Mutaciones y sus implicancias clínicas en neoplasias mieloproliferativas

Genetics and prognosis in myeloproliferative neoplasms

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Abstract

Recent advances in the understanding of the molecular landscape of chronic myeloproliferative neoplasms (MPN) have remarkably improved the diagnostic approach to these hematologic neoplasia. Furthermore, it is increasingly being appreciated how the presence of specific mutations contributes to better defining the prognosis, particularly for patients with essential thrombocythemia and primary myelofibrosis. The three phenotypic drivers mutations, involving *JAK2* (V617F, exon 12 mutations), *MPL* and *CALR*, are included as major diagnostic criteria in the WHO classification, and point to different risk categories. Mutations in genes of the epigenetic regulation and the spliceasome, considered as subclonal mutations, have no diagnostic value since they occur in a wide spectrum of myeloid neoplasms, but deserve major prognostication significance and contribute to identify categories of patients with different survival and risk of leukemia. We will address these aspects to elucidate how mutational analysis may contribute to advanced assessment of MPN patients. Further enhancement of risk stratification in MPN is possible by combining cytogenetic and/or mutation information with clinical and hematological data. Developing an integrated prognostic model would facilitate therapeutic decision making for the individual patient.

Introduction

Classical Philadelphia-negative myeloproliferative neoplasms (MPNs) include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). PV and ET can evolve to secondary forms of myelofibrosis, known as post-polycythemia vera (PPV-MF) and post-essential thrombocythemia (PET-MF) myelofibrosis, as part of their natural history. All four disorders are characterized by stem cell-derived clonal myeloproliferation[1,2] with mutually exclusive driver mutations, including Janus kinase 2 (*JAK2*) gene, gene encoding the endoplasmic protein calreticulin (*CALR*) and gene encoding the thrombopoietin receptor (MPL). Current diagnosis is based on revised 2016 WHO criteria[3] that does not include major changes to the disease categories but incorporates new knowledge of these disorders, such as the discovery of the diagnostic and prognostic *CALR* mutations, the identification of novel molecular findings with diagnostic and/or prognostic importance, as well as improved understanding of the morphologic features.

The term myeloproliferative neoplasms (MPN)[4] has been attributed by the World Health Organization (WHO) in 2008 to those relatively common hematologic neoplasia also known as Philadelphia chromosome-negative, classic, chronic myeloproliferative diseases, as initially described by W. Dameshek in 1951[5]. Phenotypically, PV is characterized by clonal erythrocytosis, ET by clonal thrombocytosis and PMF by bone marrow (BM) fibrosis. In addition, all three disorders might be associated with hepatosplenomegaly, leukocytosis, thrombocytosis, microvascular symptoms, constitutional symptoms, thrombotic and hemorrhagic complications[6]. MPN clinical course varies from one to over 30 years and may evolve from asymptomatic into progressive BM failure, symptomatic splenomegaly and acute leukemia in 3-20 % of cases as appropriate. Additional clinical manifestations include symptoms of itching and hyperviscosity in PV, progressive anemia, leukoerythroblastosis, extramedullary hematopoiesis, recurrent splenic infarcts, cachexia and symptoms of portal hypertension, including ascites and bleeding of varices in PMF.

The 2016 revised WHO classification system distinguishes prefibrotic (pre-PMF) from overtly fibrotic (overt PMF) primary myelofibrosis, based mainly on bone marrow fibrosis grade; criteria that allow to define two distinct diseases in terms of presentation and outcome[3].

These disorders have remained orphan of an unique molecular marker until 2005, when the groups leaded by W. Vainchenker, T. Green, G. Gilliland and R. Skoda concurrently described the presence of a point mutation in exon 14 of the *JAK2* gene in almost the totality of patients with PV and about 50-60% of those with ET and PMF[7-10]. This pivotal discovery, soon followed by the identification of mutations in *MPL* in some patients with ET and PMF (3-8%)[11] and by additional mutations occurring in exon 12 of *JAK2* in about half of *JAK2 V617F*-negative patients with PV[12]. More recently, mutations in *CALR* have been discovered in about 20-25% of ET and PMF patients[13,14], prompted the WHO to revise the previous diagnostic criteria by electing these mutations as major criteria. The term triple-negative (TN) has been applied to those MPN without evidence of these consistent mutations.

Collectively, owing their specific associations with MPN and the fact that the expression of the mutated genes in animal models reproduced phenotypes resembling a myeloproliferative disease, although with some differences in the models that have been developed, mutations in *JAK2*, *MPL* and *CALR* are considered as "phenotypic driver mutations" (**Table 1**). Accordingly, the presence, absence or specific type of driver mutations cannot be used for diagnostic distinction among the different MPN, which is based primarily on bone marrow morphology and peripheral blood counts[2].

Such great advancements in the knowledge of MPN genetics prompted to herald a new clinical dimension of MPNs. Later studies indicated that a number of additional mutations occur in a subset of patients, particularly those with PMF, that are usually harbored by subclones of variable size[15]. These abnormalities target genes involved in the epigenetic gene regulations, the spliceasome, or oncogenes; however, they are found also in myelodysplastic syndromes, other myeloid neoplasia and acute leukemias, therefore they have no diagnostic value, but contribute remarkably to the prognostic assessment of patients, particularly for patients potentially candidate to allogeneic stem cell transplantation (allo-SCT), and for monitoring after disease modifying therapies[16].

Table 1. Somatic mutations in classical MPNs and their clinical significance.

Gene	Mutated region	Mutational frequency	Clinical relevance
DRIVER Somatic mutations of driver genes represent a major diagnostic criterion for PV, ET, and PMF			
JAK2			• PV may progress to myelofibrosis and less commonly to a blast phase similar to acute myeloid leukemia (AML), sometimes preceded by a myelodysplastic phase; • *JAK2* (V617F)-mutant ET involves a high risk of thrombosis, and may progress to PV or myelofibrosis; • *JAK2* (V617F)- and MPL-mutant PMF have worse prognosis than *CALR*-mutant PMF; • *CALR*-mutant ET involves lower risk of thrombosis and higher risk of progression to myelofibrosis; • *CALR*-mutant PMF is associated with longer survival compared with other genotypes; • Triple-negative ET is an indolent disease with low incidence of vascular events • Triple-negative PMF is an aggressive myeloid neoplasm characterized by prominent myelodysplastic features and high risk of leukemic evolution
JAK2 V617F	Exon 14	PV ~ 96% ET ~ 55% PMF ~ 65%	
JAK2 EX12	Exon 12	PV ~ 3%	
CALR	Exon 9	PMF ~ 25% ET ~ 20%	
MPL	Exon 10	ET ~ 3% PMF ~ 10%	
NON DRIVER Somatic mutations of non-driver genes contribute to phenotypic variability, phenotypic shifts, and progression to more aggressive myeloid neoplasms..			
ASXL1	Exon 12	ET ~ 3% PMF ~ 13% BP-MPN ~ 18%	Somatic mutations are found in age-related clonal hematopoiesis. In PMF, ASXL1 mutation is an established unfavorable prognostic factor, also included in the HMR definition, that may be associated with leukemic transformation
IDH1/IDH2	Exon 4	PV ~ 2% ET ~ 1% PMF ~ 4% BP-MPN ~ 20%	Somatic mutation may represent a late event associated with leukemic transformation. IDH were included in the HMR genes
EZH2	Full gene	PV ~ 3% PMF ~ 7% MDS ~ 6%	EZH2 mutation may represent a mechanism of myelofibrotic transformation in ET and PV. Somatic mutation represents an unfavorable prognostic factor in PMF, included in the HMR genes
SRSF2	Exon 2	PMF ~ 17%	Mutation in spliceosome genes represent a pathogenetic mechanism of anemia or cytopenia. • SF3B1 accounts for the presence of ring sideroblasts in MPN patients. • SRSF2 mutation may responsible for myelofibrotic transformation and represents an unfavourable prognostic factor in PMF (HMR gene) • U2AF1 mutation may be associated with leukemic transformation
SF3B1	Exons 14, 15	PMF ~ 7%	
U2AF1	Full gene	PMF ~ 16%	

CBL	Exon 8, 9	PV ~ rare ET ~ rare MF ~ 6%	Somatic mutation may represent a late event associated with leukemic transformation
IKZF1	Full gene	CP-MPN ~ rare BP-MPN ~ 19%	
TP53	Exons 4-9	PMF 4% BP-MPN ~ 27%	Somatic mutation often represents a mechanism of leukemic transformation, typically through transition from heterozygosity to homozygosity for the mutation
DNMT3A	Full gene	PV ~ 7% PMF ~ 7% BP-MPN ~ 14%	Somatic mutations in these genes are typically associated with age-related clonal hematopoiesis. In MPN, these mutations may variably impact on clinical phenotype and predispose to disease progression
TET2	Full gene	PV ~ 16% ET ~ 5% PMF ~ 17% BP-MPN ~ 17%	
SH2B3	Exons 1-3	PV ~ rare ET ~ rare PMF ~ rare BP-MPN ~ 10%	Germline or somatic mutation may cooperate with JAK2 (V617F) or CALR mutation to give rise to an MPN phenotype

Gene abbreviations. ***JAK2***, *Janus kinase 2;* ***CALR***, *calreticulin;* ***MPL***, *myeloproliferative leukemia virus oncogene;* ***ASXL1***, *Additional Sex Combs-Like 1;* ***IDH1/2***, *isocitrate dehydrogenase;* ***EZH2***, *enhancer of zeste homolog 2;* ***SRSF2***, *serine/arginine-rich splicing factor 2;* ***SF3B1***, *splicing factor 3B subunit 1;* ***U2AF1***, *U2 Small Nuclear RNA Auxiliary Factor 1;* ***CBL***, *Casitas B-lineage lymphoma proto-oncogene;* ***IKZF1***, *IKAROS family zinc finger 1;* ***TP53***, *tumor protein p53;* ***DNMT3A***, *DNA cytosine methyltransferase 3a;* ***TET2***, *TET oncogene family member 2;* ***SH2B3***, *SH2B Adaptor Protein 3;* ***MPN***, *myeloproliferative neoplasms;* ***ET***, *essential thrombocythemia;* ***PV***, *polycythemia vera;* ***PMF***, *primary myelofibrosis;* ***MF*** *includes both PMF and post-ET/PV myelofibrosis;* ***BP-MPN***, *blast phase MPN;* ***CP-MPN***, *chronic phase MPN;* ***HMR***, *high molecular risk.*

Mutations in phenotypic driver genes: *JAK2, MPL, CALR*

The V617F mutation is located in exon 14 of *JAK2* gene that encodes for the pseudokinase domain of the protein, believed to exert auto-inhibitory regulation of the kinase activity of JAK2[17]; as a result of such "gain of function" mutation, JAK2 is autonomously (ie, in the absence of cytokines bound to cognate receptors) "on" and leads to downstream target activation through deregulated protein phosphorylation, ultimately resulting in sustained JAK/STAT signaling. Expression of mutated JAK2 in mice results in a myeloproliferative disease characterized by erythrocytosis, varying degree of leukocytosis and thrombocytosis, splenomegaly and eventual progression to myelofibrosis; transformation to leukemia is not clearly documented[18,19]. Mutations in exon 12 of *JAK2* have been identified in about 40-50% of patients with a PV phenotype who are negative for the *JAK2 V617F* mutation[12]. Greater than 20 variants have been reported to date, the most common being N542-E543del found in 30% of cases[20]. Clinically, these cases present with erythrocytosis but less marked leukocytosis and thrombocytosis compared with V617F mutated PV, although the rate of thrombosis, transformation to myelofibrosis or leukemia, and overall survival, are comparable to *JAK2 V617F* mutated PV[21].

About 3% and 8% of patients with *JAK2* wild-type ET or PMF, respectively, harbor point mutations located at codon 515 of *MPL*, the gene encoding for the thrombopoietin receptor[11]; occasional patients with somatically acquired S505N mutation, most

commonly associated with familial thrombocytosis, have also been reported[22,23]. The trypthophan residue located at position 515 is part of a short aminoacidic strech (K/RWQFP), located in the intramembrane, juxta cytoplasmic portion of the protein, whose integrity is mandatory to prevent the constitutive, ligand independent, activation of the receptor[24]; any aminoacid change at this level (the most frequent being L, K, A) induces instability of the receptor and its activation. Retroviral expression of *MPL*W515L in a mouse model resulted in an aggressive MPN-like disease with extreme thrombocytosis, rapid development of bone marrow fibrosis, splenomegaly, and shortened survival[11,25]. Patients with ET and PMF harboring any of the *MPL*W515 substitutions present more extensive thrombocytosis when compared with *JAK2 V617F* mutated, lower levels of hemoglobin[26] and, in case of PMF, are more at risk of being transfusion-dependent[27].

The discovery of mutations in the calreticulin gene (*CALR*)[28], reported concurrently by the group of Tony Green[13] and Robert Kralovics[14] at the end of 2013, has represented a major breakthrough in the diagnostic approach and has put back on the shelf, unexpectedly, the relevance of phenotypic driven mutations for prognosis assessment. Mutations in *CALR* are presented by 60-88% of patients with ET and PMF who are negative for the *JAK2* and *MPL* mutations. *CALR* mutations are represented by insertions or deletions restricted to exon 9; more than 80% of cases present two alternative mutations, either either a 52-bp deletion (Type 1; 45-53% of all cases) or a 5-bp insertion (Type 2; 32-41%). However, more than 50 variants are currently reported that can be divided in Type 1-like and Type 2-like. All mutations cause a frameshift, which lead to a unique alternative reading frame coding a novel protein C-terminus consisting of approximately 36 amino acids. Theoretically, this represents a new antigen potentially suitable for labeling and targeting; in fact, recently several anti-mutant calreticulin antibody have been developed[29,30].

JAK2 V617F mutated patients may express the mutation in a heterozygous or homozygous status, meaning that the proportion of mutated allele is lower (heterozygous) or higher (homozygous) than 50% when assayed in whole blood nucleated cells or preferably purified granulocytes that are a heterogeneous cell population. Therefore, although commonly used as such, this terminology is not strictly appropriate since the concept of hetero- or homozygosity points to the single cell rather than to a heterogeneous cell population where the proportion of clonal *JAK2 V617F* mutated cells and polyclonal normal cells is variable. Homozygosity is acquired through a process of mitotic recombination[10]. Levels of *JAK2 V617F* allele burden in blood cells greater than 50% are definitely more common in PV than in ET; in PMF, and even more in myelofibrosis developed from previous PV (PPV-MF) or ET (PET-MF), the large majority of patients have allele burden over 50%, but one quarter of the patients may present levels lower than 20%[31]. Therefore, quantifying *JAK2 V617F* allele burden is not useful for making differential diagnosis among the different MPN, although a diagnosis of ET with a high levels of *JAK2 V617F* mutated allele should prompt the clinician to re-analyze data to definitely confirm such diagnosis, since homozygosity in 2008 WHO-diagnosed ET patients involved only 4% in a large series[32]. The level of *JAK2 V617F* allele remains substantially stable over time in many patients and is not obviously modified by conventional cytotoxic drugs[33], although some decline with interferon or long-term JAK2 inhibitors treatment has been reported[34-37]. Therefore, it is not informative, nor has any clinical implications, to obtain serial measurements of *JAK2 V617F* allele during an uneventful disease as well as in patients receiving conventional treatment. With prolonged observation, some patients show progressive increase in the amount of mutated allele, and accumulation of V617F allele has been associated with progression to post-PV or post-ET MF[32,38]. In case of *MPL* mutation, the mutant allele burden ranged from 1% to 95%, being significantly higher in PMF or PET-MF than in ET. Homozygosity for *MPL* mutation was due to acquired copy-neutral loss of heterozygosity at 1p. Levels of *MPL* mutated allele burden greater than 50% were associated with occurrence of marrow fibrosis, overall suggesting a pathogenetic role of accumulation of *MPL* mutated alleles in the development of fibrosis[39]. Therefore, in the clinical practice, outside a clinical trial, it is not informative to have MPN patients sequentially evaluated for their *JAK2 V617F* or *MPL*W515L/K/A allele burden.

Some patients who maintained such molecular response even after treatment discontinuation have

been anecdotally reported, but it remains to be demonstrated prospectively whether the attainment of molecular remission represents a criterion for managing therapy, and what is the final significance of the disappearance of measurable *JAK2 V617F* allele for disease progression. In such instances of complete molecular remissions obtained with drugs (IFN, JAK2 inhibitor), one must consider the availability of diagnostic assays that should be able to reproducibly measure levels of *JAK2 V617F* allele burden with at least 10^{-4} performance[40]. This is of particular relevance in the settings of HSCT, where careful monitoring of the *JAK2 V617F* allele burden may contribute to the optimized management of patients by providing information about the disappearance of the mutated clone and the identification of subjects that are at increased risk of relapse[41,42]. The use of *JAK2 V617F* allele monitoring for timely and successful delivery of donor lymphocyte infusions has been reported[43,44]. Similarly, patients with MPL and *CALR* mutations may be evaluated for outcome after HSCT[45-47], but until now the reliability and sensitivity of available tests for these mutations remain to be fully ascertained. In this regards, the MPN taskforce of the Italian Society of Hematology (SIE) assessed the scientific literature and composed a framework of the best, possibly evidence-based, recommendations for optimal molecular methods as well as insights about the applicability and interpretation of those tests in the clinical practice[48].

The discovery of *CALR* mutations deserved a major impact on the diagnostic approach to suspected MPN targeting that 40% of PMF and ET cases that were *JAK2* and *MPL* unmutated[49]. *CALR* mutations were found very infrequently in a few cases of atypical chronic myeloid leukemia, chronic myelomonocytic leukemia or chronic neutrophilic leukemia[50] and in occasional patients with MDS, particularly with refractory anemia with ring sideroblasts[51], therefore mutated *CALR* is considered a highly specific marker of *JAK2* and *MPL* wild-type MPN[52]. Driver mutations might also influence MPN phenotype[29]. For example, *JAK2 V617F* mutated patients with ET or PMF are usually older and display higher hemoglobin and leukocyte counts and lower platelet count[53]. *JAK2* exon 12 mutated PV patients are younger and often display an isolated erythrocytosis and distinctive bone marrow morphology. In contrast, *CALR* mutated or triple-negative ET patients are younger and display male preponderance, higher platelet count, and lower hemoglobin and leukocyte counts. *CALR* mutated patients with PMF are also younger and present with higher platelet count and lower frequencies of anemia and leukocytosis. Notably, platelet count was significantly higher in type-2 vs. type-1 CALR-mutated patients and this particular observation was validated in different series[54,55].

However, analysis of *CALR* mutated patients quite unexpectedly revealed to be extremely useful in the process of prognostic assessment for ET and particularly PMF patients. In the era of "*JAK2*V617 or *MPL* mutation-only", no clear-cut impact of those mutations, as well as the respective allele burden, on disease outcome was realized. In patients with PMF, *JAK2 V617F* homozygosity was associated in some studies to shorter survival[56] and greater risk of transformation to leukemia[57], while others reported that a low *JAK2 V617F* allele burden was prognostically adverse[58,59]. In ET, patients who are *JAK2 V617F* mutated had a 2-fold higher rate of thrombosis compared with the wild-type ones, and a mutated allele burden in excess of 50% and 75% was associated with increased risk of thrombosis in ET and PV, respectively[32,60]. However, this somewhat conflicting information had not resulted in a systematic use of mutation asset for prognostication except in the case of ET. The International Prognostic Score of thrombosis includes the presence of *JAK2 V617F* positivity as one risk variable for accurate prediction of thrombosis in patients with ET[61]. Conversely, ET patients who express the *CALR* mutation are at significantly lower risk of thrombosis when compared with *JAK2 V617F* and *MPL* mutated ones[14,62,63], with a relative risk very close to the "triple negative". The positive impact of *CALR* mutation in ET may be particularly pronounced among the youngest patients; no measurable differences in outcome according to the type (1 versus 2) mutation[54]. The addition of *CALR* mutation to the variables already listed in the IPSET-thrombosis score did not affect its performance, owing the strong impact of the *JAK2*V617 mutation as prognostically adverse variable[64]. However, it is particularly in PMF that the impact of CALR mutation has been striking[65]. In the largest study, that included over 800 patients with PMF, *CALR*

mutation was associated with significant better overall survival (17.7 years) compared with *JAK2 V617F* (9.2 years) and *MPL* (9.1 years) mutated patients[66]. However, perhaps the most relevant findings is that triple negative patients are at very high risk of early death (overall survival 3.2 years) that was associated with lower cumulative incidence of anemia (Hb <10 g/dL), leukocytosis (>25x10^9/L) and thrombocytopenia (<100x10^9/L)[65]. Concurrent presence of *CALR* mutation with abnormalities in *ASXL1* partially mitigated the adverse impact of the latter[67]. More subtle effects may also be dependent on the unique molecular lesion in *CALR*, with Type 1 mutation being reported to account for better prognosis than Type 2[68]. ET and PMF negative for standard phenotype driver mutations are referred to as "triple negative". High-throughput sequencing identified non-canonical activating JAK2 or MPL mutations in almost 8-10% of triple-negative MPNs, some of which were somatic and others that were inherited, identifying patients with hereditary thrombocythemia misdiagnosed as MPN[69,70].

Life expectancy in patients with MPN is reduced than control population matched for sex and age. Median survivals are estimated at 20 years for ET, 14 years for PV and 6 years for PMF[71]. Several prognostic scoring systems have been proposed in MPNs in the last years, that are focused on the main disease-related complications, including vascular complications in PV and ET, evolution into post-PV/post-ET myelofibrosis or progression to acute leukemia (AL) and premature death in MF.

Pathogenic and prognostic role of mutations in additional genes

The molecular landscape of patients with MN is much more complex than originally believed. This is supported by evidences from several studies showing that many different mutations other than the phenotypic drivers, mainly falling in the functional categories of epigenetic regulators, genes of the spliceasome and oncogenes, may occur in a proportion of the patients. The most recurrent abnormalities reported to date are listed in **Table 1**. The most frequent such mutations in PMF were *ASXL1* (36%), *TET2* (18%), *SRSF2* (18%), and *U2AF1* (16%) and 35%, 26%, 10%, and 9% of the patients harbored 1, 2, 3, or 4 or more such variants/mutations, respectively. In PV, the most frequent mutations were *TET2* (22%), *ASXL1* (12%) and *SH2B3* (9%) and in ET, *TET2* (16%), *ASXL1* (11%), *DNMT3A* (6%) and *SF3B1* (5%); the respective percentages of patients with 1, 2, or ≥3 sequence variants/mutations were 30%, 20%, and 3% for PV and 41%, 8%, and 4% for ET. A comprehensive list of mutated genes is reported in **Table 1**.

Generally speaking, these are definitely more frequent in PMF as compared to PV and ET, while others, in particular mutations of *TP53*, tend to accumulate at the time of leukemic transformation. These same abnormalities occur with similar or even higher frequency in myelodysplastic syndromes and acute leukemias, therefore they have no specific utility for the diagnosis of MPN apart for rare instances of unusual clinical presentation and absence of the phenotypic driver mutations where finding any one of these subclonal mutations might help to assess the existence of a clonal myeloid disorder. However, recent reports indicating that mutations within some of these genes may be found in normal elderly individual advocates caution in interpreting the results[72]. On the other hand, mutations of these genes in the settings of an otherwise well characterized MPN patient deserve prognostic relevance.

However, the interpretation of the presence of additional somatic mutations, in absence of driver mutations, is made complex, in some instances, by the recent demonstration that some mutated genes can be found in normal elderly individuals. This phenomenon is referred as to "clonal hematopoiesis of indeterminate potential" (CHIP)[73,74]. In fact, clonal hematopoiesis is increasingly common in elderly people and this condition has been associated with increased risks of hematologic cancer and death.

Cytogenetic abnormalities

Karyotypic abnormalities detected by conventional cytogenetic analysis are seen in a minority of patients with PV or ET (< 10%) but approximately one third of patients with PMF[75]. Gain of chromosome 9 is seen recurrently in PV and is associated with JAK2 V617F. Interstitial deletions of 13q and 20q are seen in all MPN subtypes, suggesting the presence of one or more tumor suppressor genes in these regions. In PV, abnormal karyotype is seen in approximately 15% of the patients, at time of their diagnosis, and mostly consists of sole abnormalities. The most frequent cytogenetic abnormalities in PV include -Y,

+8, +9, del(20q) and chromosome 1q+[76]. Incidence of abnormal karyotype is approximately 7% in ET with +9, chromosome 1q+ and +8 identified as the most frequent abnormalities[77]. The most frequent cytogenetic abnormalities in PMF (and their approximate incidences) were 20q- (23%), 13q- (18%), +8 (11%), +9 (10%), chromosome 1q+ (10%) and -7/7q- (7%). In a recent study of 826 informative patients with PMF[78], approximately 43% displayed abnormal karyotype at time of their referral; among the group with abnormal karyotype, 68% consisted of sole aberrations and 14% complex karyotype.

Genetic risk stratification in polycythemia vera and essential thrombocythemia

In general, life expectancy in polycythemia vera (PV) and essential thrombocythemia (ET) is shorter compared with control population matched for age and gender. Most recent survival data from Mayo Clinic in 559 patients showed a median survivals of approximately 20 years for ET and 14 years for PV[79]. Risk factors for overall survival in ET and PV included advanced age, leukocytosis and thrombosis, in particular those in venous district for PV patients. Abnormal karyotype at diagnosis, documented in about 12% of the patients, is an additional risk factor associated to a median survival ranging from 6 to 29 years. The same holds true for somatic mutations shown to affect outcome also in other myeloid malignancies. Adverse somatic mutations, harbored in about 15% of patients, were associated with inferior survival in both PV and ET (median overall survival of 7.7 and 9.0 yrs, respectively) and include *ASXL1*, *SRSF2*, and *IDH2* in PV and *SH2B3*, *SF3B1*, *U2AF1*, *TP53*, *IDH2*, and *EZH2* in ET. These prognostically detrimental mutations predicted also leukemic and fibrotic progression; however, the number of mutations, prognostically unfavorable in myelofibrosis, mutational status of driver mutations (*JAK2*/*CALR*/*MPL*) or their allele burden did not provide additional prognostic information in either ET or PV.

The primary objective of treatment in PV and ET is to prevent thromboembolic or hemorrhagic complications occurring in about 15% of cases during disease course. In this regards, a prognostic scoring system validated in recent years is based on patient-oriented characteristics including age more than 60 years and history of thrombosis. These 2 simple variables allow to categorize patients into 2 risk groups: low risk (no risk factors) and high risk (1 or 2 risk factors)

Other risk factors have been more recently associated with an increased risk of developing major thrombotic events, including hypertension and previous arterial events, for arterial thrombosis in PV and cardiovascular (CV) risk factors, leukocytosis, and presence of *JAK2 V617F* in ET, and for venous thrombosis older age and venous events in PV or male gender in ET.

The International Working Group for MPN Research and Treatment (IWG-MRT) developed the IPSET-thrombosis score, a new prognostic model for thrombotic risk stratification in ET patients recently revised, prone to quantify the individual and combined risk contribution of CV risk factors and JAK2 mutation in both conventionally defined low- and high-risk ET. Revised IPSET-score is based on three risk factors including age greater than 60 years, history of thrombosis and JAK2 V617F mutation.

Progression to myelofibrosis represents a natural evolution of PV and ET; less than 10% of PV and 5% of ET patients evolve into MF after their first decade from diagnosis. Transformation to MF occurs late during the course of the disease, overall median time to progression from diagnosis is approximately 8-20 years in PV and 7-16 years in ET. A longer time from diagnosis strongly impacts on MF transformation rate: in PV patients a disease duration over ten years reached an hazard ratio of 15. Homozygosity for *JAK2 V617F* is usually associated with MF development, supporting the idea that accumulation of mutated alleles usually accompanies transition to myelofibrosis. In a study with 320 PV patients a mutant allele burden greater than 50% was identified as an independent risk factor for postPV-MF (PPV-MF) progression.

As regards *CALR* variant subtypes, it has been found a significantly higher risk of myelofibrotic transformation in ET patients carrying type1/type1-like than in those carrying type2/type2-like *CALR* mutations.

Leukemic transformation rates at 20 years are estimated at less than 5% for ET and 10% for PV. Risk factors for leukemic transformation have been identified in PV including advanced age, leukocytosis, and abnormal karyotype; in ET, leukocytosis ($\geq 15\times10^9$/l), extreme thrombocytosis

(≥1000 × 10^9/l), anemia, older age (≥60 years), reticulin grading, and bone marrow cellularity. In particular, combination of anemia (12 g/dl in females or 13.5 g/dl in males) and thrombocytosis (≥1000 × 109/l) defined a high-risk group of ET patients with a rate of acute leukemia transformation of 6.5% versus 0.4% in those without risk factors.

Information regarding the prognostic significance of driver mutations and/or the allele burden in leukemic evolution are not consistent. In both PV and ET statistical association has been found between *JAK2* mutation or the allele burden and the time of evolution to acute leukemia or overall survival has been described. Overall, *JAK2 V617F* is not considered a prerequisite for blast transformation and more likely additional genetic events are required in the setting of disease progression.

Genetic risk stratification in primary myelofibrosis

Primary myelofibrosis (PMF) is the most aggressive among classical myeloproliferative neoplasm; its main features are bone marrow fibrosis, splenomegaly, and variable degrees of abnormal count of leukocytes, platelets and red cells (anemia). Current diagnosis is based on revised 2016 WHO criteria that allow to define two distinct diseases in terms of presentation and outcome: prefibrotic/early (pre-PMF) and overt fibrotic (overt PMF)[3]. Despite the median survival of patients with PMF remains significantly worse than ET or PV, data derived from a large series of studies found a significant improvement over time in outcome during the last decade, increasing of almost 2 years (4.6 vs 6.5 yrs). Moreover, with respect to clinical, hematologic, and molecular phenotypes, pre-PMF is associated with better risk factors that result in prolonged overall survival compared to overt-PMF. All these features aside, prognosis is very heterogeneous with a median survival ranging from more than 15 yrs to less than 2 yrs. The most common causes of death include leukemic progression, that occurs in approximately 10-15% of cases, but many patients also die because of comorbidities including major cardiovascular events and consequences of cytopenias including infection or bleeding. Treatment is essentially clinical-oriented focusing mainly on the improvement of anemia, thrombocytopenia, constitutional symptoms and splenomegaly; the newly approved JAK1/2 inhibitor offered a new targeted therapy option for most of MF patients.

Allogeneic hematopoietic stem cells transplantation is the only treatment that is potentially curative resulting in long-term remission, however mainly because of median older age only a minority of the patients are eligible; consequently considerable interest in accurate prognostication arise in the last few years aimed to identify patients with different life expectancy in order to select those that may benefit from HSCT versus other therapeutic alternatives.

Therefore, primary myelofibrosis risk stratification is based on parameters predicting survival, and several efforts have been made to identify clinical and laboratory features that could predict patients' survival. The most widely used score is the International Prognostic Score System (IPSS), proposed in 2009 by the IWG-MRT[80] and it is applicable at the time of diagnosis; it is based on 5 variables: age >65 yrs, hemoglobin less than 10 g/dL, leukocyte count >25x10^9/L, peripheral blast cells count ≥1% and presence of constitutional symptoms. These prognostic factors formed the basis of four risk categories with non-overlapping survival curves: no factors (low risk), one factor (intermediate risk-1), two factors (intermediate risk-2) or three or more factors (high risk) where median survivals were 135 months, 95 months, 48 months and 27 months, respectively. A dynamic IPSS (D-IPSS) was afterwards proposed[81]. DIPSS uses the same prognostic variables of IPSS anytime the disease progression and is able to predict the remaining life expectancy even far from the diagnosis. The main difference in this score was to assign a doubled weight to anemia; risk categorization was accordingly modified in low (0 adverse points), intermediate-1 (1 or 2 points), intermediate-2 (3 or 4 points) and high (5 or 6 points). The corresponding median survivals were not reached, 14.2, 4.0 and 1.5 years for each category, respectively. Importantly, the DIPSS has also been shown to predict progression to acute leukemia and outcomes in patients receiving allogeneic HSCT.

Dynamic IPSS was subsequently redefined by including three new parameters (the DIPSS-plus) [82]: red cell transfusion need, platelet count less than 100x10^9/L, and "unfavorable" karyotype (included complex karyotype or sole or 2 abnormalities that included þ8, 7/7q-, i(17q), inv(3), -5/5q-, 12p-, or 11q23 rearrangement) as additional independent

risk factors. DIPSS-plus defined 4 risk categories with median survival of 15.4, 6.5, 2.9, and 1.3 years, respectively.

Most recently, the phenotypic and prognostic relevance of "driver" and "non-driver" mutations was carefully investigated in a series of collaborative studies. In one of these studies involving 617 subjects with PMF, a favorable effect of *CALR* mutations on survival and LFS was demonstrated; the study also documented the poorer survival and shorter LFS of triple-negative patients. Median survival was 17.7 years in *CALR*-mutant, 9.2 years in *JAK2*-mutant, 9.1 years in *MPL*-mutant, and 3.2 years in triple-negative patients. Furthermore, the impact of genetic lesions on survival was independent of the IPSS and DIPSS prognostic scoring systems. The prognostic advantage of *CALR* mutation in PMF regards only patients harboring type 1/type 1-like mutation, as the survival of those harboring type 2/type 2-like mutation does not differ from *JAK2 V617F*-mutated patients.

In recent years, a greater number of molecular abnormalities have been discovered to be associated with reduced survival in PMF. Mutation in at least one of *EZH2*, *ASXL1*, *IDH1/2* and *SRSF2* genes defined a "high molecular risk" (HMR) category associated with shorter survival[83]. The study also found a prognostic relevance of the number of HMR adverse mutations; median survival was 2.6 years for patients with ≥ 2 mutations, 7 years for patients with 1 mutation, and 12.3 years for those with no mutations (LMR). The prognostic significance of HMR muted genes and the numbers of mutated genes were independent of both IPSS and DIPSS-plus and were also predictive of shorter LFS. Despite genetic information is helpful in predicting survival outcome or risk of disease complications, the prognostic value of these somatic mutations or abnormal karyotype have not been integrated yet with other clinical variables in multivariable analyses. Based on these seminal observations, two new prognostic models that incorporate clinical, hematological and mutational status data have been proposed.

Prognostic score developed for PMF is routinely used also in patients with PPV- / PET-MF. However, when these score were applied in PPV/PET-MF patients in retrospective studies, they failed to accurately discriminate different prognostic groups, suggesting a potential limitation of the IPSS/DIPSS in secondary MF. Concerning the prognostic impact of driver mutations, data from a large retrospective study in 359 patients (194 with PPV- and 165 with PET-MF) showed that driver mutations do not appear to meaningfully predict survival, at variance with PMF. In fact, survival among the mutational groups was largely superimposable, even between *CALR* type1/type1-like and type2/type2-like; the only exception was for triple negative patients that, as in PMF, presented the worst prognosis. In the above study mutations in HMR genes in secondary MF did not inform prognosis nor leukemia transformation, with the exception of *SRSF2* mutations that predicted for shorter survival in patients with PET-MF.

Several studies, most limited in PPV-MF, reported a list of adverse prognostic factors for survival: unfavorable cytogenetics (abnormalities other than del13q or del20q), older age, hemoglobin <10 g/dL, evolution from PV platelet count $< 100 \times 10^9$/L, leukocyte count $>30 \times 10^9$/L and treatment with hydroxyurea at the time of diagnosis.

Recently Passamonti et al. developed a new prognostic score, the Myelofibrosis Secondary to PV and ET Prognostic Model (MYSEC)[84]. The project collected retrospectively 685 (333 PET-MF, 352 PPV-MF) consecutive patients from 16 international centers in which multivariate analysis identified independent covariates as predictor of survival. Each variable was assigned a risk point: 0.15 points per each year of age, two points to hemoglobin < 11 g/dL, to circulating blasts ≥ 3%, and to *CALR*-unmutated genotype; one point to the presence of constitutional symptoms and to a platelet count <150 x10^9/L.

According to the sum of risk points, patients were allocated in 4 risk categories: low, intermediate-1, intermediate-2 and high, with corresponding median survival times of not reached, 9.3 years, 4.4 years, and 2 years, respectively.

Impact of mutation status on response to treatment

The advent of next-generation sequencing (NGS) techniques has brought intense interest to the complex genetic landscape of myeloproliferative neoplasms (MPNs), particularly the ability of mutational data to impact on clinical outcomes in the context of novel MPN therapies are limited.

A recent study of 95 patients treated with ruxolitinib using a panel of 29 genes, excluding *SRSF2*, reported

that mutations in any of the HMR genes studied as well as a total of ≥3 mutations were associated with decreased spleen response and a shorter time to discontinuation of therapy[85].

A retrospective analysis including patients enrolled in COMFORT-II trial, which compared ruxolitinib to the best available therapy by using a 14-gene panel, found that HMR mutations HMR status did not predict to spleen or symptoms response[86].

Pardanani and al. evaluated the clinical response stratified by using a gene panel comprised of driver mutations and *ASXL1* in 100 MF patients on momelotinib and found that *CALR*-positive patients had improved spleen response, whereas mutations in *ASXL1* resulted an inferior response[87].

Recently, retrospective studies including patients treated with ruxolitinib and or momelotinib reported that those showing clonal evolution had significantly shorter survival after discontinuation[88] and those with *ASXL1* or *SRSF2* mutation also showed a shorter time to treatment failure[89]. Similar negative influence of additional somatic mutations on the clinical response was observed in *CALR* mutated ET patients treated with interferon-alfa[90]. Analysis of response by mutation status in 33 MF patients treated with imetelstat reported that presence of mutations in *ASXL1* or spliceosome mutations might adversely affect clinical response[91]. These observations suggest that the presence of additional non-driver mutations affects the response to therapy, a finding that deserves further investigation to understand the underlying molecular mechanisms and possible impact on clinical outcome.

Conclusions

The understanding of the mutation landscape of MPN has witnessed significant advances in the last few years with the discovery of phenotypic driver mutations that have revolutioned the diagnostic approach and paved the way for novel treatments with drugs targeting the key JAK/STAT signaling pathway. Patients lacking the three phenotypic driver mutations are very likely harboring novel genetic abnormalities, the objective of intensive efforts from several groups. In addition, findings of recurrent subclonal mutations have added further insights into a previously unforeseen molecular complexity, and much still remains to be learned. On this ground, emergent data support the use of some genetic abnormalities for more accurate prognostication of MPN patients, particularly with PMF, and if substantiated in prospective studies might turn out useful in the managment of MPN patients.

Conflict of interest:
The authors have nothing to declare.

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