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REVIEW

Impact of Mutational Profile on the Management of Myeloproliferative Neoplasms: A Short Review of the Emerging Data

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Abstract: Philadelphia-chromosome negative myeloproliferative neoplasms (MPN) are a heterogeneous group of clonal hematopoietic stem cell disorders characterized by an increased risk of thrombosis and progression to acute myeloid leukemia. MPN are associated with driver mutations in *JAK2, CALR* and *MPL* which are crucial for the diagnosis and lead to a constitutive activation of the JAK-STAT signaling, independent of cytokine regulation. Moreover, most patients have concomitant mutations in genes involved in DNA methylation, chromatin modification, messenger RNA splicing, transcription regulation and signal transduction. These additional mutations may arise before, in the context of clonal hematopoiesis of indeterminate potential (CHIP), or after the acquisition of the driver mutation. The clinical phenotype of MPN results from complex interactions between mutations and host factors. The increased application of next-generation sequencing (NGS) techniques to a large series of patients with MPN has expanded the knowledge of mutational landscape and contributed to define the clinical significance of mutations. This molecular information is being increasingly used to refine diagnosis, risk stratification, monitoring of residual disease and response to treatment. *ASXL1, SRSF2, EZH2, IDH1*/*IDH2* and *U2AF1* mutations are associated with a more advanced disease and reduced overall survival in primary myelofibrosis (PMF), whereas spliceosome mutations in Polycythemia vera (PV) and essential thrombocythemia (ET) adversely affect both overall (*SF3B1, SRSF2* in ET and *SRSF2* in PV) and myelofibrosis-free (*U2AF1, SF3B1* in ET) survival. This review discusses current knowledge of the molecular landscape of MPN, and how the availability of those molecular information may impact patient management.

Keywords: myeloproliferative neoplasms, polycythemia vera, essential thrombocythemia, myelofibrosis, JAK-STAT pathway, gene mutations

Introduction

The classic Philadelphia-chromosome negative myeloproliferative neoplasms (MPN) are a heterogeneous group of clonal hematopoietic stem cell diseases characterized by overproduction of one or more types of cells of the myeloid lineage.[1] According to the 2016 World Health Organization (WHO) criteria, MPN include Polycythemia Vera (PV), Essential Thrombocythemia (ET) and myelofibrosis, which may be classified as primary (PMF) or secondary to PV and ET (post-PV-MF and post-ET-MF).[2] This classification has been updated with the introduction of pre-fibrotic/early PMF, distinguishable from ET on the basis of bone marrow (BM) morphology, that has a higher tendency to develop overt MF and is characterized by a reduced overall survival

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compared to true ET[3] (Table 1). The diagnostic criteria of PPV-MF and PET-MF were developed by the International Working Group for MPN Research and Treatment – IWG-MRT[4,5] (Table 2). All MPN can transform into secondary acute myeloid leukemia, referred to as MPN-blast phase (BP), which is typically refractory to conventional chemotherapy and has a poor prognosis.[6] The 15-year risk of leukemia is estimated at 2.1% to 5.3% for ET, 5.5% to 18.7% for PV and more than 20% for PMF whereas fibrotic progression rates of ET and PV, during a similar time interval, are estimated at 4% to 11% and 6% to 14%, respectively.[7]

In Europe, the incidence of MPN varies from 0.4 to 2.8/100.000 in PV, 0.38 to 1.7/100.000 in ET, and 0.1 to 1/100.000 in PMF, while prevalence remains difficult to determine.[8] MPN have clinical heterogenicity, with some patients having normal lifespan and others developing disease progression or life-threatening complications. Median survival is around 20 years for ET, 14 years for PV and 6 years for PMF; the corresponding values for patients younger than 60 years are 33, 24 and 15 years.[9] PV and ET are characterized by cardiovascular events, mainly thrombosis and less frequently hemorrhage, a varying burden of symptoms, and an intrinsic risk of evolution to MF or BP. Treatment is mainly focused on the reduction of thrombosis risk, control of myeloproliferation, improvement of symptoms, and management of related complications. MF is a protean disease with variable levels of cytopenias, constitutional symptoms, often as a result of a hypercytokinaemia, extramedullary hematopoiesis and marrow fibrosis. Splenomegaly is present at the varying extent and can cause abdominal pain, early satiety, splenic infarction, and

Table 1 Summary of WHO Criteria for MPN

Polycythemia Vera	Essential Thrombocythemia	Prefibrotic Myelofibrosis	Primary Myelofibrosis
Major criteria			
1. HGB > 16.5 g/dL (men), >16 g/dL (women) or HCT > 49% (man) and >48% (women) or increased red cell mass. 2. BM biopsy showing hypercellularity for age with trilineage growth, including prominent erythroid, granulocytic and megakaryocytic proliferation with pleomorphic, mature megakaryocytes. 3. Presence of *JAK2*V617F or *JAK2* exon 12 mutation.	1. Platelet count ≥450 x 10^9/L 2. BM biopsy showing proliferation mainly of the megakaryocytic lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant increase or left shift in granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibers. 3. Not meeting WHO criteria for other myeloid neoplasms 4. Presence of *JAK2, CALR*, or *MPL* mutation.	1. Megakaryocytic proliferation and atypia, without reticulin fibrosis > grade 1, accompanied by increased age-adjusted BM cellularity, granulocytic proliferation, and often decreased erythropoiesis. 2. Not meeting WHO criteria for other myeloid neoplasms. 3. Presence of *JAK2, CALR*, or *MPL* mutation or presence of another clonal marker, or absence of minor reactive BM reticulin fibrosis[b]	1. Presence of megakaryocytic proliferation and atypia accompanied by either reticulin and/or collagen fibrosis grades 2 or 3. 2. Not meeting WHO criteria for other myeloid neoplasms. 3. Presence of *JAK2, CALR*, or *MPL* mutation or presence of another clonal marker, or absence of reactive BM fibrosis[b]
Minor criterion		**Minor criteria (confirmed in two consecutive determinations)**	
• Subnormal serum EPO level.	• Presence of a clonal marker or absence of evidence for reactive thrombocytosis.	(a) Anemia not attributed to a comorbid condition (a) Leucocytosis ≥11 x 10^9/L (b) Palpable splenomegaly (c) LDH increased	(a) Anemia not attributed to a comorbid condition (b) Leukocytosis ≥11 x 10^9/L (c) Palpable splenomegaly (d) LDH increased (e) Leukoerythroblastosis
Patients must meet all three major criteria or the first two major criteria and the minor criterion[a]	**Patients must meet all four major criteria or the first three major criteria and the minor criterion**	**Patients must meet all 3 major criteria, and at least 1 minor criterion.**	**Patients must meet all 3 major criteria, and at least 1 minor criterion.**

Notes: [a]Criterion number 2 (BM biopsy) may not be required in cases with hemoglobin levels > 18.5 g/dL in men (hematocrit > 55.5%) or > 16.5 g/dL in women (hematocrit > 49.5%), if major criterion 3 and the minor criterion are present. [b]Fibrosis secondary to infection, autoimmune disorder or other chronic inflammatory conditions, lymphoid neoplasm, metastatic malignancy, or toxic myelopathies. Data from Arber et al.[3]

Abbreviations: WHO, World Health Organization; HGB, hemoglobin; HCT, hematocrit; BM, bone marrow; EPO, erythropoietin; LDH, serum lactate dehydrogenase.

Table 2 Summary of IWG Criteria for PPV-MF and PET-MF

Post-Polycythemia Vera Myelofibrosis (PPV-MF)	Post-Essential Thrombocythemia Myelofibrosis (PET-MF)
Required criteria	
• Prior diagnosis of PV according to WHO criteria. • Bone marrow fibrosis ≥ grade 2	• Prior diagnosis of ET according to WHO criteria. • Bone marrow fibrosis ≥ grade 2
Additional criteria (two required)	
• Anemia or loss of phlebotomy requirement • Leukoerythroblastosis • New splenomegaly or ≥5 cm increase in spleen size • Development of at least one constitutional symptom[a]	• Anemia and decrease ≥2 g/dL in hemoglobin level • Leukoerythroblastosis • New splenomegaly or ≥5 cm increase in spleen size • Increased LDH • Development of at least one constitutional symptom[a]

Notes: [a]Constitutional symptoms include > 10% of weight loss in six months, night sweats and unexplained fever (> 37.5 °C). Data from Barosi et al.[5]
Abbreviations: IWG, International Working Group; WHO, World Health Organization; LDH, lactate dehydrogenase.

portal hypertension. Progressive MF can be associated with marrow failure and evolution to BP. Treatment, both standard and experimental, is variably effective in reducing myeloproliferation, splenomegaly, constitutional symptoms and improving the quality of life. However, some patients do not respond to treatment, and others become resistant. Nowadays, the only curative approach is allogeneic hematopoietic stem cell transplant (allo-HSCT) which has a high mortality risk and is often not considered for older patients and those with comorbidities.

Mutational Landscape of MPN

Driver Mutations: *JAK2, CALR* and *MPL*

Constitutive activation of the JAK-STAT pathway is the hallmark of MPN. Mutations in *JAK2, CALR* and *MPL* are referred as "driver mutations", based on their role in driving the MPN phenotype and are crucial for the diagnosis along with clinical and histopathological features. These three mutations are mutually exclusive, but cases of co-occurrence have been reported.[10,11] Patients with features of MPN without any one of these mutations are classified as "triple negative". Two types of *JAK2* mutations are described in MPN. The first, discovered in 2005, is a valine to phenylalanine substitution at amino acid position 617 (V617F) in exon 14;[12–15] the second, described in 2007, comprises many different mutations, particularly in-frame insertions or deletions, in exon 12 of *JAK2*.[16,17] *JAK2*V617F is detected in approximately 95% of PV cases and 50–60% of ET and PMF. Conversely, *JAK2* exon 12 mutations are extremely rare and are found exclusively in 2–3% of all PV patients, that are negative for *JAK2*V617F. *JAK2*V617F can drive all the different MPN phenotypes through ligand-independent activation of receptors for erythropoietin (EPO), thrombopoietin (TPO) and granulocyte-colony stimulating factor (G-CSF), resulting in the expansion of erythroid, megakaryocytic and granulocytic lineages, respectively.[18–20] In comparison to V617F mutation, patients with exon-12 mutations predominantly activate EPO receptor and are characterized by erythroid-dominant myeloproliferation with lower leukocytosis and thrombocytosis, and younger age at diagnosis.[21,22] The different phenotypes of *JAK2*V617F mutated MPN may reflect different host characteristics, different development stages at which the mutation arises, the presence and order of other molecular variants and characteristics of bone marrow microenvironment. The timing of mutation acquisition may affect the clinical phenotype with the "JAK2-first" more commonly occurring in PV, and "TET2-first" more commonly in ET.[23] Similar to *TET2*, patients are more likely to present with PV when *JAK2*V617F is acquired before *DNMT3A* mutation, compared with patients who first acquired *DNMT3A* mutation and more currently have an ET phenotype.[24] Moreover, allele burden has been associated with different phenotypes; homozygous mutation and higher mutant allele burden (>50%) have been described in most PV patients, and associated with increased risk of thrombosis and fibrotic evolution, whereas ET patients tend to have a lower burden.[25]

CALR encodes for calreticulin, a 46-kDa chaperone protein located in endoplasmic reticulum (ER) lumen, which has a key role in the maintenance of calcium homeostasis and protein folding.[26] To date, more than 50 different *CALR* mutations, all in exon 9, have been described in 20–25% of ET and 25–30% of PMF cases, but not in PV patients.[27,28] Two *CALR* mutations account for approximately 80% of all the subtypes; type-1 is a 52-bp deletion (c.1092_1143del p.L367fs*46) and type-2 is a 5-bp insertion (c.1154_1155insTTGTC p.K385fs*47), resulting in mutant proteins that loss the ER-retention motif (KDEL) at the C-terminus. All the other *CALR* mutations are grouped as type 1-like and type 2-like in relation to their corresponding structural similarities and effect on C-terminal. *CALR* subtypes are associated with many clinical phenotypes and outcomes in MPN. In PMF,

type-1 *CALR* mutations are more prevalent than type-2 (70% vs 13%), while in ET they are more balanced (51% vs 39%).[29] Moreover, type-1 mutations have been associated with a significantly higher risk of myelofibrotic transformation in ET.[30] In a large series of ET patients both *CALR* variants, compared to mutant *JAK2*, were associated with higher platelet and lower hemoglobin and leukocyte counts.[31] The role of mutated *CALR* in driving the clinical phenotype of MPN has yet to be fully elucidated. Recently, some studies demonstrated that mutant CALR binds to MPL within the ER, resulting in the constitutive activation of MPL, and consequently of the JAK-STAT signaling.[32–35] Moreover, MPL-CALR complex has been detected on the cell surface and this translocation appears essential for the oncogenic activity,[36,37] raising the prospect for future target therapies.

MPL is the cell surface receptor for TPO, which regulates megakaryopoiesis and platelet production through the JAK-STAT signaling and is also expressed on the hematopoietic stem cells. *MPL* mutants largely account for the marked thrombocytosis in patients with ET and PMF.[38] *MPL* gain of function mutations of tryptophan 515 (W515) in exon 10, located in the transmembrane domain of the protein, are described in 3–8% of ET and PMF cases.[39] The most common mutations are W515L and W515K. Other rare mutations at the same position, as W515R, W515A and W515G have been reported.[40] Although the *MPL*S505N allele was identified initially as an inherited mutation in familial thrombocythemia,[41] it can also be acquired as a somatic event in ET patients. Moreover, in a recent report, several second-site mutations that enhance S505N-driven activation were described.[42]

Triple-Negative MPN

Mutations of *JAK2, CALR*, and *MPL*, account for over 90% of MPN cases and are usually mutually exclusive. However, in approximately 15% of patients with essential thrombocythemia (ET) and 8–10% of primary myelofibrosis (PMF), driver mutations are absent and these patients are referred as triple-negative (TN).[43] A small number of TN MPN patients acquired *JAK2* mutations after some time from diagnosis; this acquisition may reflect the clonal expansion of a very-low burden mutation or false negative in initial testing. Approximately 10% of TN ET and PMF patients may have mutations outside of *MPL* exon 10 and *JAK2* exon 14. These "non-canonical" *MPL* mutations include T119I, S204F/P and E230G in the extracellular domain and Y591D/N in the intracellular domain.[44] Non-canonical *JAK2* mutations include V625F, F556V, R683G and E627A.[45] As demonstrated in functional studies, most of these rare variants led to constitutive activation of the JAK-STAT signaling.[44,45] Some are somatic mutations, while others represent germline variants, with the possibility that many patients may have a form of non-clonal erythrocytosis or thrombocytosis with variable family penetrance.

Additional Mutations in Myeloid Genes

High-throughput next-generation sequencing (NGS) analyses have discovered a remarkable number of additional somatic mutations with prognostic and therapeutic relevance, particularly in PMF (Figure 1). More than 50% of MPN patients harbor additional mutations.[46] These mutations are not restricted to MPN but are shared by other myeloid malignancies including acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). They are more frequent with increasing age, not only in patients with hematologic neoplasms but also in healthy individuals in the context of clonal hematopoiesis of indeterminate potential (CHIP), which is defined as a cell population associated with a recognized hematological neoplasm driver mutation at a variant allele frequency (VAF) ≥2%, in the absence of severe cytopenias or a WHO-defined disorder. Some studies demonstrated that CHIP predisposes to the development of a hematological malignancy and cardiovascular death, the latter probably resulting from proinflammatory interactions between endothelium and macrophages derived from clonal monocytes.[47] Affected genes are involved in DNA methylation (*TET2, DNMT3A, IDH1* and *IDH2*), histone modification (*ASXL1, EZH2*), mRNA splicing (*SF3B1, SRSF2, U2AF1* and *ZRSR2*), signaling pathways (*LNK/SH2B3, CBL, NRAS, KRAS* and *PTPN11*), transcription factors (*RUNX1, NFE2, PPM1D and TP53*). In Table 3 main clinical features associated with recurrent additional mutations in MPN are summarized.

Clinical and Molecular-Integrated Prognostic Scores in MPN

Polycythemia Vera and Essential Thrombocythemia

For many years, thrombosis prediction in PV and ET relied on clinical variables only (age >60 years and prior history of thrombosis). Recent findings, however, have highlighted the contribution of genetic information. While patients with PV almost exclusively carry mutations in *JAK2*, the effect of mutated *JAK2* in patients with ET was compared with

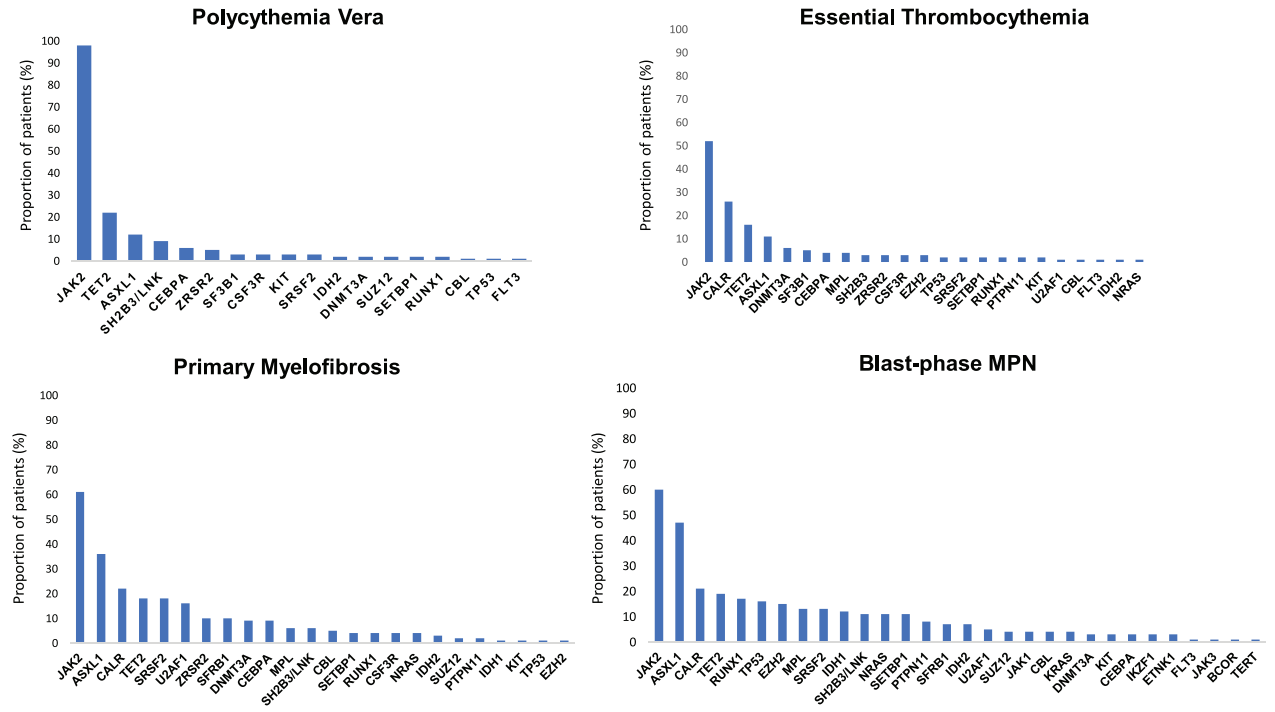

Figure 1 Mutational landscape of MPN. Results from a target next-generation sequencing analyses in patients with polycythemia vera, essential thrombocythemia, primary myelofibrosis and blast-phase MPN. The graphs show the proportion of patients for each corresponding mutation (both driver *JAK2, CALR* and *MPL* mutations and additional somatic mutations). Data from Tefferi et al[54,60] and Lasho et al.[108]

JAK2 wild-type ones, highlighting that *JAK2* wild-type patients showed a lower risk of thrombosis.[48,49] In 2012, these studies led to the development of a three-tiered International Prognostic Scoring of thrombosis in ET (IPSET-thrombosis) with the addition of *JAK2* status and cardiovascular risk factors (2 and 1 point, respectively) to age and prior thrombosis (1 point each).[50] The score was re-analyzed leading to a refined 4-tiered version, which excluded cardiovascular risk factors evaluation,[51] (Table 4) and was validated in a large independent cohort of patients.[52] Because of their very low-risk of thrombosis, ET *CALR* mutated young patients without a prior history of thrombosis and cardiovascular risk factors may not require aspirin. In these patients, as reported, aspirin might rather increase the risk of bleeding without improving the intrinsically low thrombotic risk.[51,53]

Recently, the prognostic relevance of somatic mutations was investigated in large PV and ET cohorts of patients.[54] Adverse molecular variants in PV included *ASXL1, SRSF2*, and *IDH2*, and in ET *SH2B3/LNK, SF3B1, U2AF1, TP53, IDH2*, and *EZH2*, based on age-adjusted multivariable analysis of the impact on overall, leukemia-free and myelofibrosis-free survival. Their presence was associated with inferior survival in both PV (median, 7.7 vs 16.9 years) and ET (median, 9 vs 22 years). The authors incorporated mutational information into a new prognostic model, the Mutation-Enhanced International Prognostic Scoring System (MIPSS) specific for PV and ET (Table 5). Further studies are necessary to validate these models and to determine their role in clinical decision-making.[55]

Primary and Post-PV/ET Myelofibrosis

Traditionally, two prognostic models, which include exclusively clinical parameters, are used to stratify PMF patients into risk categories with significant differences in overall survival: International Prognostic Scoring System (IPSS),[56] that is used at the time of diagnosis, and Dynamic IPSS (DIPSS),[57] applicable at any time during the clinical course. These models use five variables that independently predict inferior survival: age >65 years, hemoglobin <10 g/dL, leukocyte count >25 × 10^9/L, circulating blasts ≥1%, and constitutional symptoms. Subsequently, DIPSS was revised to DIPSS plus,[58] including red blood cells transfusion need, platelet count <100 × 10^9/L, and unfavorable karyotypes (complex karyotype or sole or 2 abnormalities that included +8, −7/7q-, i(17q), inv(3), −5/5q-, 12p- or 11q23 rearrangement).[59] In addition to the driver mutations, more than 80% of patients with PMF harbor other DNA variants in myeloid genes, as

Table 3 Recurrent Additional Mutations in MPN

Gene	Function/Mutation Types	Frequency (%)				Prognostic Impact on MPN	References
		PV	ET	MF	BP		
DNA methylation							
TET2	Demethylation through oxidation of 5-methyl-cytosine (5-mc) into 5-hydroxymetylcytosine (5-hmc), an important process in stem cell gene regulation. Heterozygous and homozygous loss-of-function mutations in catalytic domain are described.	10–20	10–15	20	25	No defined impact on survival and thrombosis	[23,54,60,108]
DNMT3A	Principal factor of DNA/histone methylation in blood stem cells. Nonsense/ frameshift and missense mutations are described, resulting in reduce methyltransferase activity.	5	5	5–15	20	Detrimental effect in MF and inferior overall survival.	[54,60,108]
IDH1/2	Mutant proteins acquired the ability to convert alpha-ketoglutarate (a-KG) to 2 - hydroxyglutarate (2-HG), favouring leukemogenesis through epigenetic dysregulation of some genes. Mutations are heterozygous and occur mostly as point missense mutations at residues R132 in *IDH1* and R140 or R172 in *IDH2*.	<2	<2	3–5	15–25	Detrimental effect in MF and inferior overall survival	[66,109]
Histone modification							
ASXL1	DNA methylation and transcription repression. Mutations are heterozygous nonsense/frameshift and occur mostly in exon 12	1–10	5–10	18–35	20–40	Risk of fibrotic and leukemic transformation	[54,61,62,66,85,110,111]
EZH2	Histone methyltransferase and transcription repression. Heterozygous and homozygous loss-of-function mutations mostly in SET2 domain are described.	1–3	0–3	0–9	13–15	Risk of fibrotic and leukemic transformation	[112,113]
mRNA splicing							
SF3B1	Subunit 1 of the splicing factor 3b protein complex. Heterozygous missense mutations in exons 14–16, hotspot K700E is the most frequent.	5	3	10	4–7	Increased risk of fibrotic transformation	[54]
SRSF2	Necessary for the splicing of pre-mRNA. It is required for formation of the earliest ATP-dependent splicing complex and interacts with spliceosomal components bound to both the 5′- and 3′-splice sites during spliceosome assembly. Heterozygous mutations and small in-frame deletions around hotspot P95 are frequent.	<3	<3	10–20	10–20	Increased risk of leukemic transformation and reduced overall survival in MPN	[61,114–117]

(*Continued*)

Table 3 (Continued).

Gene	Function/Mutation Types	Frequency (%)				Prognostic Impact on MPN	References
		PV	ET	MF	BP		
U2AF1	U2 Auxiliary factor 1, comprising a large and a small subunit, is a non-snRNP (small nuclear ribonucleoprotein) protein required for the binding of U2 snRNP to the pre-mRNA branch site. Heterozygous missense mutations around hotspot S34 and Q157 are described.	1–2	1–2	16	6	Associated with disease progression and reduced overall survival in MF	[60,69,118]
ZRSR2	Protein associates with the U2 auxiliary factor heterodimer, which is required for the recognition of a functional 3′ splice site in pre-mRNA splicing. Frameshift/nonsense and missense mutations are described.	1–2	1–2	10	5	No defined impact on survival and thrombosis	[60,108]
Signalling							
LNK/SH2B3	Adaptor protein that inhibits signalling through cytokine and tyrosine kinase receptors, including JAK2. Mostly heterozygous missense mutations are described as somatic or germline, also in the context of familial cases of MPN.	1–3	0–5	0–6	10	No defined impact on survival and thrombosis.	[54,60,108,119–121]
CBL	Mutations lead to increased STAT5 phosphorylation, cytokine hypersensitivity and cell proliferation; mostly homozygous missense substitutions.	<1	0–2	0–6	4	Reduced overall survival in MF, resistance to JAKi	[60,108,122,123]
NRAS/KRAS	Heterozygous missense mutations, particularly in codons 12, 13 and 61 led to a constitutive activation of growth signalling.	<1	<1	3–4	7–15	Reduced overall survival in MF, resistance to JAKi	[60,108,123,124]
PTPN11	Missense mutations in Src-homology 2 (N-SH2) and phosphotyrosine phosphatase (PTP) domains	<1	<1	1	2–5	Reduced overall survival in BP	[108]
Transcription factors							
RUNX1	Element of core bonding factor (CBF) heterodimer. Essential role in normal hematopoiesis. Missense, frameshift, and nonsense mutations causing loss-of-function.	<1	<1	3–4	4–13	Reduced overall survival in BP	[46]
NFE2	Mostly heterozygous frameshift mutations, leading to over-expression of wild-type protein functions.	2–3	1	1–5	11–35	No defined impact on survival or thrombosis	[125,126]
PPM1D	Encodes a protein phosphatase that regulates the DNA damage response pathway by inhibiting p53 and other tumor-suppressors through dephosphorylation.	2	1	1	NA	No defined impact on survival or thrombosis	[108,127]

(*Continued*)

Table 3 (Continued).

Gene	Function/Mutation Types	Frequency (%)				Prognostic Impact on MPN	References
		PV	ET	MF	BP		
TP53	Encodes a tumor suppressor protein that responds to different cellular stresses to regulate expression of target genes, inducing cell cycle arrest and apoptosis. Mostly missense mutations.	1	2–3	2–3	11–35	Associated with disease progression and reduced overall survival in all MPN	[60,108]

Abbreviations: PV, polycythemia vera; ET, essential thrombocythemia; MF, myelofibrosis; BP, blast-phase; MPN, myeloproliferative neoplasms; JAKi, JAK inhibitors.

discussed above, often in multiple combinations.[60] Beyond their presumed pathogenetic relevance, driver and other mutations in PMF were shown to influence overall survival and leukemia-free survival, independent of IPSS and DIPSS-plus.[61–63] The effect of driver mutations on outcome supports prognostic distinction based on the presence or absence of *CALR* type-1 mutation,[64,65] whereas additional *ASXL1, SRSF2, EZH2*, and *IDH1*/*IDH2* mutations were defined as high–molecular risk (HMR), with a prognostic relevance amplified by the number of such mutations in an individual patient.[60,61,66] Building upon the complementary nature of molecular information, the Molecular enhanced International Prognostic Score Systems (three-tiered MIPSS70 and four-tiered MIPSS70-plus) were developed using a cohort of patients younger than 70 years, potentially eligible for allo-HSCT, recruited from multiple Italian centers and the Mayo Clinic.[67] The MIPSS70-plus score additionally included unfavorable karyotype defined by any abnormal karyotype other than normal karyotype or sole abnormalities of 20q-, 13q-, +9, chromosome 1 translocation/duplication, -Y, or sex chromosome abnormality other than -Y.

Table 4 Revised IPSET-Thrombosis Model for Essential Thrombocythemia

Variables	Risk Categories	Therapy
Age ≤ 60 years old Prior thrombosis ***JAK2*V617F mutation**	Very low (Age ≤ 60 years, ***JAK2* wild type**, no prior thrombosis)	Management of CV risk factors, observation or low dose aspirin, unless contraindicated[a]
	Low (Age ≤ 60 years, ***JAK2*V617F positive**, no prior thrombosis)	Management of CV risk factors and low dose aspirin unless contraindicated[a]. Higher dose aspirin may be used if CV risk factors present.
	Intermediate (age > 60 years, ***JAK2* wild type**, no prior thrombosis)	Management of CV risk factors and cytoreductive therapy plus low-dose aspirin, unless contraindicated[a]. Higher dose aspirin without cytoreductive therapy if no CV risk factors.
	High (age > 60 years and ***JAK2*V617F positive**, or prior thrombosis)	Management of CV risk factors and cytoreductive therapy plus low-dose aspirin

Notes: [a]Aspirin is contraindicated in the presence of acquired von Willebrand's disease or active major bleedings. In bold molecular variable. Data from Barbui et al.[51]

Abbreviations: IPSET, International Prognostic Score for Essential Thrombocythemia; CV, cardiovascular.

Table 5 Clinical-Molecular Prognostic Scores in Polycythemia Vera and Essential Thrombocythemia

Prognostic Score	Variables (Points)	Risk Categories (Points)	Median Survival (Years)
MIPSS-PV Tefferi et al[55]	Leukocyte count ≥15 x 10^9/L (1) Thrombosis history (1) Age >67 years (2) ***SRSF2* mutation (3)**	Low (0–1) Intermediate (2–3) High (4–7)	24 13.1 3.2
MIPSS-ET Tefferi et al[55]	Leukocyte count ≥11 x 10^9/L (1) Age >60 years (4) Male sex (1) ***SRSF2, SF3B1, U2AF1* and *TP53* mutation (2)**	Low (0–1) Intermediate (2–5) High (6–8)	34.3 14.1 7.9

Note: In bold molecular variables.

Abbreviations: MIPSS, Mutation-Enhanced International Prognostic Scoring System, PV, polycythemia vera; ET, essential thrombocythemia.

Table 6 Clinical-Molecular Prognostic Scores in Myelofibrosis

Prognostic Score	Variables (Points)	Risk Categories (Points)	Median Survival (Years)
MIPSS70 Guglielmelli et al[67]	Hemoglobin < 10 g/dL (1) Blasts > 2% (1) Constitutional symptoms (1) Leukocytes > 25 x 10^9/L (2) Platelet count < 100 x 10^9/L (2) BM fibrosis ≥ 2 (1) **Non *CALR* type-1 (1)** **HMR[a] = 1 (1)** **HMR[a] ≥ 2 (2)**	Low (0–1) Intermediate (2–4) High (5–12)	27.7 7.1 2.3
MIPSS70 plus Guglielmelli et al[67]	Hemoglobin < 10 g/dL (1) Blasts > 2% (1) Constitutional symptoms (1) **Non *CALR* type-1 (2)** **HMR[a] = 1 (1)** **HMR[a] ≥ 2 (2)** **Unfavourable karyotype[b] (3)**	Low (0–2) Intermediate (3) High (4–6) Very high (7–11)	20.0 6.3 3.9 1.7
MIPSS70 plus v2.0 Tefferi et al[68]	Hemoglobin 8–10 g/dL (1) Hemoglobin <8 g/dL (2) Blasts > 2% (1) Constitutional symptoms (2) **Non *CALR* type-1 (2)** **HMR[a]+*U2AF1* Q157 = 1 (2)** **HMR[a]+*U2AF1* Q157 ≥ 2 (3)** **HR Karyotype[c] (3)** **VHR Karyotype[d] (4)**	Very low (0) Low (1–2) Intermediate (3–4) High (5–8) Very high (9–14)	Not reached 10.3 7 3.5 1.8
GIPSS Tefferi et al[72]	**Non *CALR* type-1 (1)** ***ASXL1* mutation (1)** ***SRSF2*mutation (1)** ***U2AF1*Q157 (1)HR karyotype[c] (1)** **VHR karyotype[d] (2)**	Low (0) Intermediate-1 (1) Intermediate-2 (2) High (3–6)	26.4 8.0 4.2 2.0
MYSEC-PM Passamonti et al[74]	Hemoglobin < 11 g/dL Blasts ≥ 3% Platelets < 150 x 10^9/L Constitutional symptoms (2) Age at secondary MF (0.15 point/year) ***CALR* unmutated genotype (2)**	Low (<11) Intermediate-1 (11- <14) Intermediate-2 (14- <16) High (≥ 16)	Not reached 9.3 4.4 2.0
MTSS Gagelmann et al[75]	Platelets < 150 x 10^9/L (1) Leukocytes > 25 x 10^9/L (1) Karnofsky PS < 90% (1) Age ≥ 57 years (1) HLA-mismatched unrelated donor (2) **Non *CALR*/*MPL* mutation (2)** ***ASXL1*mutation (1)**	Low (0–2) Intermediate (3–4) High (5) Very high (6–9)	5-years OS 83% 5-years OS 64% 5-years OS 37% 5-years OS 22%

Notes: [a]High molecular risk (HMR) include *ASXL1, SRSF2, EZH2, IDH1/2*. [b]Unfavourable karyotype defined any abnormal karyotype other than normal karyotype or sole abnormalities of 20q2, 13q2, +9, chromosome 1 translocation/duplication, -Y, or sex chromosome abnormality other than -Y. [c]High risk (HR) karyotype include all the abnormalities that are not VHR and favourable (normal karyotype or sole abnormalities of 20q−, 13q−, +9, chromosome 1 translocation/duplication or sex chromosome abnormality including -Y). [d]Very high risk (VHR) include single or multiple abnormalities of −7, inv (3), i(17q), 12p−, 11q−, and autosomal trisomies other than +8 or +9. In bold molecular variables.
Abbreviations: MIPSS, Mutation-Enhanced International Prognostic Scoring System; GIPSS, Genetically Inspired Prognostic Scoring System; MYSEC-PM, Myelofibrosis Secondary to PV and ET-Prognostic Model; MTSS, Myelofibrosis Transplant Scoring System; OS, overall survival.

Leukocytosis and BM fibrosis grade ≥2 were included in MIPSS70 but not retained in MIPSS70-plus. Both models predict LFS and OS, but MIPSS70-plus seemed to have the best performance in identifying a very high-risk category of patients, 23% of whom developed acute leukemia, due to additional cytogenetic abnormalities. Further revision, the MIPSS70 v2.0,[68] was published incorporating a new HMR mutation *U2AF1*Q157.[69] Additional refinement of risk categories was provided with different thresholds for anemia (defined severe by hemoglobin levels of <8 g/dL in women and <9 g/dL in men, and moderate by hemoglobin levels of 8 to 9.9 g/dL in women and 9 to 10.9 g/dL in men)[70] and a new three-tiered cytogenetic risk distribution with the introduction of very high risk (VHR) group for those with single/multiple abnormalities of −7, i(17q), inv(3)/3q21, 12p-/12p11.2, 11q-/11q23, or other autosomal trisomies not including + 8/+ 9.[71]

These updates were also used for the development of a Genetically Inspired Prognostic Scoring System (GIPSS) that is exclusively based on genetic (*ASXL1, SRSF2, U2AF1*Q157, absence of type-1 *CALR* mutations) and cytogenetic markers.[72] The authors demonstrated the non-inferiority of GIPSS in discrimination ability and prediction accuracy, compared to MIPSS70-plus and DIPSS. These data were confirmed in an external validation cohort showing also that GIPSS score performs equally well for both primary and secondary myelofibrosis.[73] Primarily, all these scores were developed exclusively for patients with a diagnosis of PMF and their performance in patients with secondary MF was suboptimal. Therefore, a specific Myelofibrosis Secondary to PV and ET-Prognostic Model (MYSEC-PM) was developed. This four-tiered score includes the following variables: hemoglobin level, circulating blasts, platelet count, age, presence of constitutional symptoms and *CALR* mutational status.[74] Finally, to overcome the shortcoming of clinical scores for MF in the setting of HSCT and to accurately predict outcome following transplantation, the Myelofibrosis Transplant Scoring System (MTSS) was recently formulated for patients with PMF and post-PV-ET MF. The score variables incorporated were age (≥57 years), Karnofsky performance status, platelet and leukocyte count prior to transplantation (<150x10^9/L and >25x10^9/L, respectively), HLA-mismatched unrelated donor, *CALR*/*MPL* mutation and *ASXL1* mutational status.[75] The last to be developed, based on sequencing effort of 69 genes in more than 2000 patients with MPN, including 309 with myelofibrosis, identified a prognostic role for *CBL, NRAS, RUNX1, TET2* and *P53* and a contribution from *GNAS, IDH2* and *U2AF1* in both overall survival and leukemia-free survival. This study integrated a remarkable number of demographics, clinical and genomic variables and through the random effects modelling, led to the creation of a personalized predictive individual model for disease transformation and death at each phase into a single multi-stage model.[46] A prognostic calculator of individualized patient outcome is available online (https://jg738.shinyapps.io/mpn_app/). An interactive web application is similarly available for MYSEC-PM (http://www.mysec-pm.eu/) and MIPSS70 (http://mipss70score.it). Table 6 provides a comprehensive overview of the clinical-molecular prognostic scoring systems described.

Clinical and Therapeutic Implications

Since *JAK2*V617, *MPL* and *CALR* mutations are driver abnormalities of MPN through activation of the JAK-STAT signaling, developing JAK inhibitors (JAKi) has raised a great interest. Ruxolitinib was the first JAK1/2 inhibitor that received approval in myelofibrosis based on COMFORT I/II clinical trials.[76,77] Ruxolitinib was effective in reducing splenomegaly and alleviating constitutional symptoms, with possible effects on survival. Long term follow-up studies suggested a reduction in allele burden, with rare cases of molecular remission.[78,79] Subsequently, ruxolitinib was tested in PV patients resistant or intolerant to hydroxyurea, according to European Leukemia-Net (ELN) criteria,[80] in RESPONSE[81] and RESPONSE-2[82] studies in patients with and without splenomegaly, respectively. A progressive decline in *JAK2* mutant burden in patients treated with ruxolitinib was seen in RESPONSE trial, although without definite clinical correlation.[83] Therefore, this provides a challenge to the use of *JAK2* allele burden reduction as a marker of treatment efficacy. Moreover, one small study highlighted that MF patients starting with a higher allele burden may benefit the most from ruxolitinib, showing a higher probability of spleen response if allele burden was greater than 50% at entry.[84] It is important to underline that ruxolitinib targets the kinase domain of JAK1/2 kinases, without a greater affinity against mutant *JAK2*; this explains the clinical efficacy also in *JAK2* negative patients, with similar response rates. Unfortunately, most patients with myelofibrosis on ruxolitinib will become resistant to therapy with the progression of symptoms and splenomegaly, worsening cytopenias, or evolution to BP; in the COMFORT-

II study, responding patients had a < 50% chance of maintaining response at 5 years.[78] Intriguingly, as reported by Newberry et al,[85] clonal evolution after ruxolitinib discontinuation, defined by the acquisition of at least one additional mutation, was reported in 35% of patients. Mutations mostly occurred in *ASXL1*, followed by *TET2, EZH2* and *TP53*. Moreover, overall survival after ruxolitinib discontinuation was shorter for patients with clonal evolution compared with the others (6 *vs* 16 months). One drawback of this study was the lack of a control cohort of patients.[86] A subsequent study which included 25 MF patients treated with hydroxyurea as a control group confirmed these data but also demonstrated that clonal progression is independent of the treatment.[87] However, acquisition of new mutations under ruxolitinib has important clinical correlates, since it is associated with a higher rate of discontinuation due to resistance to treatment and death.

Allo-HSCT remains the only therapeutic approach that can modify the natural history of MF, but it is associated with a relevant morbidity and mortality and only a minority of patients is eligible for such an intensive procedure. As a consequence, research aimed at the discovery of more effective drugs, also in the context of novel JAKi, is extremely active. In recent years, Interferon-α (IFNα), especially better tolerate pegylated forms (Peg-IFNα), has emerged as a promising approach in MPN, particularly in PV and ET, with high rates of both hematological and molecular remissions.[88–92] In this respect, monopegylated Ropeginterferon alpha-2b (Ropeg) has received approval in Europe as a first-line therapy for PV patients following the demonstration of its superior efficacy over hydroxyurea in the PROUD/CONTINUATION-PV trial.[92] The treatment appears to selectively target the mutant *JAK2* clone, as suggested by the high rate of reduction of *JAK2*V617F allele burden compared to *CALR* positive ones.[93] As recently reported, enhanced sensitivity of *JAK2*V617F mutated cells to IFNα was related to high expression and phosphorylated levels of STAT1.[94] Interestingly, the presence of concomitant mutations is associated with smaller mean decreases in *JAK2*V617F allele burden under Peg-IFNα treatment.[89] IFNα may have decreased ability to eradicate *TET2* clones even in cases where *JAK2*V617F-mutant clone is markedly reduced, indirectly suggesting that the genomic landscape of MPN patients can predict responses to treatment.[95] However, Peg-IFNα used at a higher dose in a cohort of 31 *CALR* positive ET patients, induced hematologic responses in all patients and median *CALR* allele burden decreased from 41% to 26%, with two patients achieving complete molecular remission.[96] In the latter study, decreases in *CALR* variant allele frequency correlated with laboratory parameters of disease burden including platelets and white blood cell counts, hemoglobin, and lactate dehydrogenase. Similar to findings in *JAK2* mutated patients, the presence of additional mutations such as *TET2, ASXL1, IDH2*, and *TP53* was associated with poorer molecular responses. These responses seem to be also durable, which is an appealing aspect of IFNα treatment. In a long-term 83-month follow up of ET and PV patients treated with Peg-IFNα, median duration of hematologic and molecular response was 66 and 53 months, respectively.[97] In addition, three patients had a complete molecular remission even after discontinuation of therapy, although in most patients, *JAK2*V617F allele burden increased after the first 2 years of treatment stop.

Imetelstat, a 13-mer lipid-conjugated oligonucleotide that targets the RNA template of human telomerase reverse transcriptase, was tested in MF and ET. A pilot study evaluated the efficacy and safety in 33 patients with intermediate-2 and high-risk MF according to DIPPS-plus score. A complete or partial response was observed in seven patients and responses correlated with the presence of *JAK2*V617F, *SF3B1* or *U2AF1* mutations and the absence of *ASXL1* mutations.[98]

Conversely, in 18 ET patients treated with imetelstat, a partial molecular response was detected in seven of eight *JAK2*V617F mutated patients. The median *JAK2*V617F mutant allele burden was reduced by 71% at 3 months after the initiation of treatment. *MPL* and *CALR* mutant allele burdens were also reduced, by 15% to 66%.[99] Additional mutations significantly reduced the depth of response and had an impact on the duration of response. Of acquired mutations with known adverse prognosis, *ASXL1, EZH2* and *U2AF1* mutations were responsive to imetelstat, while *SF3B1* and *TP53* mutations persisted.[100]

Mutations in *IDH1/2* and *TP53* are enriched in BP. These molecular findings may have clinical implications given the role of ivosidenib and enasidenib (anti-IDH1 and IDH2, respectively) in patients with relapsed/refractory *IDH*-mutated AML[101,102] and high response rate of *TP53*-mutated AML to 10-day decitabine.[103] Another promising option is venetoclax, a BCL2 inhibitor that can be used in combination with low-dose cytarabine or hypometilating agents. In a recent trial for patients with AML ineligible for standard induction chemotherapy, azacytidine plus

venetoclax was superior to azacytidine alone, also in the subset of *IDH1/2* and *TP53* mutated patients.[104] Although these therapies are promising, patients with MPN BP have a dismal outcome without allo-HSCT. Further studies are needed, but these target therapies may represent a valid therapeutic approach also as a bridge to transplantation, given the frequent refractoriness to conventional chemotherapies of MPN BP patients.

The presence of acquired mutations in the driver and/or myeloid genes in the most part of MF patients offers the opportunity to use these markers as indicators of minimal residual disease (MRD) after allo-HSCT. In some studies, the persistence of *JAK2*V617F mutation after allo-HSCT was associated with a higher incidence of relapse and a shorter overall survival.[105,106] More recently, in a series of 136 patients, Wolschke et al demonstrated that patients with detectable *JAK2, MPL* or *CALR* mutation at either day +100 or day +180 after allo-HSCT had a significantly higher risk of relapse at 5 years compared to those in molecular remission (62% vs 10%, and 70% vs 10%, respectively).[107] Based on these studies, monitoring of molecular MRD in MF patients after allo-HSCT is strongly recommended.

Conclusions

The discovery of *JAK2, CALR* and *MPL* driver mutations elucidated the genetic basis of MPN although some patients, so-called triple negative, while having clinical and histological characteristics of MPN, remain molecularly mute. The introduction of high-throughput NGS techniques expanded the mutational landscape and further raised pathophysiological knowledge. Furthermore, these molecular information let to refine the diagnosis, attribute better prognosis score, and monitor the response to treatments. The development of JAKi offered new hopes to patients with MPN allowing them to achieve significant advances in the control of symptoms and quality of life but are largely insufficient to cure the diseases and prolong survival. The last unmet needs regard the understanding of molecular mechanisms at the basis of loss of response to JAKi and the identification of new molecular abnormalities suitable for target therapy.

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