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Review Article

Mutational Analysis of Driver and Non-driver Mutations of Philadelphia Chromosome-negative Myeloproliferative Neoplasms; Diagnosis and Recent Advances in Treatment

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Abstract: Myeloproliferative neoplasms (MPNs) are hematological disorders affecting myeloid stem cells. They are classified as Philadelphia (Ph) chromosome positive-chronic myeloid leukemia, and Ph-negative polycythemia vera, essential thrombocythemia, primary myelofibrosis, chronic neutrophilic leukemia, chronic eosinophilic leukemia, juvenile myelomonocytic leukemia, and MPN unclassifiable. This review is mainly focused on the Ph-negative MPNs namely, PV, ET, and PMF. These affect both males and females with a slight male predominance, with patients mainly presenting in the seventh decade. Patients often present with thrombotic events resulting in complications that lower survival rates. The major driver mutations that have been identified in MPNs are *JAK2* Exon 14, *JAK2* Exon 12, *MPL* Exon 10, and *CALR* Exon 9. The importance of these driver mutations gives due recognition to their inclusion into the 2022 diagnostic criteria of the MPN WHO Classification. However, other non-driver mutations have also been reported, especially in triple-negative cases. These mutations lead to downstream constitutive activation of the JAK/STAT signaling pathway, as well as the MAPK, and PI3K/Akt pathways. Insights into the molecular pathogenesis of MPN and its association with *JAK2*, *CALR*, and *MPL* mutations have identified JAK2 as a rational therapeutic target. Thus, as an approach to MPN therapy, JAK2 inhibitors, such as ruxolitinib, have been shown to effectively inhibit JAK2, and are currently in clinical trials in combination with other drug classes. This review comprehensively examines the molecular markers of the main Ph-negative MPNs, as well as diagnosis and treatment options.

Keywords: Myeloproliferative Neoplasms, JAK2, MPL, CALR, Non-driver mutations, MPN diagnosis, Ruxolitinib, Polycythemia Vera, Essential Thrombocythemia, Primary myelofibrosis

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1. Introduction

Myeloproliferative neoplasms (MPNs) are hematological disorders affecting myeloid stem cells. According to the 5th edition of the WHO classification of myeloid neoplasms, MPN can be classified as Philadelphia (Ph) chromosome positive-chronic myeloid leukemia (CML), and Ph-negative polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia (CEL), juvenile myelomonocytic leukemia (JMML), and MPN unclassifiable (MPN-U) [1,2]. However, the main Ph-negative MPNs; PV, ET, and PMF, are the focus of this review. These are characterized by clonal expansion of bone

marrow stem cells resulting in unregulated proliferation of the myeloid cells; major overproduction of erythroid cells in PV, platelets in ET, and megakaryocytes secreting platelet derived growth factor (PDGF) and monocytes secreting fibroblast activation leading to fibroblast proliferation and bone marrow fibrosis in PMF [3]. This can evolve into excess cells in the peripheral blood leading to thrombosis, overt myelofibrosis, or accumulation of mutations leading to acute leukemia, making diagnosis, risk assessment, and therapeutic approaches difficult [4].

The breakthrough discovery of the *JAK2* V617F mutation, it's role in diagnosis of the MPN subtype, and the development of novel molecular diagnostic technologies, permitted identification of molecular markers. This made molecular testing the cornerstone in investigation of MPN in a routine diagnostic setting [5]. The detection of mutations in *JAK2* Exon 14, *JAK2* Exon 12, *MPL* Exon 10, and *CALR* Exon 9 are now major diagnostic criteria within the WHO classification of myeloid neoplasms, 2022. Mutations in these genes are associated with constitutive activation of the JAK/STAT signaling pathway involved in sustained cell proliferation and survival of hematopoietic cells and thus are linked to the various diagnosed MPN phenotypes. Other clonal markers include non-driver mutations in genes affecting epigenetic control (*TET2, ASXL1*, and *DNMT3A*), splicing regulators (*SRSF2, SF3B1, U2AF1*) and regulators of chromatin structure, and cellular signaling (*EZH2, IDH1, IDH2, TP53*) [6]. Here, we comprehensively review the molecular markers of the main Ph-negative MPNs, as well as the diagnosis and treatment options.

2. Epidemiology

MPNs affect both males and females with a slight male pre-dominance. It affects especially those >60 years of age [4]. Common risk factors predisposing to MPNs include smoking, family history, obesity, occupation, and ionizing radiation [3]. Based on a meta-analysis of studies, the estimated annual incidence of MPN was reported as 2.17 cases per 100,000 of the population. It reported a pooled annual incidence of 0.84 per 100,000 for PV, 1.03 per 100,000 for ET, and 0.47 per 100,000 for PMF across Europe, North America, Australia, and Asia [3, 7]. A study done in 2020 by Yassin et al [8] identified that the prevalence and incidence of MPNs were estimated to be 57-81 and 12-15, respectively, per 100,000 hospitalized patients per year over the last 4 years in Asia, including the Middle East, Turkey, and Algeria. Recent literature estimated the annual global incidence of MPN in pediatric patients to be 0.82 per 100,000 patients and found children, adolescents, and young adults (40 years or below) to represent a small portion of MPNs, although infrequent [9,10]. The National Cancer Registry, Sri Lanka (2020) [11], reported an age-specific incidence rate for MPN in males ranging from 2.6 to 6.1 cases per 100,000 individuals, while in females, it ranged from 1.3 to 2.4 cases per 100,000 individuals. These rates were observed among individuals aged between 60 and 75 years.

3. Diagnostic approach

Accurate diagnosis of the MPN sub-types is important for patient prognosis and therapeutic recommendations [12]. The revised World Health Organization (WHO) diagnostic criteria for MPN, 2022 (Table 1,2,3), highlights the importance of clinical evaluations in diagnosing PV, post-PV MF, ET, post-ET MF, pre-fibrotic/early PMF, and overt PMF. These include initial evaluation of palpable splenomegaly, thrombosis and hemorrhage, cardiovascular risks, and medical history [13]. Further investigations include hematological (i.e. complete blood count, peripheral blood smear), biochemical (i.e. serum lactate dehydrogenase, uric acid, vitamin B_{12}, ferritin, C-reactive protein), and histopathological (i.e. bone marrow morphology) investigations and cytogenetic studies [12,14].

Table 1. Simplified 2022 WHO diagnosis criteria for PV and post-PV MF; Adapted from: [6, 104].

PV	Post-PV MF
Major Criteria: • Elevated hemoglobin > 18.5 g/dL in men, 16.5 g/dL in women, or increased hematocrit (> 49% in men, > 48% in women)⁺ • Presence of *JAK2* V617F or Exon 12 mutation (< 1% sensitivity). • Hypercellular bone marrow with trilineage proliferation, including prominent erythroid, granulocytic and increased pleomorphic, mature megakaryocytes without atypia. **Minor Criteria:** • Subnormal erythropoietin level **Requirements for diagnosis:** • All 3 major OR first 2 major + minor criterion*	**Required Criteria:** • Previously diagnosed PV • Grade 2 or 3 bone marrow fibrosis. **Additional Criteria:** • Anemia • Leukoerythroblastosis. • New or increased palpable splenomegaly >5 cm from baseline • Presence of at least 2 of the following: unexplained pyrexia (>37.5°C), weight loss (>10% in 6 months), night sweats. **Requirement for diagnosis:** • All required criteria + 2 or more additional criteria

⁺*As a refinement to the 2016 WHO diagnostic criteria, increased red cell mass has been removed as a major criterion.*

**Major criterion 3 may not be required in patients with sustained absolute erythrocytosis: hemoglobin >18.5 g/dL and hematocrit >55.5% in men or hemoglobin >16.5 g/dL and hematocrit, 49.5% in women; if major criterion 2 and the minor criterion are present.*

Table 2. Simplified 2022 WHO diagnosis criteria for ET and post-ET MF; Adapted from: [6, 104].

ET	Post-ET MF
Major Criteria: • Increased platelet count ≥ 450×10⁹/L • Proliferation of enlarged, mature megakaryocytes with hyperlobulated nuclei. • Criteria for BCR-ABL1, CML, PV, PMF, MDS or other myeloid neoplasms not met. • Presence of *JAK2* (< 1% sensitivity assay). *CALR* or *MPL* (1-3% sensitivity assay) mutations. **Minor Criteria:** • Presence of clonal marker (eg, *ASXL1, EZH2, TET2, IDH1/IDH2, SRSF2, SF3B1* mutations) or absence of evidence for reactive thrombocytosis **Requirements for diagnosis:** • All 4 major OR first 3 majors + minor criterion	**Required Criteria:** • Previously diagnosed ET • Grade 2 or 3 bone marrow fibrosis. **Additional Criteria:** • Anemia and decreased hemoglobin >2g/Dl from baseline. • Leukoerythroblastosis. • New or increased palpable splenomegaly >5 cm from baseline • Increased LDH levels above the reference range. • Presence of at least 2 of the following: unexplained pyrexia (>37.5°C), weight loss (>10% in 6 months), night sweats. **Requirement for diagnosis:** • All required criteria + 2 or more additional criteria

Table 3. Simplified 2022 WHO diagnosis criteria for pre-PMF and overt fibrotic PMF; Adapted from: [6, 104].

Early/pre-fibrotic stage PMF (pre-PMF)	Overt fibrotic stage PMF
Major Criteria: • Proliferation of atypical megakaryocytes, accompanied by marrow fibrosis < grade 2*, increased age-adjusted BM cellularity, granulocytic proliferation, but without reticulin fibrosis, and decreased erythropoiesis. • Criteria for BCR-ABL1, CML, ET, PV, PMF, MDS or other myeloid neoplasms not met.	**Major Criteria:** • Proliferation of atypical megakaryocytes, accompanied by either reticulin and/or collagen fibrosis grade 2 or 3*, osteosclerosis, and extramedullary hematopoiesis. • Criteria for BCR-ABL1, CML, ET, PV, PMF, MDS or other myeloid neoplasms not met. • Presence of *JAK2, CALR* or *MPL* mutations, or in the absence of these, presence of another clonal marker (e.g.,

• Presence of *JAK2, CALR* or *MPL* mutations, or in the absence of these, presence of another clonal marker (e.g., *ASXL1, EZH2, TET2, IDH1/IDH2, SRSF2, SF3B1* mutations) or absence of reactive MF. **Minor Criteria:** Presence of at least one of the following: • Anemia • Leukocytosis ≥ 11×10^9/L. • Palpable splenomegaly. • Increased LDH. **Requirements for diagnosis:** • All 3 major criteria + at least 1 minor criterion	*ASXL1, EZH2, TET2, IDH1/IDH2, SRSF2, SF3B1* mutations) or absence of reactive MF. **Minor Criteria:** Presence of at least one of the following: • Anemia • Leukocytosis ≥ 11×10^9/L. • Palpable splenomegaly. • Increased LDH. • Leukoerythroblastosis. **Requirement for diagnosis:** • All 3 major criteria + at least 1 minor criterion

**PMF shows a higher degree of megakaryocytic atypia than in any other MPN subtype, with distinctive features including small to giant megakaryocytes with cloud-like, hypolobulated, and hyperchromatic nuclei, and the presence of abnormal large dense clusters of >6 megakaryocytes.*

4. Molecular markers for MPN

The majority of Ph-negative MPN cases are due to point mutation in *JAK2* Exon 14 (V617F) which results in constitutive activation of the JAK/STAT signaling pathway. Various studies have identified mutations in other genes including *JAK2* Exon 12, *CALR*, and *MPL* in patients with V617F-negative MPN. Hence, the WHO has identified testing for mutations in these genes as part of the diagnostic procedures for patients with MPN [6]. Different clinical phenotypes characterize the MPN subtypes, thereby making the accurate molecular diagnosis of the specific gene mutation critical to promote treatment and management outcomes.

4.1. JAK2 Exon 14 and Exon 12

The somatic gain-of-function point mutation in Exon 14, codon 617 of the erythropoietin receptor tyrosine kinase, Janus kinase 2 (*JAK2* V617F), was discovered in 2005. It was found to be majorly associated with Ph-negative MPNs and contributed to the understanding of the pathophysiology, pathogenesis, and underlying molecular drives of MPN [4].

JAK2 has seven JAK homology domains: JH1-JH7; the *JAK2* kinase activity is regulated by the pseudokinase domain present in JH2 coded by Exon 14 of the *JAK2* gene (Figure 1). A point mutation in V617F of Exon 14 causes constitutive expression of the *JAK2* kinase (JH1) due to the removal of the auto-inhibitory role of the pseudokinase domain. This mutation is present in approximately 96% of cases with PV, 55% of cases with ET, and 65% of cases with PMF [14]. The binding of hematopoietic cytokines, such as interleukins, colony-stimulating factors, interferon, erythropoietin, and thrombopoietin, to JAK2, promotes signal transduction of the JAK/STAT signaling pathway. These synergistically generate constitutive activation of the JAK/STAT pathway via transcription factors STAT3 and STAT5. They activate or repress genes important in hematopoiesis, cell division, cytokine-independent growth, and cell survival, such as *c-MYC*, *CYCLIN D2*, *ID1*, *BCL-XL* and *MCL-1* [15].

V617F mutation also constitutively activates the mitogen-activated protein kinases (MAPK) and phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathways, resulting in increased expression of mitotic proteins, cell cycle regulatory proteins, and anti-apoptotic genes [16]. The MAPK/ RAS pathway is involved in signal transduction through small GTPase RAS proteins [17]. RAS proteins are frequently mutated in human cancers, including MPN. Mutant RAS in the active GTP-bound conformation, constitutively recruits RAF, a protein-serine/threonine kinase, and activates the downstream MAPK signaling cascade to promote cell survival, proliferation, and differentiation [15].

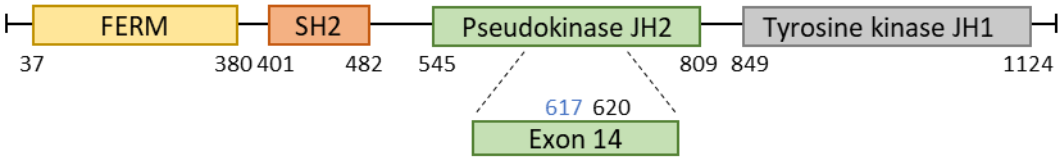

Figure 1. Structural domains of *JAK2* showing Exon 14. FERM, Ezrin, Radixin, Moesin subdomains; JH1 and JH2, JAK Homology 1 and 2 domains; SH2 indicates Src Homology 2 domain. Amino acid positions are indicated with numbers: blue indicates V617F mutation.

PI3K is a heterodimer consisting of a catalytic and regulatory subunit which upon stimulation phosphorylates and activates Akt, thereby activating the serine threonine kinase-mammalian target of rapamycin (mTOR). This mediates various cellular functions including angiogenesis, metabolism, cell proliferation, survival, and apoptosis [18]. In MPN, activating mutations in the receptor tyrosine kinases or loss of function mutation of PTEN, which plays a regulatory role on the PI3K pathway, promotes the proliferation of hematopoietic stem cells (HSCs) [19]. Furthermore, bone marrow biopsies of V617F-positive patients show increased phosphorylation of Akt which suggests hyperactivity of the pathway [20]. Dysregulation of the PI3K/Akt pathway is suggested to contribute to resistance to tyrosine kinase inhibitors; hence, making the management more difficult [21]. However, combined therapy involving inhibitors of the JAK/STAT, PI3K/Akt, and/or MEK pathways are supposed to be efficacious [22,23]. Thus, a better understanding of the interconnectedness of these pathways with MPN pathogenesis will help improve treatment and management outcomes.

Recent clinical findings have observed that *JAK2* Exon 14 V617F-negative patients have mutations in Exon 12 of the *JAK 2* gene. This region codes for amino acids 505 to 547 in the SH2 and JH2 domains of *JAK2* (Figure 2). Therefore, these patients have dysfunctional erythropoietin signaling pathways characterized by reduced erythropoietin production and isolated erythrocytosis. The most frequent exon 12 mutation involves an in-frame deletion of six nucleotides at codons 542 and 543, present in approximately 40% of *JAK2* V617F-negative PV [14, 24]. Other mutations constituting approximately 10% of *JAK2* V617F-negative PV include: E543_D544del (c.1627_1632del); F537_K539delinsL (c.1609_1616delins); R541_E543delinsK (c.1622_1627delins), and K539L (c.1615A>T or A>C; c.1616A>T) [14].

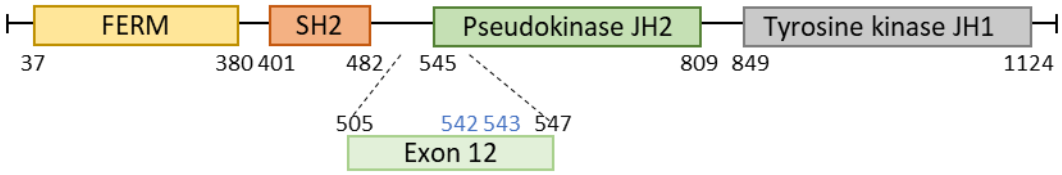

Figure 2. Structural domains of *JAK2* showing Exon 12. The amino acid position indicated in blue represents the region of most common Exon 12 mutations.

4.2. MPL Exon 10

Activating mutations in a second gene, codon 515 in Exon 10 of the myeloproliferative leukemia virus oncogene (*MPL*), known to interact with *JAK2*, was identified in both *JAK*2 V617F-positive cases and *JAK2* V617F-negative ET (8.5%) and PMF (10%) [25]. Other mutations in Exon 10 constituting approximately 1% of either PMF or ET include W515L (c.1544G>T), W515R (c.1543T>C), W515L (c.1544G>T), S505C (c.1514G>A), S505N (c.1514G>A), A506T (c.1516G>A), L510P (c.1529T>C), W515A (c.1543T>G; c.1544G>C) and A519T (c.1555G>A) [14].

The *MPL* gene (Figure 3), located on chromosome 1p34, encodes a thrombopoietin receptor which binds to thrombopoietin, the primary cytokine that regulates megakaryocyte development, platelet production, and hematopoietic stem cell homeostasis. This binding activates *JAK2*, phosphorylating *MPL* and initiating a signaling cascade that regulates cell survival, proliferation, and differentiation [14].

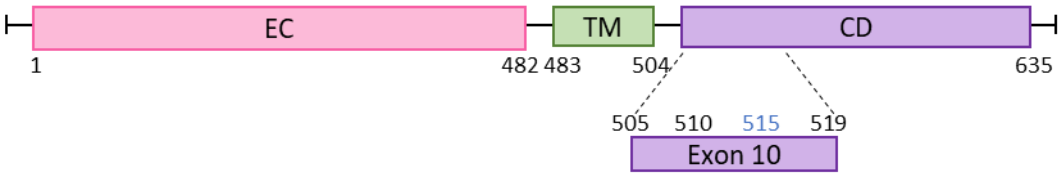

Figure 3. Structural domains of *MPL* showing Exon 10. EC, TM, and CD represent the extracellular, transmembrane, and cytoplasmic domains. The amino acid positions indicated represent the reported Exon 10 mutation hot spots; W515L or W515K in blue.

A mutation in the *MPL* gene, such as W515L guanosine to thymidine substitution at nucleotide 1544, results in the impaired function of the auto-inhibitory region and ligand-independent thrombopoietin receptor activation. This activates *JAK2* tyrosine kinase and transcription factors STAT3 and STAT5, leading to the transformation of hematopoietic cells into cytokine-independent clones, megakaryocytic hyperplasia, and marrow fibrosis [14]. This can be associated with lower hemoglobin levels at diagnosis and a high risk of transfusion dependence in patients with MF [26].

4.3. CALR Exon 9

Frameshift mutations in Exon 9 of the calreticulin (*CALR*) gene have recently been discovered as a driver mutation for MPN, particularly in patients with *JAK2* or *MPL*-negative PMF (20%) and ET (25%) [1, 27]. All *CALR* mutations are insertions or deletions in Exon 9, the last exon in the *CALR* gene. The most common mutations were type 1, a 52 bp deletion (p. L367fs*46; c.1092_1143del52), or type II, a 5 bp insertion (p. K385fs*47; c.1154_1155InsTTGTC), accounting for approximately 50% and 30% of patients with *CALR* mutations, respectively [1, 28,29]. About 3-6% of *CALR* exon 9 mutations in PMF and ET cases occur in: L367fs*52 (c.1098_1131del34), E364fs*55 (c.1092_1125del), E369fs*46 (c.1105_1150del) [30].

The wild-type *CALR* gene (Figure 4) located on chromosome 19p13.2, encodes a Ca^{2+}-binding chaperone protein, calreticulin, primarily localized to the endoplasmic reticulum (ER). The localization and retention of *CALR* are defined by the N-terminal signal sequence and the C-terminal ER retention sequence KDEL. Calreticulin is involved in Ca^{2+} homeostasis, disposal of misfolded proteins, phagocytosis, and immune response. It also mediates gene nuclear transport, apoptosis, and integrin-mediated cell adhesion. However, frameshift mutation, due to indels, changes the reading frame and results in the loss of most acidic amino acids, including the KDEL sequence found in the C-domain. This generates a new tail with low calcium buffering therefore, the resulting mutant protein is positively charged as opposed to the wild type and leaks from the ER [1, 30].

Recent studies have identified that the mutated calreticulin protein can induce the JAK-STAT signaling pathway by binding to the *MPL* receptor in a thrombopoietin-independent manner. Mutant calreticulin are 'rogue chaperones' that acquire unique oncoprotein properties, they promote clonal expansion of HSCs and megakaryocytes via binding and activation of MPL with the N-terminal lectin binding domain of CALR mutants and their positively charged tail [1, 31]. The MPL-mutant calreticulin complex requires cell-surface localization to induce autonomous activation of HSCs, this depends on full activation of mitogen-activated protein kinase, JAK2-STAT5/STAT3, and phosphoinositide 3-kinase pathways [31,32]. *In vitro* studies have also revealed that cells

co-expressing *MPL* and mutant calreticulin, respond less to thrombopoietin; suggesting that the binding of mutant calreticulin competitively hinders the binding of the normal cytokine [33].

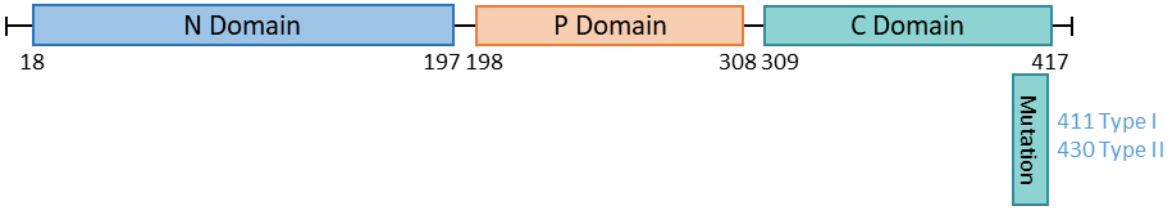

Figure 4. Structural domains of *CALR* showing the Type I (deletion) or Type II (insertion) mutations.

Other infrequent mutations in Exon 9 account for up to 15% of *CALR* mutations. The distribution of *CALR* mutation types differs according to the MPN type. When compared with *JAK2*-positive patients with ET, those with *CALR* mutation have higher platelet counts, lower hemoglobin levels, and leucocyte counts. Also, *CALR*-positive patients with PMF have a characteristic lower leukocyte count and elevated platelet count. Furthermore, it has been identified that patients with ET and PMF with *CALR* mutations have a higher survival rate compared to patients with *JAK2*-positive [27, 34].

4.4. Other non-driver mutations in MPN

Studies have shown that a small group of patients negative for the three main driver mutations (i.e. triple negative cases), acquire mutations in novel regions of the *MPL* and *JAK* genes, as well as genes involved in regular cellular processes, including epigenetic regulation, mRNA splicing, and cytokine signaling regulation [6]. These genes can acquire mutations either before or after the onset of the driver mutations and are involved in disease evolution and progression to AML. It is considered that individuals, especially men and patients with PMF, having more than one mutation in these genes are at "high molecular risk (HMR)" and have a lower survival rate [35]. Detection of this mutation requires Next Generation Sequencing (NGS), however, employing this technique in routine analysis remains a challenge [36]. Critical mutations associated with the pathogenesis of MPN are illustrated in Figure 5.

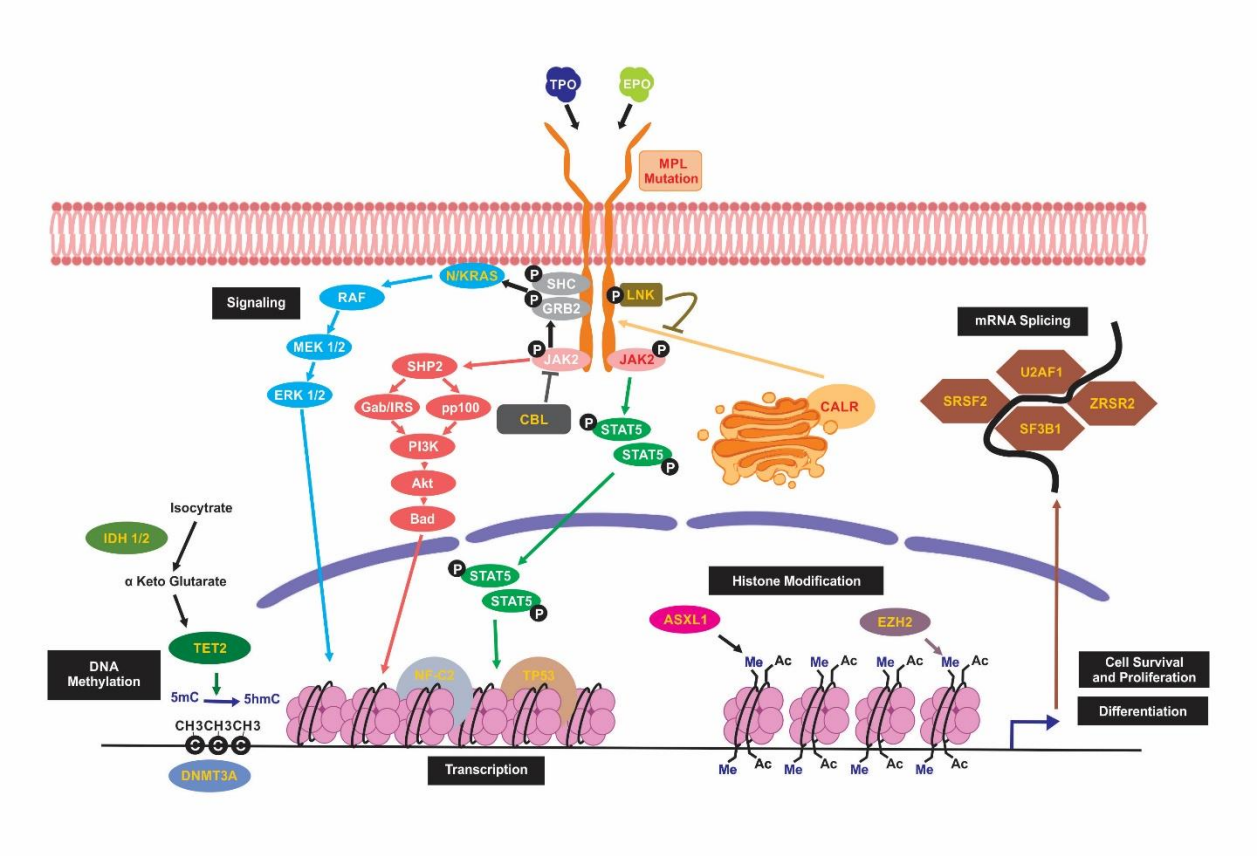

Figure 5. Acquired mutations associated with MPN pathogenesis showing constitutive activation of JAK/STAT, MAPK, and PI3K/Akt pathways; driver mutations (*JAK2*, *CALR* and *MPL*) in red; other mutations in yellow.

4.4.1. *TET2*

The *TET2* gene is involved in DNA methylation for epigenetic regulation. Mutations in the *TET2* gene, including insertions, deletions, or substitutions, result in a loss of catalytic activity and hence is associated with various myeloid neoplasms. Minimal prognostic impact has been observed from *TET2* gene mutations, especially in patients with *JAK2* V617F-positive and negative MPN [37,38]. A study by Lundberg (2014) [39] demonstrated that *TET2* gene mutation can occur before or after *JAK2* V617F mutation resulting in clonal dominance. DNA hypermethylation is associated with the loss of function of the *TET2* gene due to decreased 5-hydroxymethylcytosine (5hmC) production. This essentially affects HSCs leading to the release of self-renewal signals, incorporation of new mutations, and progression to myelomonocytic differentiation [40].

4.4.2. *DNMT2* and *DNMT3*

DNA methyltransferase 2 coded by the *DNMT2* gene is an enzyme that transfers a methyl group in epigenetic regulation. Less than 10% of cases of MPN are reported to arise from *DNMT2* gene mutations, with exon 23 mutations being more prevalent [41]. Mutations in the *DNMT3* gene are present at the early stage of MPN, in about 5-10% of reported cases [42]. Substitution at position 882 (R822H) is the most common mutation identified in myeloid neoplasms [43]. Patients with *JAK2* V617F-positive MPN with loss of the *DNMT3* gene are suggested to have increased progression to myelofibrosis, reduction of HSCs, and lowered erythropoiesis [44].

4.4.3. *IDH1* and *IDH2*

Mutations in *IDH1* and *IDH2* which code for isocitrate dehydrogenase 1 and 2 respectively, result in the generation of small molecules that interfere with histone demethylation; and consequently, block hematopoietic differentiation [45]. Although *IDH1/2* mutations have been found in low frequency (0.5-4%) in MPN cases and more prevalent in PMF than PV or ET, it has been suggested that mutation in these genes may facilitate progression to acute leukemia [46,47].

4.4.4. *EZH2*

EZH2 mutations are found in higher proportion in PMF, about 3% in ET, and infrequently in patients with PV. These mutations are suggested to result in complications in MPN including poor survival and progression to myelofibrosis [48]. Studies conducted on transgenic mice have confirmed the critical effect of co-occurrence of *JAK2* V617F and *EZH2* mutations, showing the faster progression to myelofibrosis and shorter life expectancy compared to just *JAK2* V617F alone [49].

4.4.5. *ASXL1*

ASXL1 gene codes for proteins involved in chromatin remodeling. Frameshift and nonsense mutations occur frequently in exon 13 of the gene and have a significant prognostic impact on patients with PMF, however, little evidence of this mutation has been demonstrated in patients with PV and ET [50]. This mutation has also been associated with leukocytosis, increased platelet count, and the presence of ≥1% circulating blasts [37]. About 47% of MPN cases that progressed to the leukemic phase were identified with heterogenous mutations in the *ASXL1* gene [51].

4.4.6. *SF3B1, SRSF2* and *U2AF1*

Mutations in *SF3B1*, *SRSF2*, and *U2AF1* genes which are involved in mRNA processing, have been reported in critical stages of certain rare cases with MPN. In PV and ET, spliceosome mutation is suggested to lead to poor outcomes of survival [52]. *SF3B1* mutations were first discovered in patients with myelodysplastic syndrome (MDS); however, patients with PMF have been observed to have mutation clusters in exons 12-16, with an increased risk of thrombotic events [53,54]. *SRSF2* and *U2AF1* genes are involved in the activation or suppression of mRNA splicing. In about 19% of cases of PMF that progress to acute myeloid leukemia, somatic mutations in *SRSF2* genes are frequently found coexisting with *TET2, ASXL1*, or *RUNX1*, and offer a poor prognosis for cases with MPN. Less than 2% of cases with MPN have an associated with *U2AF1* gene mutation; however, a strong indication has been reported for advanced-stage PMF and leukemic transformation [51, 55,56].

4.4.7. *TP53*

TP53 is a major tumor suppressor gene involved in apoptosis and cell cycle arrest, and its loss of function is associated with various cancers [57]. *TP53* mutation with loss of heterogeneity is associated with leukemic transformation in about 16–17% of cases of MPN [58]. MDM2 and MDM4 are upstream regulators of the *TP53* gene and are found in increased expression and copy number in cases of MPN associated with leukemic transformation [59].

5. Molecular Diagnosis of MPN

Detection of mutations in *JAK2* Exon 14, *JAK2* Exon 12, *MPL* Exon 10, and *CALR* Exon 9 are important for early diagnosis and timely management of patients with MPN. Various molecular diagnostic methods have been developed to detect these mutations in patients with MPN (Table 4). The most widely used method to identify and quantify the

causative gene mutation in MPNs involves allele-specific quantitative polymerase chain reaction (AS-qPCR). Other methods include allele-specific-polymerase chain reaction (AS-PCR), high-resolution melt curve analysis, denaturing high-performance liquid chromatography (HPLC) followed by direct sequencing, capillary electrophoresis, pyrosequencing, restriction fragment length polymorphism (RFLP), fluorescent in situ hybridization (FISH), Sanger sequencing and cloning [5, 14].

Table 4. Methods for MPN mutational analysis. Adapted from: [14].

Method	Sensitivity (%)	Advantage	Disadvantage
Sanger sequencing	20	All mutations detected; bidirectional confirmation	Poor sensitivity; takes time; not quantitative
Restriction Fragment Length polymorphism	1-10	Less expensive	Not quantitative; relatively low sensitivity; requires post-PCR modifications; high + samples required for good results
Pyrosequencing	5-10	Easy to perform; semiquantitative; less expensive	Only target mutations detected; relatively low sensitivity
Melt curve analysis	5-10	Easy to perform; semiquantitative; less expensive	Only target mutations detected; relatively moderate sensitivity; high + samples required for good results
Allele-specific PCR	0.1-1	Highly sensitive and easy to perform	Only target mutations detected; not quantitative
Locked nuclei acid qPCR	0.1-0.01	Highly sensitive; quantitative	Only target mutations detected
Allele-specific qPCR	0.1-0.01	Highly sensitive; quantitative	Only target mutations detected

Although used extensively, most of these methods, such as PCR-Sanger sequencing, have relatively poor sensitivities of greater than 5% of mutant alleles and a limitation of comprehensive coverage of the targeted genomic region [14], hence affecting the efficiency of the analysis. Next Generation Sequencing (NGS) has the potential to efficiently detect the common, novel, or rare mutations in *JAK2* Exon 14, *JAK2* Exon 12, *CALR* Exon 9, and *MPL* Exon 10, with deep sequencing of the targeted amplicons [5]. It is increasingly being used in clinical laboratories but there is only limited validation of the use of NGS along with PCR in detecting MPN-associated mutations, especially in the diagnosis of patients with triple-negative MPNs [12, 14].

5.1. Cytogenetic analysis of MPN

Cytogenetic investigation of MPN involves inter-phase FISH and karyotype analysis, preferably with a bone marrow aspirate specimen or with a peripheral blood or bone marrow core biopsy specimen [12]. Karyotype analysis distinguishes MPN from other closely similar myeloid malignancies presenting with thrombocytosis, such as MDS and chromosome 5q deletion, thereby aiding prognosis. In triple-negative patients with MPN, karyotype analysis is critical for confirming clonal hematopoiesis. Cytogenetic analysis of PV and ET patients is suggested to be of significance only in cases with triple-negative MPN [60]. In PMF cases, karyotype analysis is crucial and may reveal its association with thrombocytopenia, showing greater than 1% circulating blasts, leukopenia, and reduced hemoglobin levels. Common findings of cytogenetic studies are gains of chromosomes 8 and 9, del(20q), del(13q), and abnormalities of chromosome 1, including duplication of 1q. Other less frequent lesions include –7/del(7q), del(5q), del(12p), +21, and der(6)t(1;6)(q21;p21.3) [61,62,63].

6. Treatment

6.1. Standard Treatment

The increasing insight into the molecular pathogenesis of MPN and the finding that *JAK2*, *CALR*, and *MPL* mutations activate JAK2 signaling has revealed rational therapeutic targets such as JAK2 inhibitors [64]. The standard treatment, particularly for PV, is therapeutic phlebotomy to achieve hematocrit <45%, sometimes combined with other therapies. Antiplatelet drugs such aspirin, and cytoreductive chemotherapy, such as, hydroxyurea (Hydrea®), α-interferon or anagrelide (Agrylin®), are used instead of or in combination with phlebotomy for patients with a history of thrombosis to suppress the excess production of blood cells [65].

Aspirin is indicated in the treatment of patients with moderate to high-risk PV and ET, and is shown to control microvascular symptoms such as erythromelalgia, transient neurological symptoms, ocular disturbances, migraines, and seizures. Hydroxyurea, is often used to treat older patients with high-risk PV and ET (>60 years old), and sometimes patients with low/intermediate-1 risk MF. It limits the bone marrow's ability to make blood cells and thereby reduces the platelet count and the incidence of thrombosis or leukocytosis [66].

Interferon may be used to stimulate the immune system and slow down the production of RBCs in patients not achieving sufficient thrombocyte control by cytoreductive therapy. BESREMi®, a mono-pegylated long-acting interferon was the first interferon, specifically approved in 2021 by the U.S. Food and Drug Administration (FDA) to treat adults with high-risk PV, regardless of their treatment history. In addition, interferons can be used to treat patients with high-risk ET and low-risk MF, but are not recommended for patients with high-risk MF [67]. Anagrelide is frequently used to lower platelet counts in patients with high-risk ET demonstrating intolerance or experiencing complications with hydroxyurea. There is no single treatment option that can effectively treat all patients with MPN. The choice of treatment including the use of platelet-lowering agents, is based on risk factors including age, history of bleeding or thrombosis, vascular risk, severity of symptoms, and drug tolerance [65].

6.2. Allogeneic Stem Cell Transplantation (ASCT)

Allogeneic stem cell transplantation is curative for all MPN subtypes, however, this is limited to a subset of patients with MF, due to the associated expense and risk of transplant-related morbidity and mortality, such as hepatobiliary toxicities. It involves the transfer of HSCs from donor to patient, essentially to replace the defective stem cells with healthy cells. This has reported an overall survival (OS) rate of 41% and a disease-free survival rate of 32% at 10 years in a cohort of patients with MF who underwent ASCT [68].

A favorable OS and low non-related mortality rate have been reported in patients with *CALR* and *MPL* mutation-positive PMF, post-PV MF, and post-ET MF, who underwent ASCT [69,70]. However, identification of HMR mutations may aid in ASCT selection as a therapeutic option for patients with PMF, as some studies have associated lower survival rates in patients with one or more HMRs [71,72]. Thus, treatment decisions regarding ASCT should be individualized based on mutational status, MF risk assessment, and transplant eligibility criteria.

6.3. JAK inhibitors (JAKi)

The discovery of the *JAK2* V617F oncogene mutation contributed to the exploitation of the JAK2 tyrosine kinase as an important therapeutic target. Small-molecule inhibitors for JAK2 were developed for treatment of patients intolerant or refractory to hydroxyurea, particularly in patients with PV, and numerous drugs that inhibit JAK2 are currently in clinical trials [65, 73].

The JAK1/2 inhibitor, ruxolitinib (Jakafi®) was approved in 2012 by the FDA. It represents a current standard of care for the treatment of symptomatic intermediate to high-risk MF, including PMF, post–PV MF, and post–ET MF. It targets and partially inhibits the activity of JAK2 and the related protein JAK1 with clinical benefits in splenomegaly, mutant allele burden reduction, and symptom control [74,75]. Further, it increases overall survival and reduces the incidence of death in patients with HMR. However, it does not prevent the onset of these HMR in patients initially negative for these mutations [76]. It is suggested that the administration of ruxolitinib be discontinued if there is no reduction in symptoms [13]. Figure 6 shows a schematic representation of the mechanism of action of ruxolitinib.

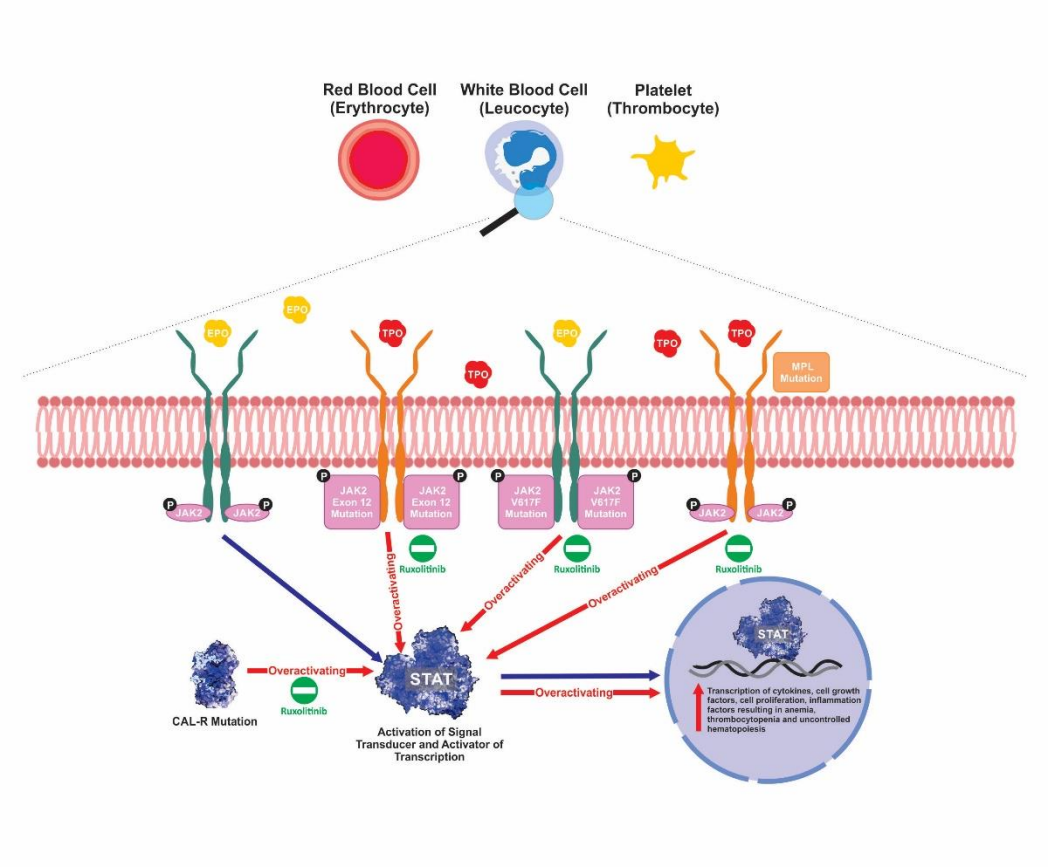

Figure 6. Inhibitory effect of Ruxolitinib on MPN progression; blue arrows indicate the normal JAK/STAT pathway and red arrows indicate the overactivation of the JAK/STAT pathway due to driver mutations.

Fedratinib (Inrebic) was approved by the FDA in 2019 for treating adult patients with intermediate-2 or high-risk primary or secondary myelofibrosis [13]. It is an oral kinase inhibitor with activity against wild-type and mutationally-activated JAK2 and FMS-like tyrosine kinase 3 (FLT3) (JAK2/FLT3) with analogous type 1 mode of JAK2 binding. It inhibits the JAK2-selective mutation than the other JAK family members JAK1, JAK3, and TYK2 [65]. The drug has also shown activity in reducing splenic size and symptom burden in patients with intermediate/high-risk MF resistant or intolerant to ruxolitinib [77]. Ruxolitinib, fedratinib, or clinical trial can be considered for patients with high-risk MF, having platelet count $\geq 50 \times 10^9$/L, who do not meet the criteria for ASCT [78,79]. In addition, exposure to ruxolitinib before ASCT is suggested to be associated with positive outcomes [80]

Pacritinib (Vonjo), also FDA-approved, shows promising profiles for anemic and thrombocytopenic patients with intermediate or high-risk MF with platelet counts below 50×10^9 /L [13]. Pacritinib is yet another oral kinase inhibitor with activity against wild-type JAK2, mutant *JAK2* V617F, and FLT3, and contributes to cytokine and growth factor-mediated signaling, to regulate hematopoiesis and immune function [65].

The disease-modifying potential of JAK2 inhibitors, however, remains limited and is further impeded by the loss of therapeutic responses in a substantial proportion of patients over time. The combination of novel compounds with JAK2 inhibitors is of specific interest to enhance the therapeutic efficacy of molecularly targeted treatment approaches, especially for patients with MF with poor survival rates [65, 81]. Clinical studies are currently evaluating approaches of JAK2 inhibition in combination with inhibition of MEK-ERK or PI3K signaling, as well as interference with apoptosis regulation by Bcl-2/Bcl-xL inhibition using navitoclax. *In vivo* studies in mice showed that combined inhibition of JAK2, PI3K, and mTOR in *JAK2* V617F mutated cells causes reduction of both JAK2 and PI3K-mediated STAT5 phosphorylation, impairment of the clonogenic potential of *JAK2* V617F mutated hematopoietic progenitor cells, reduced splenomegaly and myeloid cells infiltration [15].

Patients suffering from excessive splenomegaly, and ineligible for treatment with JAK2 inhibitors, may require splenectomy, splenic irradiation, or partial splenic artery embolization for long-term control of disease symptoms, such as anemia and thrombocytopenia [82]. Other innovative treatment approaches under clinical investigation include telomerase inhibition, interference of the telomere function, MDM2 inhibition, and impact on the TP53 tumor suppressor function. Recent studies with epigenetic drugs such as histone deacetylase inhibitors, givinostat, panobinostat, and hypomethylating agents, azacitidine and decitabine, were effective in treating PV, MPN transformed to AML and minimally effective in treating MF. These efforts will add valid options to the molecular targeted treatment approaches for patients with MPN [65].

6.4. Regulation of Apoptosis

Increased level of tumor necrosis factor-alpha (TNFα) is a characteristic feature of the pathobiology of MPN, especially in patients with MF, promoting the survival of malignant cells over normal cells [81]. Second mitochondria-derived activator of caspases (SMAC) mimetics or inhibitors of apoptosis (IAP) antagonists promote cancer cell death by activating caspases and cytokine-mediated apoptosis in tumor models with high TNFα expression. IAP antagonism by SMAC occurs through the binding of the N-terminal tetrapeptide (AVPI) of SMAC to IAP at selected domains. Hence, small molecule compounds that mimic the AVPI motif of SMAC have been designed to overcome IAP-mediated apoptosis resistance of cancer cells [83].

LCL-161 is a small molecule SMAC mimetic that inhibits multiple IAP family proteins. This oral agent is under phase II study in patients with primary, post-PV, or post-ET and MF (NCT02098161). Preliminary results of 44 patients enrolled in this clinical trial showed approximately 30% ORR (objective response rate), defined as complete remission (CR) + partial remission (PR) + clinical improvement [84,85].

BCL-xL inhibitor, such as navitoclax, is a high-affinity small, orally available molecule that inhibits the anti-apoptotic activity of BCL-xL. Navitoclax is under phase II clinical evaluation in combination with ruxolitinib for reduction of splenic size in patients with MF (NCT03222609) [86].

6.5. Targeting the Hematopoietic Micro-Environment

Heat shock protein-90 (Hsp90), is a chaperone protein found in normal and cancer cells. It stabilizes proteins against heat stress, facilitates protein degradation, and plays a prominent role in cancer. Oral PU-H71, an Hsp90 inhibitor is recently under trial being

assessed for the safety and tolerability in patients with MPN previously treated with ruxolitinib (NCT03935555) [87].

Histone deacetylase inhibitors (HDACi) function as a therapeutic agent by down-regulating JAK2 through acetylation and interference of the chaperone protein function of Hsp90. Ongoing trials are evaluating the synergism of ruxolitinib in combination with HDACi, such as Givinostat and panobinostat [88,89].

6.6. TP53 modulation

TP53 is an unstable protein due to continuous MDM2-mediated degradation. It is a principal mediator of growth arrest and apoptosis. Upon oncogene activation, MDM2-mediated degradation is blocked and p53 stabilizes. Idasanutlin, an MDM2 antagonist, is under phase II study as monotherapy in patients with PV who are intolerant to therapy with hydroxyurea (NCT03287245), and in phase I in patients with PV and ET (NCT02407080) [90,91].

Another MDM2 antagonist, (KRT-232) is under phase II study in phlebotomy-dependent patients with PV, assessing independence of phlebotomy and reduction in splenic size as compared to ruxolitinib (NCT03669965) [92]. It is also under phase II study in patients with MF, unresponsive to treatment with JAKi (NCT03662126) [88, 93].

6.7. Inhibitors of Fibrosis

A determining feature of PMF is progressive bone marrow fibrosis which is associated with poor prognosis. Treatment with a fibrocyte inhibitor may interfere with the development of marrow fibrosis. A fibrocyte inhibitor, serum amyloid P (SAP; pentraxin-2), significantly prolongs survival and decelerates the development of marrow fibrosis in mouse models [94]. PRM-151, a recombinant form of SAP, is under phase II trial, for bone marrow response rate (reduction in bone marrow fibrosis score by at least one grade according to WHO criteria), in patients with MF (NCT01981850) [95]. It acts at sites of tissue damage, thereby preventing and reversing fibrosis. Preliminary results showed 7 of 13 evaluated patients having a morphologic bone marrow response, with PRM-151 having durable safety and efficacy at 72 weeks [81].

A selective aurora kinase-alpha inhibitor, alisertib, eradicates atypical megakaryocytes and reduces marrow fibrosis. Alisertib was found to reduce splenomegaly and symptom burden in 29% and 32% of patients with MF, respectively, during a phase II clinical study, it also normalizes megakaryocytes reducing fibrosis in 5 of 7 patients [81, 96].

6.8. TGF-β inhibitor

Transcription growth factor-beta (TGF-β) is a cytokine involved in the fibrotic phase of MF. Sotatercept is an activin receptor IIA ligand trap. It sequesters TGF-β ligands, such as GDF11, secreted by the bone marrow stromal cells and improves erythroid differentiation, improving anemia [97]. A phase II study of sotatercept, to assess the response to anemia (increase in hemoglobin and decrease in red blood cell transfusion requirement) in patients with MPN-associated MF and anemia (NCT01712308), showed response to anemia in 36% of the evaluated patients [98].

Luspatercept, another erythroid maturation agent, works by neutralizing selected TGF-β ligands, thereby facilitating late-stage erythropoiesis [99]. A phase II multicenter clinical trial is ongoing, evaluating luspatercept for anemia response in patients with MPN-associated MF and anemia (NCT03194542) [100].

Furthermore, AVID200, an engineered TGF-β inhibitor found to decrease the fibrogenic stimuli leading to MF, is under phase I/Ib study in patients with intermediate-2 or high-risk MF (NCT03895112) [81, 101].

6.9. Telomerase Inhibitors

Imetelstat, a competitive and potential telomerase inhibitor, inhibits multiplication and triggers apoptosis of cancer stem cells by binding to the RNA component of telomerase. This therapeutic agent under phase II clinical study, showed molecular responses in patients with ET unresponsive to previous therapies [102]. Imetelstat is under phase II clinical trial in patients with intermediate or high-risk MF, and previously treated with JAKi (NCT02426086), evaluating splenic and total symptom score reduction. Preliminary results showed complete remissions and a median survival of 19.9 months [81, 101].

6.10. BET Inhibitors

Epigenetic mechanisms enhance the inflammatory milieu in MPN via activation of NF-kB signaling. Hence, bromodomain and extra-terminal (BET) protein inhibition is a potential approach to therapy. BET inhibitor, CPI-0610, is under phase II clinical study as monotherapy or in combination with ruxolitinib for response of spleen size and independence of RBC transfusion rate in patients with MF (NCT02158858) [81, 88, 103].

Table 5 summarizes the therapeutic agents under clinical trials for treatment of MPN.

Table 5. Clinical trials involving therapeutic agents as monotherapy or in combination with ruxolitinib (Adapted from: [81]).

Agent	MPN-subtype	Mechanism of action	Phase	Clinical trial
LCL-161	Primary, post-PV or post-ET and MF	IAPi	II	NCT02098161
Navitoclax	MF receiving ruxolitinib	BCL-xLi	II	NCT03222609
PU-H71	MF receiving ruxolitinib	HSP-90i	II	NCT03935555
Panobinostat	MF receiving ruxolitinib	HDACi	Ib	NCT01433445
Idasanutlin	Hydroxyurea intolerant PV, ET and MF without prior JAK2i	MDM2 inhibitor	I and II	NCT03287245 and NCT02407080
KRT-232	Phlebotomy-dependent PV, and MF with JAK2i failure	MDM2 inhibitor	II	NCT03669965 and NCT03662126
PRM-151	MF	Pentraxin-2 inhibitor	II	NCT01981850
Alisertib	MF	Aurora Kinase A inhibitor	II	NCT02530619
Sotatercept	PMF and MPN-associated anemia	TGFβi	II	NCT01712308
Luspatercept	PMF and MPN-associated anemia	TGFβi	II	NCT03194542
AVID200	MF	TGF-βi	I/Ib	NCT03895112
Imetelstat	MF with JAK2i	Telomerase inhibitor	II	NCT02426086
CPI-0610	MF	BET inhibitor	II	NCT02158858

The majority of patients with MPN, with regular monitoring and treatment, live for many years without developing major symptoms. Prolonged survival can, however, be challenged in a minority of patients due to the development of serious thromboembolic disorders, post-PV MF, and cancers, including acute leukemia. Hence, treatment aims to

achieve control of symptom and prevention of the development of thrombotic and hemorrhagic complications [13].

7. Future Directions

Correlative studies have revealed associations of specific gene mutations with clinical presentation, progression dynamics, and outcome. More studies are needed to validate and confirm the prognostic and diagnostic significance of non-driver mutations of MPN, as this might help to achieve satisfiable distinguishable features from other myeloid malignancies and drive the development of precision therapies to increase survival outcomes of patients. Several pre-clinical combination treatments are currently being explored and monotherapy with JAK inhibitors is under improvement. There is also an increasing urgency to adopt sensitive and specific NGS-based technologies in routine clinical practices to facilitate accurate diagnosis of patients with MPN, particularly for those with triple-negative MPNs.

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