) [49].

Chk is mainly expressed in the brain, hematopoietic cells, colon tissue, and smooth muscle cells [3].

The binding of SH2-kinase and SH2–SH3 linkers to the amino-terminal lobe of the kinase domain stabilizes the active conformation. CSKs function as the major endogenous negative regulators of SFKs, as a result of CSK phosphorylation of

the auto-inhibitory tyrosine residues in the SRC family kinase's C-terminal tail. Although its physiological importance is not known, several other signaling proteins such as paxillin, P2X3 receptor, c-Jun, and Lats can also serve as substrates of CSK [3].

These proteins have a critical role in the regulation of cell functions, such as growth, migration, differentiation, and immune response. Recent studies suggest that CSK can have a function as tumor suppressor through the inhibition of SFK oncogenic activity [3].

3. Myeloproliferative neoplasms and their association with non-receptor tyrosine kinase families

Myeloproliferative neoplasms (MPNs) are clonal hematopoietic malignancies resulting from the transformation of hematopoietic stem cells, leading to abnormal amplification of physiological signal transduction pathways and proliferation of one or more myeloid lineages. The *Word Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues* classified MPNs as chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF) [50], chronic neutrophilic leukemia, and chronic eosinophilic leukemia not otherwise specified and MPNs unclassifiable [51]. In addition to primary (de novo), myelofibrosis can be secondary to PV (post-PV) or ET (post-ET) [52]. In the last revision of the WHO classification, in 2016, some changes were introduced, and mastocytosis ceased to be listed under the heading of MPNs [53].

Dameshek (1900–1969) was the first to conceptualize these groups of disorders, in 1951, highlighting the clinical and morphologic similarities between CML and Philadelphia-negative MPNs (PN-MPNs), namely, PV, ET, and PMF [54]. He realized that these disorders are caused by hyperproliferation in the bone marrow of more than one hematopoietic lineage, which proliferates "as a unit," and introduced the term "myeloproliferative disorders," indicating that these entities may correspond to a continuum of related syndromes. Moreover, he also postulated that the proliferative activity could be the result of a "hitherto undiscovered stimulus." However, the finding that bone marrow and peripheral blood cells from MPN patients can produce erythroid colonies in vitro without the stimulus of growth factor addition indicated the cell independent nature of these disorders [55].

But the "story" about MPNs had begun a few years before. Previously in 1845, John Hughes Bennett (1812–1875), an English pathologist working in Edinburgh, had described CML, and in 1879, a German surgeon, Gustav Heuck (1854–1940), underlined the morphological distinguishing features between PMF and CML, namely, the presence of bone marrow fibrosis, osteosclerosis, and extramedullary hematopoiesis in the former. Some years later in 1892, Louis Henri Vaquez (1860–1936), a French physician, was the first to describe PV, about a patient with marked erythrocytosis and hepatosplenomegaly, and in 1903 William Osler (1849–1919) took another step forward, distinguishing PV from both relative polycythemia and secondary polycythemia. The first description of ET is credited to Emil Epstein (1875–1951) and Alfred Goedel, two Austrian pathologists, who in 1934 published a case report of a "hemorrhagic thrombocythemia" in the absence of marked erythrocytosis.

In 1960, Peter Nowell (b. 1928) and David Hungerford (1927–1993), two American scientists working in Philadelphia, established the association between the Philadelphia (Ph) chromosome and CML [56], in contrast to PN-MPNs (PV, ET, and PMF).

Finally, the description of all four classic MPNs as clonal stem cell diseases was achieved by Philip Fialkow (1934–1996), an American physician scientist, through his studies developed between 1967 and 1981, on X chromosome inactivation patterns in women with PV, ET, PMF, and CML carrying a polymorphic variant of the X-linked glucose-6-phosphate dehydrogenase (G-6-PD) gene [9, 57–59].

To better understand the pathophysiology of these disorders, the role of tyrosine kinases in all the process is crucial to elucidate some of the underlying mechanisms.

Hematopoiesis is the process by which multipotent bone marrow-based stem cells (HSC) differentiate and mature into fully formed blood cells (namely, lymphoid, erythroid, megakaryocytes, and other myeloid cells), in response to external stimulus, such as erythropoietin (EPO), thrombopoietin (TPO), granulocyte-macrophage colony-stimulating factor (GM-CSF), other stimulating growth factors, and several interleukins. Growth factors initiate signal transduction pathways (e.g., JAK-STAT pathway), which lead to the activation of transcription factors, and elicit different outcomes depending on the combination of factors and the cellular stage of differentiation.

In a healthy adult person, approximately 10^{11}–10^{12} new blood cells are produced daily in order to maintain steady-state levels in the peripheral circulation. Besides bone marrow, in some cases and if necessary, the liver, thymus, and spleen may resume their hematopoietic function, in a process called extramedullary hematopoiesis, causing these organs to increase in size substantially.

3.1 JAK-STAT signaling pathway

Due to their essential roles as intracellular signaling effectors of hematopoietic cytokine receptor activation, the Janus kinase (JAK) family of tyrosine kinases have aroused much interest since their discovery more than 20 years ago [60].

JAK proteins (presented above) can link several intracellular domains of cytokine receptors and participate in a variety of cellular mechanisms [9].

Furthermore, a seven-member family of transcription factors named signal transducers and activators of transcription (STAT) are also involved in many cytokine signaling pathways. In 1994, Darnell and colleagues identified the first two members of the family, STAT1 and STAT2, by purification of factors linked to interferon (IFN)-stimulated genes, and the other family members were described subsequently [18]. These proteins act as transcriptional factors when they form homo- and heterodimers, among them, by phosphorylation at tyrosine residues in their SH2 domain, induced by upstream JAK proteins, activating different genes and regulating downstream the JAK/STAT signaling pathway [18].

The Janus kinase/signal transducers and activators for transcription (JAK/STAT) pathway regulate a large plethora of biological processes including cellular proliferation, differentiation, cell migration, and apoptosis [18].

All of these proteins are constitutively present in the cytoplasm without previous stimuli but can be quickly activated from the cellular membrane to the nucleus, by the binding of cytokines, growth factors, or hormones on cell surface receptors (**Table 1**) [18].

Typically, Janus kinases function through their interaction with cytokine receptors that lack intrinsic kinase activity. Cytokines initiate signaling when ligand binding occurs (e.g., EPO, TPO) to the appropriate cytokine receptor (type 1 or type 2 cytokine receptors, e.g., EPO-R, MPL), which results in juxtaposition of JAKs, and bind to their specific cellular surface receptors, inducing several important conformational changes mainly oligomerization or multimerization of their receptors. JAK anchorage to the cytoplasmic domain of the cytokine receptor and phosphorylation of a tyrosine residue in the receptor follows, creating a docking site

		Cytokine or factor
JAK family	JAK1	IL-2, IL-4, IL-6, IL-7, IL-9, IL-10, IL-11, IL-13, IL-15, IFN-α, IFN-β, IFN-γ, CT-1
	JAK2	IL-3, IL-6, IL-11, IL-12, IL-13, IFN-γ, CT-1, growth hormone, prolactin, erythropoietin
	JAK3	IL-2, IL-7, IL-9, IL-15, IL-4
	TYK2	IL-6, I-11, IL-12, IL-13, CT-1, IFN-α, IFN-β, IL-10
STAT family	STAT1	IL-2, IL-6, IL-10, IL-27, IFN-α, IFN-β, IFN-γ
	STAT2	IFN-α, IFN-β
	STAT3	IL-6, IL-10, IL-27, LIF, growth hormone
	STAT4	IL-12
	STAT5 a/b	Prolactin, growth hormone, thrombopoietin
	STAT6	IL-4, IL-13

Adapted from Becerra-Díaz et al. [18]

Table 1.
Cytokine and factor stimuli for JAK and STAT family activation.

for the recruitment and activation of cytoplasmic signal transducers and activators of transcription (STATs: STAT3 and STAT5 in the case of JAK2, which is associated with PN-MPNs and will be taken as an example), through their SH2 domain. While STAT proteins are attached to the cytokine receptor, JAK proteins undergo autophosphorylation at a tyrosine residue, detaching the STAT protein from the cytokine receptor so that the STATs form homo- and heterodimers through their SH2 domain that will translocate to the nucleus. There, they bind to the promoter region of genes via specific DNA-binding domains to promote gene transcription.

The net result of STAT3 and STAT5 activation is apoptosis inhibition and a proliferative activity [61], playing an important role in growth factor-induced myeloid differentiation. STAT3 regulates cell growth through regulation of cyclins promoting cell cycle progression, as cyclin D1, and induces Bcl-2, resulting in an anti-apoptotic signal. Moreover, STAT3 may promote cellular differentiation by upregulating the expression and enhancing the transcriptional activity of CCAAT/enhancer-binding protein alpha (C/EBPα), a key transcription factor that drives myeloid differentiation [62]. STAT3 was also shown to play an important role in megakaryopoiesis, mainly through the expansion of megakaryocytic progenitor cells.

Normal differentiation of neutrophils, promoted by G-CSF, is disturbed by expression of a dominant negative form of STAT5. It has been suggested that STAT5 may induce the survival of myeloid progenitors via transcriptional upregulation of the anti-apoptotic protein BclxL and Pim kinase, inhibiting apoptosis of megakaryocytes, and mediates cell growth through induction of cyclin D1, thereby allowing myeloid differentiation to proceed [63].

EPO is secreted by interstitial kidney cells in response to reduction in blood oxygen concentration, transported to the bone marrow where it binds its receptor, EPO-R, and transmits an intercellular signal through a receptor conformational change, which stimulates an increased production of red blood cells [64–66]. The *JAK2* FERM domain constitutively binds to the EPO-R. EPO-induced EPO-R conformational change facilitates cross-phosphorylation and activation of the JAK2 proteins [67].

The amino-terminal extracellular TPO-R domain has a similar structure to EPO-R, which is critical in ligand binding, resulting in a significant overlap between

EPO- and TPO-stimulated pathways. As in EPO signaling, TPO stimulation causes the JAK2-dependent phosphorylation of STAT3 and STAT5, activation of the MAP kinase pathway, and activation of the PI3K/Akt survival pathway indirectly and can induce transcription of the pro-survival factor BclxL through STAT5- and PI3K-dependent pathways, promoting megakaryocyte differentiation. Overall, discovery of STAT, MAP kinase, and PI3K pathway stimulation downstream of the TPO-R gave a framework to understand the considerable overlap in phenotypic response to TPO and EPO [68, 69].

JAK2 also serves as an endoplasmic reticulum chaperone for the EPO and TPO receptors, transporting them to the cell surface, and increases the total number of TPO receptors by stabilizing the mature form of the receptor, enhancing receptor recycling, and preventing receptor degradation [70]. On the other hand, nuclear JAK2 is involved in epigenetic modifications [18, 60, 71, 72].

The JAK/STAT pathway is tightly regulated and inhibited at multiple levels by several protein families—tyrosine phosphatases, suppressors of cytokine signaling (SOCS), and protein inhibitors of activated STATs [9]:

1. SOCS, most notably SOCS1 and SOCS3, and CBL interact with activated JAKs and phosphorylated receptors or mark JAK for proteasomal degradation. CIS, SOCS1, SOCS2, and SOCS3 are members of the SOCS protein family. The synthesis of SOCS is induced by activated STATs resulting in a negative feedback loop, through interaction with activated JAKs and consequent inhibition of STAT recruitment to the binding sites [73, 74].

2. Hematopoietic cells express SHP1. SHP1 belongs to the family of phosphotyrosine phosphatases (PTP); PTP dephosphorylates activated JAKs, STATs, and cytokine receptors [75].

3. Protein inhibitors of activated STATs (PIAS) interact with activated STATs, inhibit their dimerization, and prevent their binding to target DNA [72].

4. LNK sequesters JAK2 by direct binding [72].

Mutations in all four JAKs have been associated with human diseases. Inherited mutated JAK alleles lead to inactivated JAK3 and TYK2 in human immunodeficiency syndrome, while somatic mutations in JAK1, JAK2, and JAK3 result in constitutively active kinases in myeloproliferative diseases and leukemia/lymphomas [60, 72].

A qualitative difference in the signaling state of STAT proteins has been described in PN-MPNs. ET progenitors have high phosphorylation levels of STAT1 and STAT5, whereas PV progenitors have only phosphorylated STAT5. The reasons behind this and other phenotypic differences are unclear but are potentially the result of a complex interplay between acquired and inherited variations, and possibly environmental exposure, all unique to each MPN patient [76].

3.2 Philadelphia chromosome-negative myeloproliferative neoplasms (PN-MPNs)

PN-MPNs (PV, ET, and PMF) are characterized by the clonal proliferation of one or more myeloid cell lineages (erythrocytic, granulocytic, or megakaryocytic), predominantly in the bone marrow, without altering the hematopoietic stem cell hierarchy, and involving JAK-STAT pathway. There is evidence of a normal and

effective maturation, resulting in increased peripheral blood erythrocytes, granulo-cytes, and platelet counts [77].

Among the different PN-MPN entities, there is a frequent overlap of clinical, laboratory, and morphological data. Leukocytosis with neutrophilia, excessive megakaryocytic proliferation with thrombocytosis, myelofibrosis, and spleno-megaly and hepatomegaly associated with the presence of extramedullary hemato-poiesis can occur in any of these diseases.

PN-MPNs are considered as rare disorders, since their combined incidence is lower than 6 per 100,000 individuals per year [78]. Among the existent registries in the European Union, PN-MPNs have an annual incidence rate per 100,000 individuals per year ranging from 0.4 to 2.8 for PV (while the literature estimated 0.68–2.6), from 0.38 to 1.7 for ET (in the literature 0.6–2.5), and from 0.1 to 1.0 for PMF [79, 80]. There are few European studies reported on MPNs' prevalence [80]. However, according to the American data published in 2014, the prevalence per 100,000 individuals of PV (44–57) and ET (38–57) was much higher than that of MF (4–6) or subgroups with MF features (post-PV MF = 0.3–0.7; post-ET MF = 0.5–1.1) [81].

These groups of disorders occur in middle- or advanced-age adults, with a medium age of diagnosis of 65–67 years for PV, 65–70 years for ET, and 67–70 years for PMF [82]. However, it can be diagnosed in younger individuals, particularly if there is a familial predisposition [83]. Some reports indicate that ET is more com-mon in women (particularly at younger ages) and PV in men, while in PMF both genders are nearly equally affected [51, 84, 85].

As demonstrated by European and international studies [86, 87], the distinction of MPNs in three nosological entities have a relevant prognostic significance. By and large, PN-MPN patients have a reduced life expectancy compared with general population, with PMF having the lowest overall survival (5.7 years), followed by PV with 15 years survival in 65% of cases and ET with an overall survival of more than 18–20 years [78, 88].

Despite insidious clinical onset, all PN-MPNs are at risk of clonal evolution and mortality. This is generally attributed to disease progression that may end in medul-lary failure (myelofibrosis or ineffective hematopoiesis) or transformation into other hematologic malignancies (the most common being acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS)) or the occurrence of bacterial infections and cardio- and cerebrovascular diseases, especially in younger patients [89, 90]. Fortunately, mortality due to these complications has been decreasing in the last few years [78].

3.2.1 Driver genes and other mutations

Until 2005 little was known about the etiology of PN-MPNs. The discovery of somatic mutations in Janus kinase 2 gene (*JAK2*), a member of the Janus kinase family located at chromosome 9 and first identified in 1993, was crucial. The identi-fication of exon 14 V617F gain-of-function mutation, made by several independent groups of investigators [91–94], was one of the major genetic insights into the pathogenesis of the PN-MPNs and transformed the understanding of these disor-ders. It turned out to be the most important and most frequently recurring somatic mutation involved in PN-MPN pathogenesis, with the highest frequency (up to 95%) in PV, and 50–60% in ET and PMF patients (**Figure 2**) [9, 23, 55, 72, 95–99].

Although there is no gold standard and the choice of methodology is dependent on the application, quantitative real-time PCR is a useful method for detecting V617F mutation in *JAK2* gene [100].

After *JAK2* V617F discovery in the majority of PN-MPN patients, there may have been an assumption of genetic uniformity, but the fact that approximately 50% of ET and PMF patients are *JAK2* V617F negative prompted the search for other putative genes in the JAK-STAT signaling pathway that could be mutated in these patients. In 2006, Pikman and colleagues [101] identified the mutations of thrombopoietin receptor (TPO-R) in myeloproliferative leukemia (MPL) virus oncogene. Moreover, a small proportion of patients with PV are *JAK2* V617F negative when tested by sensitive allele-specific assays [102], led only 1 year later, in 2007, to the identification by Scott and colleagues of a set of *JAK2* exon 12 mutations in *JAK2* V617F-negative patients with PV [103]. Although there is no gold standard and the choice of methodology is dependent on the application, quantitative real-time PCR and high-resolution melt-curve analysis are useful methods for detecting this type of mutation in *JAK2* gene [100].

One of the most recent discoveries was made by Kralovics in 2013, with the identification of calreticulin (CALR) mutation in 73% of MPN patients who do not bear the *JAK2* or *MPL* mutation (**Figure 2**) [106]. The identification of these other driver mutations (*JAK2* exon 12, *MPL*, and *CALR*) contributed to a better clarification of the pathophysiology of these disorders, their diagnostic tools, and therapeutic management [9, 91–94, 103, 107, 108]. In the majority of PN-MPN cases, *CALR, MPL*, and *JAK2* mutations are mutually exclusive, although rare exceptions can occur [70, 109].

It soon became clear that this group of diseases was far more genetically heterogeneous and complex than CML. Mutations other than in those driver genes and other genetic alterations have also been described in PN-MPNs and have shown to contribute to the establishment of the WHO diagnostic criteria, prognosis, and risk stratification in PN-MPNs [9, 90, 110, 111]. The majority of those mutations fall into one of the two categories—activation of the JAK-STAT pathway (*JAK2* V617F, *JAK2* exon 12, *MPL, LNK*, and probably *CALR*) [112] and aberrant epigenetic modification (*TET2, ASXL1*, and *EZH2*) [113]. A combination of mutations in these genes and environmental factors is likely the decisive factor of the development of each one of these disorders.

3.2.2 Molecular pathophysiology

The receptors of bone marrow progenitor cells are highly sensitive to EPO (stimulates erythroblasts), TPO (induces proliferation and differentiation of
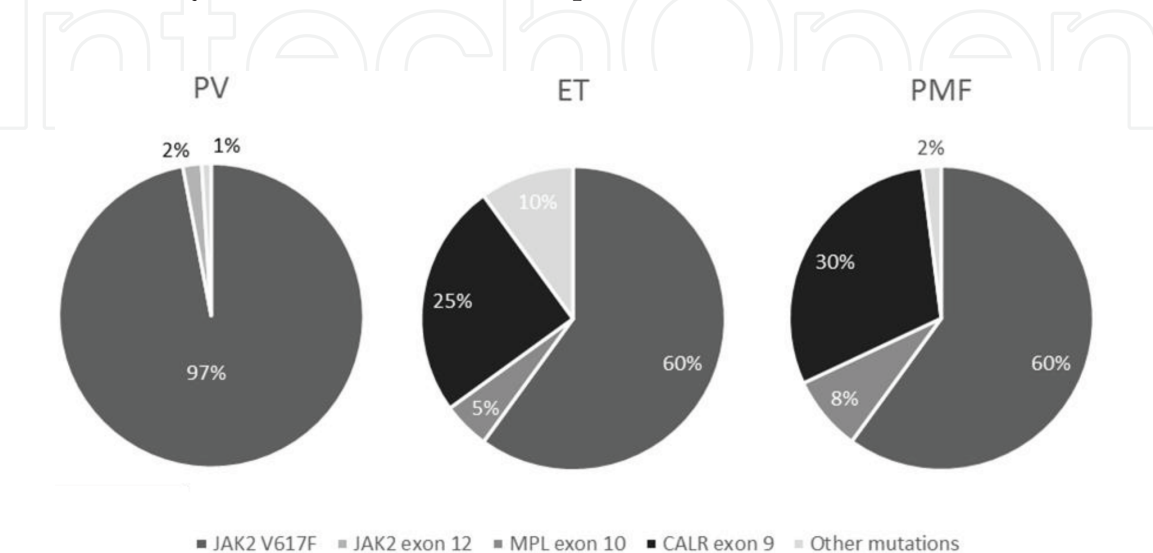

Figure 2.
Variation frequency of driver and other mutations in PN-MPNs [78, 104, 105].

15

megakaryocytes), stem cell factor (SCF, promotes proliferation and self-renewal of multipotent hematopoietic primordial cells), granulocyte-stimulating factor (GSF, stimulates proliferation and differentiation of granulocytes), and interleukins. Cytokine hypersensitivity leads to monoclonal stimulation of the erythropoiesis, megakaryopoiesis, and granulopoiesis.

*JAK*2 serves as the cognate tyrosine kinase for the EPO and TPO receptors and can also be used by the G-CSF receptor, all of which lack an intrinsic kinase domain [9, 70]. Moreover, JAK2 is crucial for normal hematopoiesis, as demonstrated by abnormal erythropoiesis developed in JAK2-deficient mice [114]. It includes two main domains: one is an enzymatically active kinase domain (JAK homology 1 (JH1)), and the other corresponds to a catalytically inactive pseudokinase domain (JH2), which promotes an inhibitory affect that induces the inhibition of the kinase activity of JAK2 [114–116].

The most frequent mutation associated with PN-MPNs, *JAK2* V617F, is present in myeloblasts, granulocytes, erythroblasts, and all EPO-independent erythroid colonies. It consists of a gain-of-function missense mutation with a G to T (guanine to thymidine) substitution at nucleotide 1849, in exon 14 of the *JAK2* gene, resulting in the substitution of valine with phenylalanine at codon 617 in the inhibitory JH2 domain [102]. When V617F mutation occurs, the result is an increased activity in myeloid progenitor cells, which leads to proliferation and excessive production of mature cells [114, 116–119].

JAK2 V617F activates signaling through the three main myeloid cytokine homodimeric receptors (EPO-R, MPL, and G-CSFR), which are involved in erythrocytosis, thrombocytosis, and neutrophilia, respectively. On the other hand, *CALR* or *MPL* mutants are restricted to MPL activation, explaining why *JAK2* V617F is associated with PV, ET, and PMF, whereas *CALR* and *MPL* mutants are found in ET and PMF [120].

In addition, expression of *JAK2* V617F results in constitutive activation of downstream signaling pathways including the JAK-STAT, MAPK/ERK, and phosphatidylinositol-3-kinase (PI3K/AKT) pathways [91–94] and later by interaction with p85, a regulatory subunit of PI3K, promoting proliferation and survival. Activated PI3K activates AKT, which in turn activates mammalian target of rapamycin (mTor) on Ser2448, which directly phosphorylates ribosomal p70S6 kinase (p70S6k). p70S6K and mTor are involved in angiogenesis by activation of vascular endothelial growth factor (VEGF) [61, 72]. It is known that this pathway is commonly activated in leukemia and lymphoma and is involved in inhibiting apoptosis in normal human erythroblasts. The PI3K/AKT pathway also induces the phosphorylation of BAD, a pro-apoptotic member of the Bcl2 family, via phosphorylated AKT (pAKT) and p70S6k, thus inhibiting BAD function and resulting in inhibition of apoptosis. BclxL is also activated by this pathway, resulting in inhibition of megakaryocyte apoptosis [61].

On the other hand, an increased activation of Ras-Erk signaling pathway was also demonstrated in PV patients. Ras is activated and activates Raf-1, which mediates the activation of MEK, which in turn activates extracellular signal-regulated kinase (ERK), one of members of the MAPK families. ERK phosphorylation also results in the inhibition of apoptosis, by blocking the function of BAD and activation of Bcl2. Therefore, due to the inactivation of the pro-apoptotic factor BAD and activation of BclxL and Bcl2, AKT and ERK together with *JAK2* V617F mutation suppress apoptosis and promote cellular survival, upregulating megakaryocytes and erythropoiesis [61].

In contrast to its effect on the EPO receptor, *JAK2* V617F appears to increase the quantity of immature MPL while increasing MPL degradation through ubiquitination and reducing its cell surface expression [70].

Several studies have shown that expression of *JAK2* V617F results in transformation of Ba/F3 cells, characterized by IL-3-independent growth, unlike wild-type JAK2 [91]. Due to *JAK2* V617F mutation and other mutations, hematopoietic progenitor cells can proliferate without the presence or induction by cytokines, resulting in factor-independent growth of the erythroid cell line and activation of signal transduction [102], mostly in PV homozygous cases. Yet, the presence of receptors is essential, leading to enhanced functional activity and increased sensitivity to cytokines and hematopoietic growth factors, such as interleukin 3 (IL-3), stem cell factor (SCF), granulocyte-macrophage CSF, and insulin-like growth factor-1 [23, 114, 121].

Recently, in 2017, Yao et al. demonstrated that activation of *JAK2* mutants can differentially link to selective cytokine receptors and change the signaling motifs, evidencing the molecular basis for phenotypic variants elicited by *JAK2* V617F or exon 12 mutations. On the basis of these findings, receptor-JAK2 interactions could evidence new targets of lineage-specific therapeutic tools against MPNs, which may be considered in other cancers with aberrant JAK-STAT signaling [122].

Recent data also indicate that the *JAK2V617F* allele might escape negative feedback by SOCS3 [72].

Unlike V617F where only a single codon is affected, exon 12 frameshift mutations comprise more than 40 different small deletions/duplications and substitutions of one or more amino acids between phenylalanines F533 and F547 (e.g., lysine for leucine at codon 539—K539 L), which are located in a linker between the JH2 pseudokinase and the SH2 domains [123]. However, just like *JAK2* V617F mutation, also exon 12 mutant alleles induce cytokine-independent/hypersensitive proliferation in EPO receptor (EPO-R) expressing cell lines and constitutive activation of JAK-STAT signaling [102]. The *JAK2* exon 12 mutations contribute primarily to erythroid myeloproliferation, associated with increasing levels of phosphorylated JAK2, STAT5, and Erk1/2 compared to patients with wild-type *JAK2*, and even higher activated JAK2 and ERK1/ERK2 levels than patients with the *JAK2* V617F mutation [61, 103, 124].

Although the complete cellular and molecular mechanisms involved in the pathophysiology of PN-MPNs have not yet been fully clarified [97, 107, 125–131], hyperactive JAK/STAT signaling pathway appears to be a constant, even in the presence of *CALR* mutations and the so-called "triple-negative" MPNs (nonmutated *JAK2*, *CALR*, and *MPL*), where the driver gene mutation is still unknown [55, 112].

3.3 *JAK2* mutation's role in Philadelphia chromosome-negative myeloproliferative neoplasms and other disorders

In humans, *JAK2* V617F occurs at the stem cell level and is present in hematopoietic stem cell progenitors from affected individuals, but not usually in the germline, suggesting that this mutation is acquired as a somatic disease allele in the hematopoietic compartment [102]. It is believed to be myeloid lineage specific because it is present in erythroid and granulocyte-macrophage progenitors. *JAK2* V617F is not specific for an individual PN-MPN, nor does its absence exclude MPNs. Although the prevalence of *JAK2* V617F mutation differs among PN-MPNs, one of the most challenging aspects of the study of these disorders still is the explanation of phenotypic heterogeneity and mechanism of progression of the PN-MPNs [97].

About 25–30% of patients with PV and 2–4% with ET [102, 132] are homozygous for the *JAK2* V617F allele (loss of heterozygosity) as a result of mitotic recombination and duplication of the mutant allele, promoting uniparental disomy (UPD). Uniparental disomy of chromosomal locus 9p24, including *JAK2*, had previously been detected in PV, before identification of the *JAK2* V617F allele

[102]. Mitotic recombination is more likely to occur in PV patients with muta-tion in exon 14 of the *JAK2* gene than in those with exon 12 mutations [133] and is an early genetic event in the development of PV, but not ET [102]. Although *JAK2* V617F homozygous subclones can be identified both in PV and ET patients, expression of a dominant homozygous subclone is almost exclusive in PV patients (~80% in PV and 50% in ET) [78, 119], originated by additional genetic or epigenetic events or, e.g., low levels of circulating erythropoietin in consequence of elevated hematocrit [119].

Although in the heterozygous state *JAK2* V617F-bearing receptors are still responsive to growth factors, in JAK2 V617F homozygosity, these receptors become autonomous with respect to growth factor [70], as referred earlier.

Almost all patients diagnosed with PV negative for *JAK2* V617F mutation are exon 12 positive (95% vs. 2–4%, respectively) [53, 103, 134–141]. Some studies have reported that Chinese PV patients have a relatively lower *JAK2* V617F mutation frequency (82%), in line with a Portuguese study [23], while the mutations in *JAK2* exon 12 are much more pervasive (13%), when compared to Westerns and other East Asians [139, 142].

Unlike *JAK2* V617F, which can be detected in any of the PN-MPNs, *JAK2* exon 12 mutations are almost exclusive of *JAK2* V617F-negative PV patients [24, 103]. PV patients who present *JAK2* exon 12 mutations, unlike those who are V617F positive, are not commonly homozygous [70, 103, 124, 138]. PV patients with the *JAK2* exon 12 mutations are usually younger than those with the *JAK2* V617F mutation and have a phenotype usually more benign than that of JAK2V617F, usually without panmyelosis [53], with normal leukocyte and platelet counts [61, 70]. Although *JAK2* V617F and exon 12 mutations express through the same C-terminal tyrosine kinase of JAK2, they originate very different phenotypic outcomes. These patients appear to be associated with a distinct syndrome, with higher hemoglobin concen-trations, without concomitant leukocytosis or thrombocytosis (or minimal throm-bocytosis), and isolated bone marrow erythroid hyperplasia [124], independently of the mutational variant [24, 124, 140]. The reasons for these various abnormal phenotypic readouts also remain unclear and are likely to be complex [124, 140]. The fact that exon 12 mutations are more frequently associated with erythrocytosis is consistent with their absence in ET but possible existence in PMF or AML second-ary to PV [138]. However, there are exceptions as evidenced in some clinical reports [24]. Despite the phenotypical diversity, the clinical course and outcome seem overlapping between *JAK2* V617F and *JAK2* exon 12-positive patients, with conver-gent incidences of thrombosis, myelofibrosis, leukemia, and death [140]. There are also reports of the coexistence of *JAK2* V617F and *JAK2* exon 12 mutations as two separate clones [70, 140].

As published by Rumi and Cazzola [78], patients with the wild-type genotype for *JAK2* are extremely rare. However, a recent study [23] demonstrated a preva-lence of 12.8% of patients with that genotype. This finding is consistent with the fact that the *JAK2* mutation expression alone may not be sufficient to induce the PV phenotype. However, larger studies are required to confirm this hypothesis.

Some reports have also suggested *JAK2* V617F clonal involvement of B [143, 144], T [143], and NK lymphocytes [83], also confirming the stem cell nature of *JAK2* V617F MPNs [102]. Lower frequencies of V617F mutation occur in PN-CML, chronic myelomonocytic leukemia, juvenile myelomonocytic leukemia, and rare cases of AML (megakaryocytic and in combination with other well-defined genetic abnormalities, such as BCR-ABL1) [145]. There is also evidence of association with certain solid tumors (generally non-hematological types) [51, 114, 117, 146–148]. Other mutations in the JAK2 pseudokinase domain (including point mutations involving R683) have been detected in about 20% of Down syndrome-associated

and other acute lymphoblastic leukemia and AML. A number of JAK2 fusion proteins, such as TEL-JAK2, PCM1-JAK2, and BCR-JAK2, lead to activation of JAK kinase activity and have also been associated with myeloid and lymphoid leukemia or atypical CML [60, 72].

Along with other driver mutations connected with clonal expansion of hematopoietic cells, *JAK2* V617F mutation might also represent a feature of the aging hematopoietic system in individuals without a malignant disease [149, 150]. There is increasing evidence that *JAK2* V617F is relatively frequent in the aging healthy population and is presently estimated to be 0.5% [120]. These individuals usually present higher erythrocyte, platelet, and leucocyte counts and are more likely to develop a hematological cancer. Aging is generally associated with a deregulation of hematopoietic stem cells, which lose their function and become myeloid-biased and less quiescent as a consequence of intrinsic and environmental changes, with *JAK2* V617F hematopoietic stem cells having higher competitive properties in this context [120, 150].

3.3.1 Prognosis and predictive factors

Besides mutations and other molecular defects, various factors, such as gene burden and individual genetic background, may be responsible for predisposition for developing an MPN, as well as influence their heterogeneity [78, 97].

Several published data have shown the contribution and influence of *JAK2* V617F mutation allelic burden in the definition of phenotype and prognostic impact in PN-MPNs [151, 152]. *JAK2* V617F allelic burden corresponds to the ratio between mutant and wild-type *JAK2* in hematopoietic cells and is on the basis of a stronger activation of intracellular signaling pathways [153]. Between MPN patients there is a variability in the number of cells carrying the *JAK2* V617F mutation, and there is a variability in the alleles that carry the mutation.

It is recognized that the allele burden tends to be higher in PV (due to the higher number of homozygous cases) and PMF, associated with the presence of acquired UPD, with defined hematological and clinical markers indicative of a more aggressive phenotype [153]. Indeed, a lower allele burden is generally observed in ET patients [97, 119, 152, 154, 155], but when it increases, some of them transform over time to PV or PMF. Importantly, ET patients positive for the *JAK2* V617F mutation have a "PV-like" phenotype compared to ET patients without this genetic abnormality. However, patients carrying *JAK2* V617F mutation do not have a higher risk of evolution to post-PV and post-ET myelofibrosis than patients without the mutation [61].

Another possible explanation concerns the concept of a "pre-JAK2" phase in which additional somatic mutations or inherited predisposing alleles present before the mutation are responsible for the clonal hematopoiesis, determine the phenotype, influence the risk of progression to AML, and might even be responsible for generating the mutation or act synergistically [55, 61]. In fact, although *JAK2* V617F mutation is crucial to the pathogenesis of PV, ET, and PMF, the existence of the same allele in three clinically distinct entities suggests that there might be additional inherited or acquired genetic predisposition. Indeed, a familial tendency has been identified in 72 families, which is consistent with an inherited genetic predisposition to MPNs [156].

On the other hand, the role of the *JAK2* V617F mutation in the pathogenicity of the various MPNs may differ among different MPNs, involving the *JAK2* V617F mutation more often than others (e.g., ET vs. PV), which would indicate other oncogenic mutations or factors that may be determinant for certain cases other than *JAK2* V617F [97, 119, 157, 158].

Moreover, mutations in epigenetic regulators, transcription factors, and signaling components modify the course of the disease and can contribute to disease initiation and/ or progression [55]. Some studies performed in mice and humans led to the "host genetic factor" concept, acting as modifiers in combination with the mutation, for instance, single nucleotide polymorphisms (SNPs) [90, 110, 111, 159, 160]. Even gender could be an independent modifier, with women having a lower allele burden than men [61].

Also, the coexistence of autonomous *JAK2* mutant and *JAK2* wild-type clonal populations in the same patient can be an explanation. It is observed that *JAK2*-positive AML patients are preceded by evolution to myelofibrosis during their disease course, in contrast to *JAK2* wild-type AML, which is preceded by chronic-phase ET and PV patients [61].

On the other hand, the role of the JAK/STAT signaling pathway in the pathogenesis of MPNs and other cancers is questionable when taking into account the example of rare families hosting germline mutations leading to weak JAK expression. The mutations induce a hereditary thrombocytosis, but hematopoiesis is polyclonal, and there is no generation of hematological malignancies or solid tumors, indicating that JAK/STAT activation alone does not drive malignant disease [147].

In PV and ET, risk factors influencing survival include older age, leukocytosis, and thrombosis. In ET, the *JAK2* V617F mutation is associated with increased risk of thrombosis, leading to inclusion into the International Prognostic Score of Thrombosis for ET-thrombosis score [90, 94, 161]. Expansion of *JAK2*-mutated allele promotes the transformation of PV and ET to secondary myelofibrosis [153]. Furthermore, the presence of two or more mutations is associated with a worse survival and predicts shortened leukemia-free survival [162].

JAK2 V617F has not been correlated to an increased risk of transformation to AML [90]; nevertheless, *JAK2* V617F-positive patients with MPN diagnosis can transform to *JAK2* V617F-negative AML [163].

The pathogenesis of thrombosis in PN-MPN patients is complex, involving clinical factors such as age, previous history of thrombotic events, obesity, hypertension, and hyperlipemia, as well increased blood cell counts (i.e., leukocytosis, erythrocytosis, and thrombocytosis), high hematocrit, and *JAK2* mutation [164]. The most important risk factor for future arterial and venous thrombosis in MPNs is the previous history of arterial and venous thrombosis, respectively [9]. The influence of the *JAK2* V617F mutational status and allele burden on the thrombotic risk has been evaluated and established in several studies among PN-MPNs [90]; however, regarding the presence of *MPL* mutation, the published results are discrepant [164]. Older (age > 60 years) patients are no longer considered "high risk," unless they have a history of thrombosis or are *JAK2*-mutated [9, 164].

In patients with ET, the frequency of thromboembolic events in different studies ranges from 10 to 30% at diagnosis and between 8 and 31% during follow-up [165], and the rate of fatal and nonfatal thrombotic events ranged from 2 to 4% patient-years, with a predominance of arterial events [164], whose risk is higher in patients with *JAK2* and *MPL* mutations [90, 166].

Risk factors for fibrotic transformation in PV include *JAK2* V617F allele burden of >50%; in ET they include advanced age and anemia, with the presence of *JAK2* V617F being associated with a lower risk of fibrotic transformation and *CALR* with a higher risk [9]. *JAK2* V617F mutational status may have prognostic significance in PV, ET, and PMF [102]. In PV, despite the phenotypic differences, the clinical course seems similar between *JAK2* V617F and *JAK2* exon 12-positive patients, with similar incidences of thrombosis, myelofibrosis, leukemia, and death [24, 140]. *JAK2/CALR* mutational status did not affect survival in ET [9]. In PMF and ET, triple-negative patients appear to have a less favorable prognosis than patients with

a driver mutation (*JAK2*, *CALR*, or *MPL*), whereas patients with *CALR* mutations tend to have a better prognosis than patients with *JAK2* or *MPL* mutations.

Another important concern refers to the increased risk of generation of new non-hematological and nonmyeloid neoplasms in MPNs, with an incidence ratio of 1.2–1.4 and 3.4, respectively, compared to the general population [90, 167]. There is evidence that this risk is higher when *JAK2* V617F mutation is identified and other patient-related factors may be also present.

3.3.2 Therapy management

The discovery of the *JAK2* mutations and their relation with the subsequent activation of the JAK-STAT pathway was crucial to the understanding of the pathogenesis of PV, ET, and PMF. This knowledge has led to the development of small-molecular *JAK* inhibitors to target autoimmune disease/immunosuppression (anti-*JAK1*, *JAK3*) and MPNs and leukemia/lymphoma (anti-*JAK2*, *JAK1*), which have been tested in several clinical trials, suggesting an overall reduction in JAK-STAT signaling and pro-inflammatory cytokines [141, 168, 169]. About 10 compounds were studied for MPNs, rheumatoid arthritis, psoriasis, and inflammatory bowel disease, all of them targeting the ATP-binding site of JAKs, but none is absolutely specific for any JAK [88]. Nevertheless, ruxolitinib (a *JAK1*, *JAK2* inhibitor, trade name Jakavi®) has been approved by the Food and Drug Administration (FDA) in November 2011, for use in myelofibrosis, and tofacitinib (a *JAK1*, J*AK3* inhibitor) has been approved for use in rheumatoid arthritis. The first two randomized controlled trials (Comfort I and II) on the effect of the JAK2 inhibitor ruxolitinib versus placebo and versus the best available therapy in intermediate-2 and high-risk PMF showed a decrease in spleen size and symptom burden in the experimental arm of both studies. In Comfort I, a survival benefit was also observed in the ruxolitinib arm compared to patients on placebo [170, 171]. Although ruxolitinib was recently approved for use in hydroxyurea-resistant PV, its role in routine clinical practice remains controversial [9, 52, 95, 172, 173].

The treatment options of PMF patients are currently limited, with stem cell transplant being the current treatment of choice for genetically or clinically high-risk disease. PMF patients may benefit from *JAK2* inhibition with immediate clinical value in the management of symptoms, through directly modulating the pro-growth signals of the JAK-STAT pathway, suppression of hematopoietic progenitor cell proliferation, and from downregulating specific pro-inflammatory cytokines produced by the affected clone [70, 113].

Ruxolitinib treatment substantially alleviates symptomatic splenomegaly and constitutional symptoms and improves quality of life in a significant proportion of patients with primary or post-PV/ET myelofibrosis [88]. Surprisingly, treatment with ruxolitinib is also effective in patients without mutated *JAK2*, suggesting that other, still unknown, underlying mechanisms are responsible for the increased JAK/STAT pathway activity in PN-MPN patients. On the other hand, there is no convincing evidence of reduction in mutated allele burden, disease modification, nor progression to AML [9, 174].

The identification of *JAK2* represented a milestone for the following studies and for today's knowledge, but the ongoing discovery of other mutations in MPNs will make possible the establishment of new drug targets and prognostic biomarkers that will for certain improve clinical practice and patients' outcome. All in all, it remains to be fully clarified whether *JAK2* mutations may be considered as "driver mutations" for MPNs or if they can act as "passenger mutations" which may alternate place with the former and have "driver" functions [129].

4. Conclusions and future perspectives

Non-receptor tyrosine kinases play an important role in the development of human malignancies, including hematological and others, and of inflammatory, and autoimmune diseases, through their profound involvement in the regulation of several vital cellular mechanisms, including cell proliferation, differentiation, maturation, apoptosis, and survival.

Targeting dysregulated NRTKs may prevent the process of tumorigenesis. The screening and clinical use of tyrosine kinase inhibitors, in combination with conventional treatments, have allowed the potential of targeted-based cancer therapy using specific cancer cell molecules, which are less toxic than traditional cytotoxic chemotherapy. The establishment of effective strategies in cancer research and patient care is mandatory.

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Author details

Ana Azevedo[1,2]*, Susana Silva[1] and José Rueff[1]

1 Faculty of Medical Sciences, NOVA Medical School, Centre for Toxicogenomics and Human Health, Genetics, Oncology and Human Toxicology, NOVA University of Lisbon, Lisbon, Portugal

2 Department of Clinical Pathology, Hospital of São Francisco Xavier, West Lisbon Hospital Centre, Lisbon, Portugal

*Address all correspondence to: ana.azevedo@nms.unl.pt

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