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REVIEW
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The Role of New Technologies in Myeloproliferative Neoplasms

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The hallmark of *BCR-ABL1*-negative myeloproliferative neoplasms (MPNs) is the presence of a driver mutation in *JAK2*, *CALR,* or *MPL* gene. These genetic alterations represent a key feature, useful for diagnostic, prognostic and therapeutical approaches. Molecular biology tests are now widely available with different specificity and sensitivity. Recently, the allele burden quantification of driver mutations has become a useful tool, both for prognostication and efficacy evaluation of therapies. Moreover, other sub-clonal mutations have been reported in MPN patients, which are associated with poorer prognosis. *ASXL1* mutation appears to be the worst amongst them. Both driver and sub-clonal mutations are now taken into consideration in new prognostic scoring systems and may be better investigated using next generation sequence (NGS) technology. In this review we summarize the value of NGS and its contribution in providing a comprehensive picture of mutational landscape to guide treatment decisions. Finally, discussing the role that NGS has in defining the potential risk of disease development, we forecast NGS as the standard molecular biology technique for evaluating these patients.

Keywords: *BCR-ABL1*-negative myeloproliferative neoplasms (MPNs), myelofibrosis (MF), *JAK2* mutations, calreticulin (*CALR*), *MPL* (W515K/L), *ASXL1* mutation, High molecular risk (HMR) mutations, next generation sequencing (NGS)

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INTRODUCTION

Myeloproliferative neoplasms (MPNs) are clonal disorders of the hematopoietic stem cell, mainly characterized by proliferative bone marrow with varying degrees of reticulin/collagen fibrosis, extramedullary hematopoiesis, abnormal peripheral blood count, and constitutional symptoms that are secondary to abnormally expressed inflammatory cytokines (1). Among the so-called "*BCR-ABL1*-negative MPNs" polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) are included. However, unlike chronic myeloid leukemia (which is always characterized by the *BCR–ABL1* fusion gene), they have not yet been associated with any specific genetic abnormalities.

The discovery in 2005 of the *JAK2*V617F point mutation (2–5) and the subsequent identification of other specific abnormalities, such as *JAK2* exon 12 (6, 7), *MPL* exon 10 (8–10) and *CALR* exon 9 (11, 12), gave an improvement in understanding their genetic basis. All of them are now included in the molecular diagnostic and prognostic algorithms for MPNs, leading to several revisions of the diagnostic criteria for these diseases. In addition, they are used as markers of disease burden and as

a measure of assessing response to various therapeutic interventions that can target the mutant clone.

The MPN driver mutations are often mutually exclusive and, after the detection of the common *JAK2*V617F mutation, generally no further testing is performed. Nevertheless, in recent years, several reports have suggested that driver mutations indeed do rarely coexist (13–16), but additional studies are needed to clarify the clinical implications of double-mutated cases.

Importantly, additional sub-clonal driver and non-driver mutations in genes such as *ASXL1*, *SRSF2*, *EZH2*, *IDH1*, and *IDH2*, among others, have been identified as being associated with disease progression (17, 18). A wide choice of techniques is currently available for the detection of MPN mutations, and a continuous evolution of molecular diagnostic applications and platforms is now ongoing.

JAK2

JAK2 is a non-receptor tyrosine kinase, which, upon ligand binding to specific cytokine receptors, is phosphorylated and activated, leading to regulation of gene expression involved in cell proliferation and survival. The *JAK2*V617F mutation is a G to T somatic mutation at nucleotide 1849 in exon 14, resulting in the substitution of valine to phenylalanine at codon 617, which triggers constitutive activation of downstream signaling and uncontrolled cell growth (**Figure 1A**).

V617F mutation in exon 14 of *JAK2* gene is present in the majority of patients with PV (more than 95%) and in 60% of those with ET or PMF (2–5). Rare insertions and deletions in exon 12 have been described in 2 to 3% of patients with PV (6, 7) (**Figure 1A**). The most widely used method for *JAK2*V617F detection is based on allele-specific PCR (2). Quantitative PCR methods (qPCR) are preferred over qualitative ones because of greater reproducibility and sensitivity and because of the need of quantifying the mutated clone in MPN patients.

Actually, V617F allelic burden at diagnosis provides important prognostic information, being found to be associated with phenotypic presentation and severity of MPNs (19–21), the risk of thrombotic events (20, 22) and progression to secondary myelofibrosis (MF) (23, 24). In particular, in PMF patients *JAK2*V617F mutation is associated with clinical characteristics which include older age, higher hemoglobin level, leukocytosis, and lower platelet count (20; (25) and a low *JAK2*V617F allele burden may represent a favorable prognostic factor (26). With regards to PV, a higher *JAK2*V617F mutant allele burden has been associated with more frequent thrombotic complications (20), pruritus and fibrotic transformation (27). Moreover, V617F allelic burden measured during the follow-up is currently used in the course of treatment with alpha-interferon and *JAK1/2* inhibitors (28–30), as well as for minimal residual disease (MRD) evaluation after allogeneic stem cells transplantation (31). In fact, although the ideal therapy should be able to eradicate the malignant MPN clone, this aim has not been reached with the current available treatments in contrast to the striking efficacy of tyrosine kinase inhibitor monotherapy in chronic myeloid leukemia (32).

Several quantitative methods have been developed in the years, most of them based on real-time allele-specific PCR, with sensitivity ranging from 0.05 to 1%, and specificity of 100% (33–36). Digital PCR (dPCR) has also been proposed with the aim to achieve an absolute quantification of the target gene without the need for a standard curve, with comparable or higher performances compared to qPCR (37–39). Recently, Next Generation Sequencing (NGS) has been shown to allow the detection of the V617F mutation with comparable performances, but weaker sensitivity to qPCR, with the advantage of detection of new potentially pathogenic *JAK2* variants (40).

Regarding *JAK2* exon 12 variants detection, different approaches can be adopted; Sanger Sequencing (SS) is the most frequently used because of the rarity and heterogeneity of these mutations (41, 42). Nested High-Resolution Melting (HRM) curve analysis has been proposed as highly sensitive screening method eventually followed by SS for the precise characterization of the mutation (36, 43).

MPL

The myeloproliferative leukemia virus oncogene (*MPL*) is located on chromosome 1p34 and encodes for the thrombopoietin (TPO) receptor, thus assuming a crucial role in the regulation of megakaryocyte growth and survival. In 2006, a somatic activating mutation in exon 10 of this gene, *MPL*W515L, was described in *JAK2*V617F-negative ET/PMF (44, 45). This mutation is characterized by a G to T transition at nucleotide 1544, resulting in a tryptophan to leucine substitution at codon 515 of the transmembrane region of *MPL*, inducing constitutive activation of the TPO receptor in a cytokine-independent fashion (**Figure 1B**).

Activating mutations in *MPL* are reported in ∼5–10% of all PMF patients and 1–4% of those with ET (44–46). All of them cluster in exon 10 and in the majority of cases affect a tryptophan in position 515 (W515L, W515K, W515A, and W515R).

The methods applied for the detection of *MPL* mutations can be grouped according to different strategies: targeted identification of specific mutations or sequencing of the entire exon 10. Targeted analysis include allele-specific PCR, allele-specific qPCR and Amplification Refractory Mutation System (ARMS)-PCR; among them qPCR displays the highest sensitivity (0.1–0.5%) (8, 9, 47–49). A multiplexed allele-specific PCR assay for the four most frequent *MPL* exon 10 mutations (W515L, W515K, W515A, and S505N) has been developed, with 100% specificity and 2.5% sensitivity (50). Analysis of the entire exon 10 allows the detection of all the known and potentially new mutations and can be achieved by SS, pyrosequencing or HRM curve. The sensitivity of these approaches is lower, reaching 2–5% for the latter method (10, 51).

Calreticulin

Calreticulin (*CALR*) mutations were reported for the first time at the end of 2013 (11, 12). These mutations represent the second most common genetic abnormality in MPNs after *JAK2*V617F, even though they are absent in PV patients. On the other hand, *CALR* mutations partially addressed the molecular gap in *JAK2*/*MPL*-unmutated ET and PMF patients, accounting for 20–25% of the overall somatic mutations. The remaining cases (i.e.,

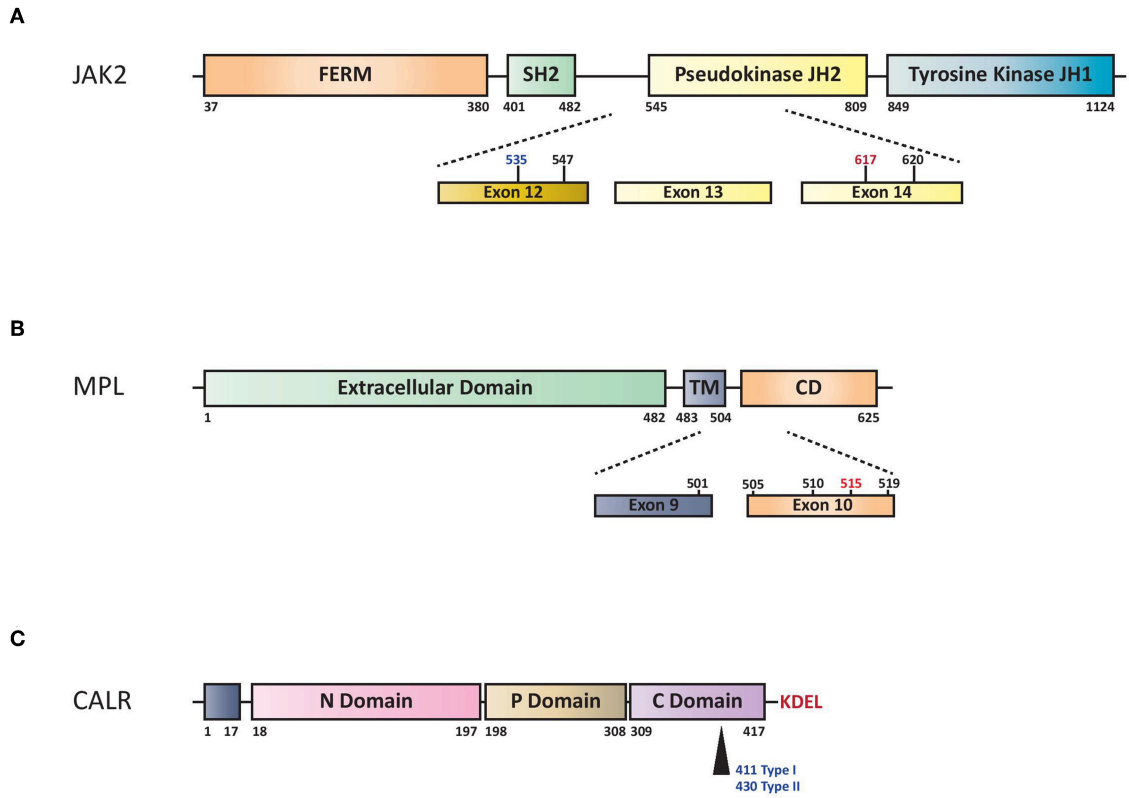

FIGURE 1 | (A) Schematic representation of structural domains of the *JAK2*. FERM indicates 4.1 Ezrin, Radixin, Moesin domain; JH1 and JH2 signal (JAK Homology 1 and 2) domains refer to tyrosine kinase and pseudo-kinase domains, respectively. SH2 indicates Src Homology 2 domain. Numbers represent amino acid positions within the *JAK2* protein: red indicates V617F mutation, in black other SNP mutations and in blue the region for insertions/deletions. **(B)** Structural domains and mutations of the MPL. Extracellular domain indicates NH2 amino terminal region, TM transmembrane domain and CD cytoplasmic domain. Dashed lines highlights exons reported mutations of MPL. W515L or W515K are indicated in red while the others hot spots variant mutations in black. Numbers represent amino acid positions. **(C)** Structural domains of CALR protein. CALR includes NH2 domain (N-Domain) and Proline-rich domain (P-Domain) with chaperone lectin-like function. COOH domain (C Domain) has aminoacids responsible for Ca^{++}-buffering. Arrow indicates Type I (deletion) or Type II (insertion) alterations that determine loss of KDEL aminoacids and generation of a new tail with low calcium buffering.

negative for *JAK2*, *CALR*, and *MPL*) are termed "triple-negative," representing the 5–10% of all *BCR-ABL1*-negative MPN patients.

CALR is a multi-functional Ca2+ binding protein with chaperone activity mainly localized in the endoplasmic reticulum. The localization and retention of *CALR* are defined by the N-terminal signal sequence and the C-terminal ER-retention sequence KDEL (**Figure 1C**). It is involved in numerous intracellular (cytoplasm and nucleus), cell surface, and extracellular functions such as protein quality control, calcium metabolism, immune response, phagocytosis, cell adhesion and others (52). *CALR* mutations were phenotypic drivers in the pathogenesis of MPNs (53). Recently, studies concerning the role of *CALR* mutated proteins demonstrated that they are able to bind to the *MPL* receptor inducing JAK-STAT activation and the positive aminoacid charge of the mutant C terminus is required to mediate this interaction (54–57).

More than 50 different *CALR* mutations have been described so far, with type 1 (L367fs*46) resulted from 52-bp deletion and type 2 (K385fs*47) from 5-bp TTGTC insertion accounting for ∼80% of all the cases. More type 1 (53%) than type 2 (32%) abnormalities are found in MPN patients (11), whereas the remaining cases are classified as either type 1-like, type 2-like, or "other type," based on their structural similarities to the classical mutations. Their distribution is 57% type 1(-like) and 39% type 2(-like) in ET and 83% type 1(-like) and 15% type 2(-like) in PMF (58). All these abnormalities frequently consist of insertions or deletions involving exon 9 of the gene, generating a frameshift to a unique alternative reading frame; it results in a novel C-terminus peptide sequence enriched for positively charged residues. Furthermore, the mutated protein lacks the KDEL signal, leading to a partial dislocation of *CALR* from the endoplasmic reticulum (11).

In PMF patients the favorable prognostic impact is limited to *CALR* type 1/type 1-like mutations, whereas type 2/type 2-like are associated with a worse prognosis, similar to that of *JAK2*-positive patients (59). On the contrary, in ET *CALR* type 1 and type 1-like mutations are associated with an higher risk of MF transformation (58).

As previously published for *JAK2* mutations, association between *CALR* mutation allele burden and disease phenotype has been reported (60, 61). In particular, an association between *CALR* allele burden, leukocyte and platelet counts, hemoglobin and lactate dehydrogenase levels was described. Furthermore, the median *CALR* allele burden remained steady over time; interestingly, differently from *JAK2*-positive cases, acute myeloid leukemias (AML) evolving from *CALR*-mutant MPNs commonly maintained their mutational profile (61). Additional studies are needed to better clarify the *CALR* mutant allele burden clinical implications.

Given their high frequency in MPNs, screening for *CALR* mutations is recommended in all *JAK2*V617F-negative patients with suspected ET or PMF (36, 62, 63). Several methods have been proposed to detect *CALR* mutations, including SS, fragment length analysis, real-time qPCR, HRM, ddPCR, pyrosequencing and NGS (64–69). Due to the heterogeneity of *CALR* insertions and deletions detected, direct SS is considered to be the primary method of testing. Indeed, SS of exon 9, providing specific sequence change information, allowed to identify the exact type of *CALR* mutation and detailed procedures, described in previous publications, were the most used conditions in diagnostic routine screening laboratories.

Role of Next-Generation Sequencing in MPNs

Nowadays, NGS has played an important role in understanding the genetic alterations of different human cancers. There are several number of available NGS platforms using different sequencing technologies, which perform sequencing of millions of small fragments of DNA in parallel (70). Nevertheless, different sequencing chemistry and methods for signal detection, the obtained results are comparable. Bioinformatics analyses are used to piece together these fragments by mapping the individual reads to the human reference genome.

This method provides several advantages compared to different sequencing methods. First of all, NGS is a high-throughput method as it detects concomitant mutations in the same run. Then, the analysis requires low input of DNA/RNA sample as compared to traditional sequencing methods (e.g., SS or Pyrosequencing). Moreover, NGS discriminates genomic aberrations, which are screened simultaneously, such as single/multiple nucleotide variants (SNVs), small and large insertions and deletions (ins/dels) and copy-number variations (CNV) with high sensitivity and accuracy, so reducing data analysis and clinical reporting time (**Table 1**) (70).

In the last few years, NGS have been applied to many hematological disorders such as for establishing T-cell clonality, recurrent cytogenetic translocations and prognosis of Philadelphia chromosome-positive acute lymphoblastic leukemia (ALL) (70). In addition, NGS approaches can be used in diagnostic samples to understand the possible evolution of MRD in clonal *IGH* and *TCR* rearrangements in lymphoproliferative disorders (71). A comparison study between the most common technologies used to lymphoproliferative disorders, underlined that NGS method was able to identify mutant or clonal DNA in few tumor circulating cells. For this reason, NGS may be used to improve MRD in post-therapy monitoring (72).

In MPNs, it was demonstrated that *JAK2* V617F allele burden identified patients with different clinical course: in PV and in ET, variant allele frequency of *JAK2* V617F, was associated with a higher ratio of fibrotic transformation. In patients in PMF high variant allele frequency was correlated to recurrent thrombosis event, while low variant allele frequency was associated to short leukemia-free survival and overall survival, probably due to acquisition of additional driver mutations. In fact, new NGS panels are used as predict methods to describe alterations of several genes involved in transcriptional regulation and cell signaling pathways (**Table 2**). These information can be used to predict the risk leukemic transformation, to guide target treatment and to speculate a personalized clinical trial (32, 73, 84). Using the high sensitivity and specificity of NGS technology, several groups analyzed and deeply studied the genetic and molecular profile of MPN subjects, with the aim of assessing not only non-driver mutations, but also infrequent variants of driver mutations (**Table 2**). In MPN patients negative for *JAK2*, *CALR*, and *MPL* (triple-negative, TN), mutations in *LNK, TET2, DNMT3A, IDH1/2, CBL,* and *ASXL1* genes but also atypical mutation in *MPL* (S204P) have been identified (83, 85). In this contest, Chang *et al.* identified 30 missense mutations in 12 of 16 triple-negative MPN patients. In particular, in this study NGS platform was able to identify low allelic burden and atypical mutations of *JAK2* (*JAK2*V626F and *JAK2*F556V) and *MPL* (*MPL*S204P and *MPL*Y591N) (86).

Furthermore, Cabagnols et al. noticed that an atypical mutation of *MPL* (*MPL*S204P) was associated with other alterations in *ASXL1* and *SRSF2* genes. The subjects with more than one alteration were classified as myelodysplastic syndrome (MDS) with thrombocytosis rather than ET. The *MPL*S204P is a weak gain-of-function mutation that induces constitutive STAT activation and more prolonged ligand-induced STAT phosphorylation than wild-type *MPL*, while *MPL*Y591N is associated with *MPL*W515A and in a mouse model induced a more aggressive MPN behavior than that associated with a single *MPL*Y591N mutant (87).

Additionally, applying NGS methodology on a group of 197 MPN patients, Lundberg *et al.* observed several alterations concerning genes involved in DNA methylation (*TET2, DNMT3A, IDH1*) and chromatin structure (*EZH2, ASXL1)* regulation. Moreover, they analyzed the impact of somatic mutations on clinical outcomes and found that the concomitant somatic mutations in *TP53* or *TET2* were correlated to both reduction of overall survival (OS) and increased risk of leukemic evolution (88). Also Agarwal et al., using a customized 26-gene NGS panel in a series of 171 MPN patients, highlighted that alterations in *ASXL1, EZH2,* and *IDH1/2* were associated with an increasing risk of disease progression and a shorter OS in both ET and PMF patients. In this work the authors found that among *JAK2*-mutated patients, 88% of subjects presented a single *JAK2* mutation while the remaining 12% showed additional mutations in *TET2*, *ASXL1,* and *SF3B1* genes.

Finally, very recently Grinfeld *et al.* (89) sequencing coding exons from 69 myeloid cancer genes in a large and

TABLE 1 | Comparison of Real Time PCR, Digital PCR, SS, and NGS technologies in clinical molecular diagnostics.

	Benefits	Critical points	Sensitivity
Real time PCR	Detection of known mutations Validated Methods	High input of DNA/RNA No simultaneous screening of multiple genes in multiple samples	1%
Sanger sequencing	Detection of known and unknown mutations Validated methods	High input of DNA/RNA	10–20%
Digital PCR	Low input of DNA/RNA Detection of known mutations Cost-effective for rapid genotyping e monitoring	No simultaneous screening of multiple genes in multiple samples	0.1–1%
NGS	Low input of DNA/RNA Massively parallel sequencing Decreased sequencing cost/gene Detection of known and unknown mutations Simultaneous screening of multiple genes in multiple samples	Validation studies require High-complexity workflow and analyzing results Genome data analysis is time-consuming	1%

PCR, Polymerase Chain Reaction; SS, Sanger Sequencing; NGS, Next Generation Sequencing.

representative series of 2035 MPN patients, identified different genetic subgroups with distinct clinical phenotypes, including blood counts, risk of leukemic transformation, and event-free survival. Integration of 63 clinical and genomic variables allowed the authors to develop a prognostic model (https://cancer.sanger.ac.uk/mpn-multistage/) capable of generating personally tailored predictions of clinical outcomes in MPN patients, even within individual categories of existing prognostic schemas. In particular, they identified a first subgroup with *TP53* disruption or aneuploidy characterized by a poor outcomes and high risk of transformation to AML; a second subgroup with chromatin or spliceosome mutations that showed an increased risk for transformation to MF and shorter event-free survival. Patients who are not included in these two subgroups were defined according to their dominant phenotypic driver mutation and were the following: patients with *CALR*, *MPL*, heterozygous *JAK2*, and homozygous *JAK2* or *NFE2* mutations. The remaining two subgroups included instead patients with no detectable driver mutations and those with additional driver mutations not identified in the other six subgroups. Thus, the model provides considerable discriminatory power that accurately generalizes to other real-world cohorts.

Focusing on *CALR*-mutated cases, 56/62 (90%) of subjects showed single mutation in *CALR* gene while 10% had additional mutations in *TET2* and *ASXL1* (*CALR* type 1 mutations were more represented in this cohort of patients than type 2 mutations). On the contrary, no additional mutations were noticed in *MPL*-mutated patients (90). In addition, Song *et al.* analyzed 135 MPN patients by NGS and found that *JAK2*, *ASXL1,* and *TET2* were frequently mutated in PMF, PV, and ET; interestingly, the comparison between mutational and cytogenetic profiles identified a possibility role in triaging and guiding different treatments (91).

Different studies have demonstrated the utility of NGS technologies to analyze the genetic profiles in AML patients. This technique is applied not only to study primary but also secondary AML, a heterogeneous group of disorders arising from MDS or MDS/MPN. In fact, secondary AMLs are characterized by several cytogenetic abnormalities and genetic alterations. In particular, Hussaini et al. (92), examining 187 subjects with a diagnosis of AML, highlighted the frequency of related-gene mutations, where *ASXL1* was the highest mutated gene, followed by *TET2, RUNX1, DNMT3A, TP53, IDH2, NRAS, FLT3,* and *NPM1*. Moreover, NGS analysis identified co-mutated genes (*ASXL1* with *RUNX1* or *TET2* or *NRAS*) and was able to discriminate somatic mutations associated with MDS/AML.

The prognostic impact of certain co-occurring mutations has been associated with MPNs disease progression as well as with the development of secondary AML (74, 93). Accordingly, the value of NGS at present lays also in the risk stratification of leukemic evolutions, in particular in critical and difficult cases, thus guiding treatment decision. In this respect, in PMF patients *ASXL1* seems to be the worst sub-clonal mutation (25, 45). Indeed, the presence of this mutation or any other among *SRSF2* and *IDH1/2* gives a High Molecular Risk (HMR) to the patient and is included in the Mutation-Enhanced International Prognostic Score System (MIPSS70) and MIPSS70-plus, where the number of HMR mutations is also weighted together with driver mutation status and clinical data. The presence of two or more HMR mutations has been associated with highly adverse prognosis and rapid leukemic transformation (75, 76). The association of HMR and worse prognosis has also been confirmed in a retrospective series of elderly MF patients (77). Similarly, in the Genetically Inspired Prognostic Scoring System (GIPSS), only the molecular features are taken into consideration and *IDH1/2* are substituted by *U2AF1* mutations (78). In the same way, in ET and PV patients *IDH2*, *U2AF1*, *EZH2*, *TP53*, *SH2B3,* and *SF3B1* mutations were all reported to give an adverse prognostic value (93).

Finally, somatic mutations with frequency ranging from 10 to 20% have been described in elderly subjects without any clinical evidence of myeloid diseases. In 2016, Bartels et al. used a customized NGS panel with 23 genes mutated in both MDS and MPNs to profile 192 formalin-fixed and paraffin-embedded (FFPE) patient samples. In this study, the authors found overall 269 pathogenic mutations in 125 of 185 analyzed patients and several of these exhibited more one-gene variants. Although they used FFPE bone marrow samples, the study demonstrated that NGS improved diagnostic accuracy and contributed to understanding the development, progression and therapy of

TABLE 2 | Mutated genes in *BCR-ABL1*-negative myeloproliferative neoplasms (MPNs).

Gene	Pathway relevance	Type of mutation	Frequency of mutation (%, PV, ET, MF)	Prognostic significance	References
ASXL1	Epigenetic regulation	Missense	3–12% in PV 4–11% in ET 22–38% in MF	Adverse in PV and MF	(17, 18, 25, 30, 32, 45, 73–82)
DNMT3A	Epigenetic regulation	Missense	6% in ET 5–10% in MF	None	(18, 32, 73, 74, 76, 77, 82)
EZH2	Epigenetic regulation	Missense	2–12% in PV 3% in ET 12% in MF	Adverse in TE and MF	(18, 25, 32, 73–77, 79, 80, 82)
IDH1	Epigenetic regulation	Missense	10% in PV 1% in ET 1–4% in MF	Adverse in MF	(18, 25, 32, 74–77, 79, 80, 82)
IDH2	Epigenetic regulation	Missense	4% in PV 1–3% in MF	Adverse in PV and MF	(18, 25, 32, 74–77, 79, 80, 82)
TET2	Epigenetic regulation	Insertion/ Deletion Nonsense or Missense	10–25% in PV 16% in ET 17% in MF	Adverse in TE	(18, 32, 73, 74, 76, 77, 82)
SF3B1	mRNA processing	Missense	3% in PV 5% in ET 10% in MF	Adverse in TE	(18, 32, 74, 77, 82)
SRSF2	mRNA processing	Missense	9% in MF <2% ET	Adverse in PV and MF	(18, 25, 32, 74–77, 80–82)
U2AF1	mRNA processing	Missense	1–2% in TE 10–17% in MF	Adverse in TE and MF	(18, 32, 74, 77, 82)
ZRSR2	mRNA processing	Missense	5% in PV 3% in ET 10% in MF	Not known	(18, 32, 74, 77, 82)
CEBPA	Transcriptional regulation	Mutations	6% in PV 4% in ET 9% in MF	Adverse in MF	(18, 32, 82)
RUNX1	Transcriptional regulation	Nonsense Missense Insertion/ Deletion	<5% (PV, ET, MF)	Adverse in MF	(18, 32, 74, 76, 77, 82)
TP53	Transcriptional regulation	Missense or Mutation	<5% (PV, ET, MF)	Adverse in TE	(18, 32, 74, 76, 77, 82)
CBL	Cell signaling pathways	Missense	4% in MF	Adverse in MF	(18, 32, 73, 74, 77, 82)
KIT	Cell signaling pathways	Mutations	3% in PV 2% in ET 1% in MF	Adverse in MF	(18, 32, 82)
NF1	Cell signaling pathways	Deletion	Rare in MF	Not known	(18, 32, 74, 77, 82)
NRAS/KRAS	Cell signaling pathways	Missense	1% in ET 1–4% in MF	Not known	(18, 32, 74, 77, 82)
SH2B3/LNK	Cell signaling pathways	Deletion or missense	9% in PV 3% in ET 11–18% in MF	Adverse in TE and MF	(18, 32, 73, 74, 77, 82, 83)

For each gene the function, type of molecular alteration, frequency and prognostic significance, if known, are reported.

myeloid diseases (94). NGS technology provide additional information to define the potential risk of development a myeloid malignancy, understand the clinical course, select the appropriate target therapy and predict potential drug-resistance mechanisms.

CONCLUSIONS

The discovery of *JAK2*V617F mutation in *BCR-ABL1*-negative MPNs by four different international cooperative groups in 2005 (2–5) led to significant insights on the pathogenesis of these disorders. In fact, this mutation results in a gain-of-function with activation of cytokine and growth factor receptors, and thus of the downstream JAK-STAT pathway (79, 95–98). The *JAK2* point mutation in exon 12, present in a small percentage of patients with PV, is able to induce the MPN phenotype through the same pathogenic mechanism (6, 7).

In 2006 the *MPL*W515L/K was reported in ET and PMF patients (44, 45) and demonstrated to be able to aberrantly activate JAK-STAT pathway through a gain-of function similar to that of *JAK2*, thus leading to megakaryocytic proliferation (8–10).

More recently, in 2013 two different groups demonstrated a spectrum of mutations in *CALR* gene that cause frameshifts of one base pair in the last coding exon with a generation of a protein with new C terminus and a tail of 36 aminoacids (11, 12). Although it was soon clear that JAK-STAT signaling pathway was consistently activated in *CALR* mutated cells (53, 99), only recently it has been demonstrated that the mutant *CALR* protein is able to bind to the *MPL* receptor and activate it independently of the TPO presence itself (54–57). Consistently with the fact that all these mutations cause JAK-STAT pathway activation, ruxolitinib, the first *JAK1/2* inhibitor, is able to exert clinical results in MF patients independently of the type of mutation (80).

As *BCR-ABL1*-negative MPNs are initiated by a driver mutation in one of *JAK2*, *CALR* or *MPL* genes in more than 95% of cases, there is a key relevance in diagnosis, prognosis and therapy (12, 61, 81). Due to the fact that these mutations are mutually exclusive, a sequential search for diagnosing purposes starting from *JAK2* is suggested (36, 62–64). Furthermore, each of these mutations has a different prognostic impact. In ET, *JAK2* is taken into consideration in the IPSET prognostic scores (100, 101). In *JAK2*-positive PMF, allele burden is relevant, with a worse prognosis for patients with <50%, while type 1/type 1-like *CALR* mutations has a favorable impact (22, 26). In the new MYSEC-PM prognostic score developed for secondary MF, *CALR* mutations absence is associated with a worse outcome (102).

Since NGS studies have been performed in *BCR-ABL1*-negative MPNs, especially in PMF, other mutations than the driver ones have been reported with few particularly relevant for prognosis and prediction of response. Sub-clonal mutations in genes of the spliceosome machinery, regulators of chromatin structure and histone modification and epigenetic regulators of DNA methylation are now taken into consideration in the new prognostic scores together with driver mutation status and clinical data in MIPSS70 and MIPSS70-plus (75) or in the GIPSS, where only the molecular features are evaluated (45).

NGS analysis during the disease natural history is of key importance to underline the eventual clonal evolution, thus uncovering an aggressive course and possibly suggesting a change in the therapeutic strategy (103, 104). Thus, in a near future, due to the progressive lowering of costs and availability in more reference laboratories, NGS will become the standard to evaluate *BCR-ABL1*-negative MPN patients.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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