Pathogenesis and management of high molecular risk myeloproliferative neoplasms

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Abstract

Classical myeloproliferative neoplasms (MPN) are clonal stem cell disorders characterized by driver mutations that affect the constitutive activation of JAK-signaling. Mutations additional to an MPN-driver occur in a large number of patients and have been shown be associated with disease presentation and progression. In this review, we outline the current hypotheses regarding how clonal evolution in MPN is thought to occur and the functional mechanisms as to how concomitant somatic mutations (i.e., mutations in genes other than the 'driver' genes) contribute to disease progression. We discuss the definitions of high molecular risk MPN, provide an overview of how concomitant mutations influence the clinical management of MPN and suggest how the rapidly developing genetic risk stratification can be utilized to improve clinical outcomes.

Introduction

The classical *BCR::ABL*-negative myeloproliferative neoplasms (MPN), polycythemia vera (PV), essential thrombocythemia (ET) and myelofibrosis (MF), are characterized by driver mutations that affect the constitutive activation of JAK-signaling, such as mutations in Janus kinase 2 (*JAK2*), calreticulin (*CALR*) and the thrombopoietin receptor (*MPL*). While the MPN phenotype is often dominated by excessive production of mature myeloid cells, these driver mutations are initiated and maintained in hematopoietic stem cells (HSC); thus, MPN are considered clonal stem cell disorders. Most patients present in a chronic phase of the disease (i.e., PV, ET, pre-fibrotic MF), with elevated peripheral blood parameters and accompanying systemic inflammation. Relevant clinical challenges include: (i) symptom control, (ii) prevention of thromboembolic complications, and (iii) prevention of disease progression. Symptom control can be achieved by supportive measures, cytoreduction or symptom-oriented therapy (e.g., JAK-inhibitors), and prevention of thromboembolic events can be achieved using acetylsalicylic acid or anticoagulants, and cytoreductive measures, such as phlebotomy or pharmacological agents (e.g., hydroxyurea, interferon or JAK-inhibitors). Patients can also present with more advanced phases of the disease resulting from excessive fibrotic deposition in the bone marrow (i.e., fibrotic phase of MF) and exhibit aggravated symptoms, splenomegaly and cytopenias that require pharmacological and supportive interventions. Up to 40% of MPN patients experience disease progression during their lifetime, either from chronic phase to fibrotic MF, or chronic phase/MF to an accelerated phase (10-20% blasts) or overt acute myeloid leukemia (AML) (≥20% blasts), also referred to as blast-phase MPN. Progression is frequently associated with clinical deterioration and shortened overall survival. Allogeneic hematopoietic stem cell transplantation (HSCT) is the only potentially curative therapy currently available for MPN; however, due to high morbidity and mortality rates, it is only indicated for younger/fitter patients at higher risk of disease progression.

Next-generation sequencing technology has facilitated increased resolution of the mutational landscape that underpins the process of malignant transformation in humans. This is exemplified by AML, in which particular patterns of mutational co-occurrence define prognostically relevant subclasses.[1] Somatic co-mutations of relevance in myeloid cancers, in addition to MPN-drivers, are present in approximately 50% of patients with chronic phase MPN.[2-5] Of note, these studies highlight genetic subgroups associated with outcomes, independent of clinical phenotypes. The number

of mutations increases with disease progression and correlates with progression to MF and accelerated/blast phase MPN.[2,4] The concept and definition of high molecular risk (HMR) mutations have facilitated the use of these mutations in addition to clinical disease parameters to predict patients' outcomes and inform treatment decisions.

In this review, we outline the current hypotheses regarding how clonal evolution in MPN is thought to occur and the functional mechanisms as to how concomitant somatic mutations (i.e., mutations in genes other than the 'driver' genes, *JAK2*, *CALR* and *MPL*) contribute to disease progression. We discuss the definitions of HMR MPN and provide an overview as to how concomitant mutations influence the clinical management of MPN and suggest how this rapidly developing genetic risk stratification can be utilized to improve clinical outcomes.

Mechanisms of mutational acquisition

Chronic phase MPN is a neoplastic state consequent to a single oncogenic driver, such as *JAK2* V617F, which is necessary but insufficient for secondary transformation. However, the complexity and phenotypic diversity in the pathogenesis of MPN cannot always be explained by progressive consequences of a single genetic driver event and thus may be related to the presence of concomitant somatic mutations. Clonal dynamics of MPN are further complicated by the understanding that the MPN-driver mutation is not always the initiating mutational event.[6] Therefore, the acquisition of additional somatic mutations in MPN must be considered as a function of both an MPN-driver and pre-existing somatic mutations (e.g., as in clonal hematopoiesis).

Mediated by myeloproliferative neoplasm drivers

In HSC, the *JAK2* V617F mutation accelerates cell division and is associated with increased DNA damage.[7,8] Increased DNA damage can be considered a precursor to the genetic instability inherent to most cancer genomes and has been established as causal in malignant transformation. In chronic phase MPN, this state of DNA damage may provide a selective pressure for the loss of key regulators of DNA-damage checkpoints, such as p53. Alternatively, it could facilitate an increased rate of mutagenesis, leading to the emergence of mutations that confer a further selective advantage. Current evidence suggests that this is mediated in a cell-intrinsic manner through the ability of JAK2 activation to directly drive downstream PI3K-AKT signaling.[7] However, *Jak2*-mutant cells may also generate an inflammatory microenvironment,[9] which enhances mutagenesis,[10] and specifically provides a selective advantage for the loss of p53.[11]

In contrast, longitudinal studies in serial human samples found a low mutation rate of one mutation per 66 patient-years,[12] arguing against a strong hypermutable state in MPN. Consistently, individuals exclusively harboring *JAK2* mutations may experience long-term stability of the mutated clone or even clonal regression. Lineage tracing approaches to assess the time course of clonal expansion provided the first evidence that driver mutations can be acquired decades before the clinical manifestations of MPN,[13,14] as in some cases, *JAK2* mutations could already be detected in cord blood. However, data investigating the behavior of mutated cells from 385 older individuals found growth trajectories of *JAK2*-mutated clones to be particularly erratic, with only 58% displaying stable growth.[15] The reason for this behavior remains unclear.

Mediated by age-related clonal hematopoiesis

Somatic mutations acquired prior to the driver (e.g., a *JAK2* mutation) may provide a 'fertile ground' for malignant transformation.[8,12] Clonal hematopoiesis is the situation in which the maturing cell progeny derived from a single HSC ancestor, or "clone", dominates the hematopoietic compartment of an individual. Clonal hematopoiesis occurs as a consequence of declining clonal diversity during aging and may not be associated with a neoplastic state, leading to the terminology of age-related clonal hematopoiesis (ARCH) or clonal hematopoiesis of indeterminate potential (CHIP). Mutations with relevance in myeloid malignancies include *DNMT3A*, *TET2* and *ASXL1* (the so-called DTA mutations), which account for over 90% of cases of ARCH. Due to its association with increased risk of future development of myeloid malignancies, including MPN, ARCH can thus represent a pre-neoplastic state.[16] Consistently, DTA mutations are found in both MPN-driver-positive and -negative cells in post-MPN AML.[3,4] Although they influence the balance towards self-renewal over lineage commitment, there is no definitive evidence that DTA mutations lead to enhanced mutational acquisition. *Dnmt3a*-null murine HSC show progressive loss in clonal diversity leading to increased variant allele frequency of existing somatic variants that have been maintained in expanded clones.[17] DTA mutations may, therefore, rather facilitate continued clonal expansion in the presence of a mutagenic stimulus, such as inflammation.[18]

Pathways of clonal evolution in myeloproliferative neoplasms

Although the chronic, accelerated and blast phases of MPN can be appreciated as a linear trajectory, this pathway of disease progression is not uniform. Patients can present with MF without a prior diagnosis of PV or ET, or accelerated/blast phases without a prior diagnosis of MF. Furthermore, disease progression to MF or accelerated/blast phases is not an inevitable outcome in PV or ET, with chronic phases sometimes lasting for many decades. The linear directionality to the evolution of MPN is further challenged and complicated by the fact that an MPN-driver mutation may not always represent the foundational event in MPN and

can be either present or absent in post-MPN AML blasts.[3,4] MPN may therefore emerge and progress along separable evolutionary paths dictated by the order of mutational events, being either linear, branching or parallel (Figure 1). The validity of these potential paths can be determined by the answers to two key questions.

The first question is: is it possible that an MPN driver mutation is lost from a cell as it undergoes leukemic transformation? *JAK2* V617F homozygosity can be detected in patients with MPN and gene dosage influences therapy response and the clinical phenotype. Homozygosity is presumed to occur as a consequence of mitotic recombination, which would result in the generation of both *JAK2* homozygous mutant and wild-type progeny from a *JAK2* V617F heterozygous founder. However, single nucleotide polymorphisms within and telomeric to a mutant *JAK2* locus can only be identified in *JAK2* V617F homozygous clones, both supporting the occurrence of mitotic recombination and indicating that *JAK2* wild-type loss of heterozygosity progeny do not expand appreciably in MPN patients.[19] Furthermore, in *JAK2* wild-type post-MPN AML, loss of heterozygosity, determined by single nucleotide polymorphism genotyping, was not detected in leukemic blasts,[19,20] providing evidence against loss of *JAK2* V617F during blast transformation.

The second question is: can AML evolve in the context of MPN from an independent clonal precursor? Combined analysis of *JAK2* V617F granulocytes and *JAK2* wild-type leukemic blasts from the same patient has demonstrated inactivation of the same parental X-chromosome.[20] Also, shared somatic mutations in *JAK2* V617F cells and *JAK2* wild-type leukemic blasts (like those with DTA mutations), support the hypothesis that MPN and post-MPN AML share a common clonal ancestor (Figure 1, branching evolution). However, the possibility that MPN and AML may arise in the same individual independently (Figure 1, parallel evolution) cannot be excluded, especially considering the inflammatory microenvironment that occurs in MPN.

Together, these findings suggest that transformation to MPN-driver-expressing AML is most likely a consequence of linear evolution whereas MPN-driver-negative AML is rather a consequence of branching or parallel evolution (Figure 1). Pathways of branching and parallel evolution in MPN do, however, appear to be less efficient, given that retrospective analysis across multiple independent cohorts of post-MPN AML suggest that MPN-driver-positive leukemia accounts for approximately 80% of this disease subset.[4,5,21]

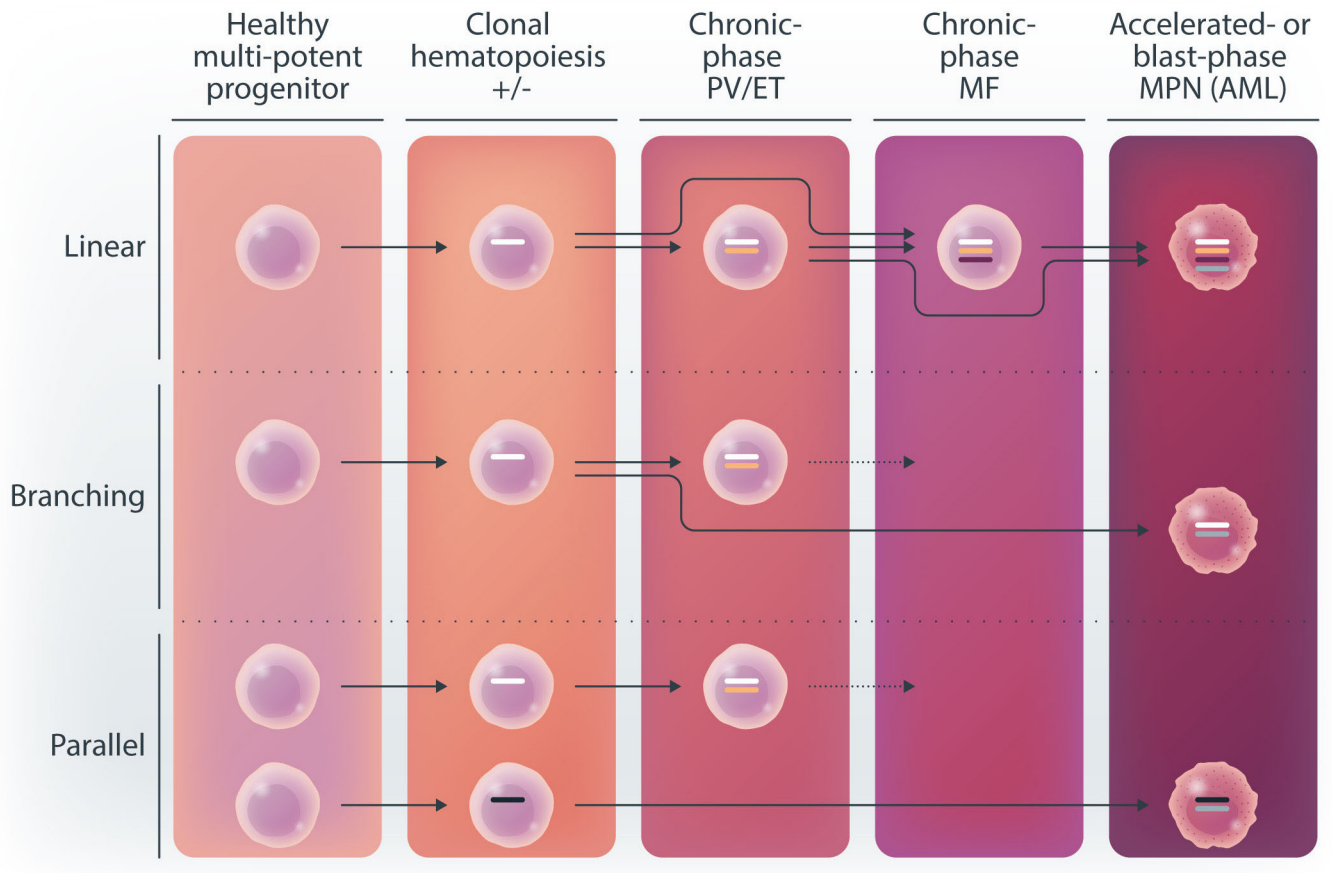

Figure 1. Potential paths of clonal evolution in myeloproliferative neoplasms. The combinations of mutations that occur in chronic phase myeloproliferative neoplasms (MPN) and disease progression to accelerated or blast-phase MPN (acute myeloid leukemia, AML) can be explained by the theories of linear, branching and parallel evolution. Linear evolution pertains to the sequential acquisition of multiple mutations within the same clone. In this scenario, the founder mutation may represent the MPN-driver or a somatic mutation known to drive clonal hematopoiesis. Branching evolution pertains to the emergence of MPN and AML in separate clones with a common clonal ancestor harboring a driver of clonal hematopoiesis. Parallel evolution pertains to the emergence of MPN and AML in separate clones with no common ancestor. The acquisition of an MPN or AML driver may be preceded by a driver of clonal hematopoiesis. It is noted that prior presentation of polycythemia vera or essential thrombocythemia is not required for the emergence of myelofibrosis, and prior presentation of myelofibrosis is not required for the emergence of AML. PV: polycythemia vera; ET: essential thrombocythemia; MF: myelofibrosis.

Functional consequences of mutational heterogeneity

Somatic mutations that occur in MPN include epigenetic regulators, splicing factors and regulators of transcription.[2] The majority of functional studies on concomitant mutations in MPN have employed genetically modified mouse models, with engineered alleles either constitutively expressed or conditionally activated and restricted to the hematopoietic system either through tissue-specific Cre-recombinases or generation of bone marrow chimeras.

Epigenetic regulators

Epigenetic regulators, including DTA, are frequently mutated in MPN.[2-5,21] As in ARCH, DTA mutations are implicated in self-renewal of MPN HSC,[22-25] counteracting the reduced HSC quiescence[26] and limited long-term replicative potential mediated by *Jak2* V617F expression. Accordingly, both *Tet2* and *Dnmt3a*-loss are associated with increased expression of stemness-related genes.[22,23] However, additional DTA mutations occur more frequently in patients diagnosed with MF, than in those with PV or ET,[2] suggesting co-existing DTA mutations also modify the MPN phenotype, rather than just maintain it. These observations can be reconciled by considering MF as a function of time, which can be accelerated by co-occurring DTA mutations. Consistent with this, MF is diagnosed at a higher mean age than ET and PV, and transgenic expression of *JAK2* V617F is sufficient to drive MF in mice, albeit with incomplete penetrance, in aged cohorts.[27,28] However, the acceleration of MF onset with *Dnmt3a*-loss in combination with a conditional *Jak2* V617F allele is also associated with increased expression of genes involved in tumor necrosis factor-α signaling,[22] suggesting *Dnmt3a*-loss may directly promote fibrosis. These transcriptional changes with *Dnmt3a*-loss are not as apparent *in vitro*,[22] suggesting an important interplay with the bone marrow microenvironment. Similarly, heterozygous *Asxl1*-loss accelerates MF latency in the context of a *JAK2* V617F transgenic allele.[25,28] Notably, *Tet2*-loss does not drive MF in mice. It is unclear whether this reflects discrete functional consequences of the individual DTA mutations, or rather nuances in the respective experimental models of MPN.

Mutations in other epigenetic regulator genes are also relevant in MPN. *Ezh2*-loss and expression of gain-of-function mutations in *IDH1* and *Idh2* increase the repopulation potential of *Jak2* V617F HSC. *Ezh2*-loss exacerbates the fully penetrant MF phenotype in both *JAK2* V617F transgenic and knock-in strains. This is associated with a bias towards megakaryocyte differentiation at the expense of erythropoiesis.[29,30] The increased fibrosis is recapitulated with megakaryocyte-restricted loss of *Ezh2* using *Pf4*-Cre.[29] The terminal disease phenotype in both *IDH1* and *Idh2* co-mutated strains, however, appears to be comparable to that of *Jak2* V617F alone.[31] Interestingly, co-existing *IDH* mutations also reduced erythroid bias without a compensatory increase in megakaryocytes. In contrast, *Asxl1*-loss in combination with *Jak2* V617F promotes megakaryocyte differentiation but not at the expense of erythropoiesis,[25] whereas *Tet2*-loss does not alter lineage commitment.[23] Together, these findings suggest that MF may be driven by dysregulation of epigenetic modifiers, with lineage-specific consequences, primarily within the megakaryocyte lineage. The order of mutation acquisition is also important in MPN pathogenesis. To date, this has not been tested in mice directly. Using primary patients' samples in colony assays, studies have demonstrated that *TET2* mutations acquired prior to an MPN-driver reduce mature cell expansion, associated with a less severe disease presentation. This is consistent with *TET2* mutations shifting the balance towards self-renewal over differentiation. When *TET2* mutations are acquired subsequent to an MPN-driver, however, they facilitate HSC expansion while mature cell expansion is largely mediated by the MPN-driver-only clone.[6] This contrasts with functional studies in mice demonstrating exacerbation of the disease phenotype with co-existing *Tet2*-loss compared to *Jak2* V167F alone.[23] The order of *DNMT3A* mutational acquisition in relation to an MPN-driver is also associated with distinct cellular and clinical phenotypes.[32]

Splicing factors

Mutations in genes encoding components of the spliceosome have been identified in MPN, notably in *SRSF2*, *U2AF1*, *SF3B1* and *ZRSR2*, some of which confer inferior clinical prognosis. Surprisingly, co-expression of *Srsf2* P95H reduces MPN severity in *Jak2* V617F knock-in mice, as evidenced by reduced splenomegaly and blood cell counts.[33] Furthermore, *Srsf2* P95H co-expression can attenuate MF and reduce the expansion and repopulation capacity of HSC.[33] However, serial transplantation studies demonstrated the capacity of *Srsf2* P95H co-expression to prevent *Jak2* V617F stem cell exhaustion by extending the long-term replicative capacity of the stem cells.[34] These functional studies may suggest additional cooperating factors are necessary for spliceosome mutations to alter MPN disease pathology.

Recently, global analysis of the *Jak2* V617F-mediated phosphoproteomic landscape has identified molecules related to mRNA splicing and processing as relevant targets of *Jak2*-dependent post-translational modification. Inactivation of non-mutated splicing factors sensitized Jak-inhibitor persistent cells to apoptosis and resulted in RNA mis-splicing, intron retention and eventually disruption of relevant oncogenic signaling pathways. Genetic and pharmacological inactivation of these molecules and pathways induced regression of the malignant clone and molecular remission.[35] Therefore, post-translational modification of (unmutated) splicing factors may contribute to clonal persistence and progression of MPN.

Transcriptional regulators

Mutations in the transcription factors *RUNX1* and *TP53*

have been associated with post-MPN AML.[3,4,36] Strikingly, none of the individual aforementioned concomitant mutations investigated in murine functional studies appears sufficient in combination with *Jak2* mutations for leukemic transformation, with the exception of *Trp53*. Loss of *Trp53* function in combination with a *JAK2*-driver mutation drives a fully penetrant AML[3,37,38] following a preceding MPN disease phase.[37] Here, megakaryocytic-erythroid progenitors are the leukemia-initiating cell population able to generate AML directly in secondary recipients. In contrast, transplantation of more primitive LSK (lineage-negative, $Sca1^+$, kit^+) cells only generates leukemia after a phase of MPN disease.[37] This finding suggests that *Trp53* loss alone may not be sufficient to drive leukemia on a *JAK2*-mutated background. Consistent with this, p53 inhibition in chronic phase *JAK2*-mutated MPN increases DNA damage in erythroblasts without affecting these cells' survival or proliferation.[7] It has also been shown that bone marrow from *Jak2* V617F/*Trp53*-null leukemic mice harbors recurrent chromosomal copy number variations, which are absent in the MPN phase.[37] Furthermore, megakaryocytic-erythroid progenitors isolated from *Jak2* V617F/*Trp53*-null leukemic mice are transcriptionally distinct from those isolated from chronic phase *Jak2* V617F/*Trp53*-null MPN mice, suggesting that the altered expression of genes contained in these regions of recurrent chromosomal losses and/or gains may be responsible for leukemic transformation.

Further functional studies of two-way genetic interactions will continue to delineate the nature of cooperation between MPN driver and concomitant mutation(s) that determine progression to myelofibrosis and AML in *JAK2*, *CALR* and *MPL*-mutated contexts. Beyond *TP53*, mutational burden represents the best genetic predictor of inferior outcomes in chronic phase, accelerated phase and blast phase MPN. Therefore, the functional consequences of three- and four-way genetic interactions with all MPN drivers on MPN disease progression will be valuable to expand the understanding of the pathogenesis of HMR MPN.

Influence of mutational heterogeneity on current clinical management of myeloproliferative neoplasms

We now discuss how molecular risk is defined clinically and how it influences the clinical management of MPN. Current clinical guidelines and consensus documents[39-41] provide algorithms for the management of patients with MPN, separated into the clinical phenotypes of PV, ET and MF. The canonical driver mutations (*JAK2*, *MPL* and *CALR*) occur as a sole genetic abnormality in 45% of MPN, but more frequently co-occur with concomitant (passenger) mutations, especially in MF.[2] Approximately 5-10% of patients with ET or MF lack a canonical driver and are termed 'triple-negative'.[2,42] Patients with triple-negative MF may harbor non-driver mutations, especially within spliceosome and chromatin-modifying genes and have inferior survival compared to MF patients with a canonical driver.[42,43] These 'additional' mutations (regardless of driver status) have prognostic influence and implications for response to therapy and survival. The clinical definition of HRM differs for PV, ET and MF. It derives from NGS-profiling studies of cohorts of patients, which have identified genetic mutations associated with inferior overall survival, leukemia-free survival and (for PV and ET) MF-free survival. A large study incorporating 2,035 patients (including 1,321 with ET, 356 with PV, 309 with MF and 49 with other MPN) demonstrated that mutations in chromatin-modifier genes (e.g., *ASXL1*, *EZH2*) and spliceosome genes (e.g., *ZRSR2*, *SRSF2*), defined a genomic subgroup with inferior prognosis.[2] In this cohort, 63 clinical and genomic variables were identified as significant for prognosis and integrated to design a prognostic model. This model is directly accessible via an online calculator (*https://cancer.sanger.ac.uk/mpn-multistage/*), facilitating 'personalized' predictions of prognosis for individuals and has been approved as a medical device within the United Kingdom (*https://blood.predict.nhs.uk/*). Studies such as this highlight the heterogeneous landscape of concomitant mutations as well as the complexity of integration into prognostication and selection of therapeutic approaches in clinical practice.

Polcythemia vera

Box 1. Management of polycythemia vera in a snapshot.
In PV, hyperproliferation of erythropoiesis is predominant, resulting in high hematocrit levels, and the therapeutic focus is on reducing the risk of venous and arterial thromboembolic complications and mitigating symptoms. This is achieved by anti-platelet therapy (100 mg of aspirin daily) in all PV patients[44] and cytoreduction using venesection and/or cytoreductive therapies targeting a hematocrit of <0.45.[45] The indication for medical cytoreductive therapies is predominantly based on stratification of thrombosis risk, integrating risk factors such as age ≥60 years, prior thromboembolic event, cardiovascular risk factors and leukocytosis,[41] although other factors including platelet counts >1,000x10^9/L, symptomatic splenomegaly, microcirculatory disturbances and persistently high frequency of phlebotomy (>6 times/year) also represent indications. Hydroxyurea remains the most frequently used drug worldwide for primary PV therapy. Limitations, including skin and mucosal toxicity, poor symptom control, and potential leukemogenic risk, have prompted investigation of other therapies.

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Ropeginterferon α2b has shown superiority in clinical trials[46] and was recently approved for PV treatment in several countries. For second-line treatment after hydroxyurea refractoriness or intolerance, ruxolitinib is effective for cytoreduction and symptom control compared with best available therapies.[47,48] Progression to secondary MF occurs in 10-15% of cases and to secondary leukemia in up to 15% of patients with PV and 25% of those with post PV-MF[43] thus representing significant causes of morbidity and mortality in PV.

Influence of high molecular risk on prognostication in polycythemia vera

Concomitant mutations in PV can predict a higher risk of reduced survival and disease progression, as well as thrombosis. Mutations in *ASXL1*, *SRSF2* and *IDH2* were associated with reduced overall survival and leukemia-free survival,[49] with *SRSF2* integrated into the weighted prognostic score, Mutation-Enhanced International Prognostic Scoring System for PV (MIPSS-PV), which stratifies patients into low, intermediate and high risk for survival.[50] The Multiple Factor-Based Prognostic Score (MFPS) is an assessment tool used to predict the risk of thrombosis in PV patients. This scoring system includes factors such as age (≥60 years), cardiovascular risk factors, history of thrombosis and presence of specific high-risk mutations (e.g., *DNMT3A*, *ASXL1*, *BCOR*, *BCORL1*), assigned a weighted score. Patients are classified into low-risk (0-1 points), intermediate-risk (2-3 points), and high-risk (≥4 points) groups. This system has shown better predictive power compared to previous models.[51]

Influence of high molecular risk on response to therapy

Further groups investigated the impact of concomitant mutations and clonal evolution on responses to specific therapies in PV and/or other MPN types. The effect of frequent concomitant mutations (*TET2*, *ASXL1*, *DNMT3A*, *EZH2*, *IDH1* and *IDH2*, sequenced by Sanger sequencing) on response to interferon in PV and ET was investigated.[52] In this study, 17% of patients achieved a complete molecular response (defined as undetectable *JAK2* V617F based on an assay with a sensitivity of 5%) over a median follow-up of 42 months. In those failing to achieve complete molecular response, there was a trend towards higher frequency of concomitant mutations at baseline and more frequent acquisition of new mutations (64% *vs*. 0% in those who achieved complete molecular response). In patients with complete molecular response, concomitant mutations were also cleared. Genomic predictors of response were investigated in the DALIAH trial (low-dose interferon-α *vs*. hydroxyurea) including 202 patients with PV, ET and MF.[53] Patients had routine re-sequencing at 24 months (N=135). Treatment-emergent mutations were detected in 32 patients (24%) at 24 months. In interferon-α-treated patients, these were most commonly DTA mutations. Interestingly, *TP53* and *PPM1D* were commonly acquired mutations in hydroxyurea-treated patients, paralleling findings of *TP53* and *PPM1D* clonal hematopoiesis emerging after cytotoxic therapy in patients with solid cancers.[54,55] The emergence of *DNMT3A* mutations was the only factor described in the study to be associated with failure to achieve complete hematologic response to interferon-α. The authors speculated that *DNMT3A*-mutated clones were likely pre-existing at baseline and were selected for by the interferon-α therapy, perhaps due to aberrant self-renewal described as a consequence of cooperation between *JAK2* V617F and *DNMT3A* mutations.[22] Recent preclinical studies in *JAK2* V617F and *Dnmt3a*-mutant murine hematopoiesis provided the first evidence that combined treatment with interferon-α and hypomethylating agents (5-azacitidine) enhances clonal regression, overcoming the adverse effect of the *Dnmt3a* co-mutation.[56] In a randomized phase III study comparing the effects of ropeg-interferon α2b against best available therapy, PV patients harboring *ASXL1* mutations showed relevant responses when treated with interferon-α.[57] In the MAJIC-PV study, ruxolitinib produced a higher rate of molecular response (defined as a 50% reduction in *JAK2* V617F allele frequency), which was associated with improved progression-free survival, event-free survival, and overall survival. Here, the presence of *ASXL1* mutations was associated with worse event-free survival (adjusted hazard ratio = 3.02 compared to those without these mutations).[48] An additional study supported the negative impact of additional baseline mutations on progression-free survival event rate during ruxolitinib treatment, and further highlighted the association between acquisition of new variants, especially in *ASXL1*, during treatment and reduced molecular responses and increased progression to MF.[58]

Implications of high molecular risk on management of polycythemia vera

Currently, the presence of HMR mutations does not alter management recommendations or target thresholds for cytoreductive therapies. While there is increasing information about predictors of response to therapies such as interferon, there is no clear recommendation to select therapies based on concomitant mutational profile. Given that concomitant mutations can be cleared with the driver mutation in some cases, it could be speculated that selection of therapies that have increased chance of inducing molecular remissions might be preferable. Allogeneic HSCT is currently not recommended for the treatment of PV that has not clinically progressed to secondary MF or AML, even in the presence of HMR mutations. Nevertheless, genomic features are highly predictive of progression from chronic phase to the accelerated phase or blast phase[2] and MIPSS-PV high-risk patients have a predicted median overall survival of 4.6 years.[59] Thus, close monitoring for

clinical symptoms and signs of clonal progression, especially in the setting of HMR is warranted to facilitate early referral of eligible patients for allogeneic HSCT.

Essential thrombocythemia

Box 2. Management of essential thrombocythemia in a snapshot. *ET phenotypically affects the megakaryocytic cell line and is characterized by peripheral thrombocytosis and hyperproliferation of large, hyperlobulated megakaryocytes in the bone marrow. ET should be distinguished from pre-fibrotic MF or PV. Reactive thrombocytosis represents a further differential diagnosis and should be strongly considered in 'triple-negative ET'. The primary therapeutic goal in the treatment of ET is to prevent thromboembolism and bleeding events (due to extreme thrombocytosis-related secondary von Willebrand syndrome), as well as to reduce disease-associated symptoms.[39] The indication for treatment is based on the thrombosis risk profile guided by established and guideline-anchored risk stratification systems, dividing patients into low, intermediate and high risk for thrombosis.[41] Numerous studies have shown that the presence of a JAK2 mutation is associated with a significantly higher risk of thrombosis compared to the presence of CALR mutations. JAK2 mutation is incorporated as a risk factor into scores such as the International Prognostic Score of thrombosis in Essential Thrombocythemia (IPSET-thrombosis) score.[60] Treatment strategies include low-dose aspirin for intermediate- and high-risk patients with microcirculatory disturbances, in those without contraindications due to bleeding risk, although prospective studies for this recommendation are lacking. Data suggest that low-risk patients with a CALR mutation do not benefit from antiplatelet therapy.[61] Cytoreductive medication is specifically indicated for high-risk patients and options include hydroxyurea, anagrelide and peg-interferon (where approved).[62] Ropeg-interferon is being evaluated in an international multicenter trial of patients with ET.[63] Data on ruxolitinib from two studies in relatively small patient populations with hydroxyurea-refractory or -intolerant ET showed a reduction in platelets and leukocytes and an improvement in ET-associated symptoms, but no significant improvement in the hematologic complete remission rate or the rate of thrombosis, hemorrhages, or leukemic transformation.[64,65]*

Influence of high molecular risk on prognostication in essential thrombocythemia

In ET, mutations in *SH2B3*, *SF3B1*, *U2AF1*, *TP53*, *IDH2*, and *EZH2* were associated with inferior overall survival.[49] In a further analysis, *SF3B1* and *SRSF2* predicted reduced overall survival, while mutations in *SF3B1* and *U2AF1* predicted reduced MF-free survival and those in *TP53* predicted inferior leukemia-free survival. These genes have been integrated into the MIPSS-ET score that significantly stratifies patients into low, intermediate and high risk for survival.[50]

Implications of high molecular risk on management of essential thrombocythemia

Overall, the risk of progression in ET is considerably low. Most data on risk factors were obtained from patients diagnosed before the World Health Organization (WHO) 2016 classification of MPN separated ET from the pre-fibrotic phase of MF. For ET, prevention of thromboembolic complications is therefore the major clinical challenge. Monitoring of molecular risk mutations may be indicated.

Myelofibrosis

Box 3. Management of primary myelofibrosis in a snapshot. *The early phase of primary MF is associated with an increase in megakaryocytic and granulocytic proliferation. Later, bone marrow fibrosis accompanied by progressive splenomegaly and pancytopenia may be the predominant phenotype. According to the current WHO 2022 classification, the pre-fibrotic phase is distinguished from overt (fibrotic) MF. Therapeutic strategies are based on risk of progression and symptom burden. In addition to dynamic risk scores with emphasis on clinical and hematologic parameters, molecular and cytogenetically driven predictors are currently gaining relevance to allow reliable risk stratification especially for younger patients (Table 1). The curative option of allogeneic HSCT is recommended for intermediate-2- or high-risk patients, especially for younger patients with primary MF and those without relevant comorbidities. Here, pretreatment with JAK-inhibitors is beneficial, especially for patients with splenomegaly and symptom burden. Symptom-oriented treatment with JAK-inhibitors or experimental therapies in clinical trials are available, if allogeneic HSCT is not indicated or possible.*

Influence of high molecular risk on prognostication in primary myelofibrosis

Risk stratification tools for MF (Table 1) predict overall survival and leukemia-free survival and are used to determine treatment approaches for patients. Newer risk classifications such as the Mutation-enhanced International Prognostic Scoring System-70 (MIPSS70)[66] and its iterations[67] and the Genetically inspired International Prognostic Scoring System (GIPSS) incorporate molecular and other genetic data into risk stratification scores that help to identify patients at high risk of disease progression who were previously not identified by clinical markers. These represent evolutions from more traditional prognostic scores which solely assess clinical and hematologic factors, such as the International Prognostic Scoring System (IPSS)[68] and

Dynamic IPSS (DIPSS)[69] and with the additional prognostic influence of karyotype, DIPSS-plus.[70]

In early studies, the prognostic influence of molecular mutations in MF using Sanger sequencing analysis of a limited panel of genes including *EZH2*, *TET2*, *DNMT3A*, *CBL*, *ASXL1*, *IDH1/2*, *SRSF2* and *MPL* was highlighted.[71] A*SXL1*, *EZH2*, *SRSF2*, *IDH1* and *IDH2* were associated with a high risk of death or leukemic transformation. The number of mutations was also predictive, with two or more being associated with inferior leukemia-free survival.[72] The mutation status of these five genes (coined HMR mutations) as well as absence of *CALR* type 1/-like mutations, were then combined with clinical variables in the MIPSS70 model, stratifying patients into low, intermediate and high-risk patients. Further iterations incorporated karyotypic information (MIPSS70-plus)[66] and added the *U2AF1* Q157 hotspot variant as an additional HMR variant (MIPSS70-plus version 2.0),[73] which is now widely used aided by an online calculator (*http://www.mipss70score.it/*). GIPSS is solely based on genetic mutations and karyotype abnormalities, without the inclusion of clinical variables such as symptoms, blood counts, or the degree of fibrosis.[74]

Additional high-risk mutations have been identified with expanded molecular characterization. A study using a 77-gene NGS panel to molecularly profile patients with MF (N=479, of whom 305 with primary MF and 174 with secondary MF [70 post-PV and 104 post-ET]) was performed by the French Intergroup of Myeloproliferative Neoplasms (FIM).[75] Within this cohort, four prognostic groups with significantly different overall and leukemia-free survival rates were identified including: (i) *TP53*-mutated (median overall survival, 20 months), (ii) presence of ≥1 high-risk mutation (*EZH2*, *CBL*, *U2AF1*, *SRSF2*, *IDH1*, *IDH2*, *NRAS* or *KRAS*) (median overall survival, 49 months), (iii) *ASXL1* without *TP53* or other high-risk mutation as listed above (*ASXL1*^{mut}-only) (median overall survival, 90 months) and (iv) other mutational profiles (median overall survival, 116 months). When assessed in multivariate analyses with clinical and hema-

Table 1. Prognostic stratification scores in myelofibrosis.

Prognostic score	For use			Patient characteristics	PB/BM parameters[a]	Karyotype	Molecular features
	At Dx	After Dx	SMF				
IPSS	Yes	-	-	Age >65 years Constitutional symptoms	Hb <10 WBC >25 Circulating blasts ≥1%	-	-
DIPSS	-	Yes	-	As for IPSS	As for IPSS (weighting on Hb <10)	-	-
DIPSS-Plus	-	Yes	-	As for IPSS	As for DIPSS. Plus, need for RBC transfusion Platelets <100	Yes[b]	-
MIPSS70	Yes	-	-	(validated in age ≤70 years) Constitutional symptoms	Hb <10 WBC >25 Platelets <100 Circulating blasts ≥2% BM fibrosis ≥MF-2	No	Absence of *CALR* type 1/-like mutation Presence of ≥2 HMR mutations[c]
MIPSS70-Plus	Yes	-	-	(validated in age ≤70 years) Constitutional symptoms	Hb <10 Circulating blasts ≥2%	Yes - high risk	Absence of *CALR* type 1 mutation Presence of ≥2 HMR mutations[c]
MIPSS70-Plus v2.0	Yes	-	-	-	As for MIPSS70-Plus with adjusted Hb thresholds	Yes - high and very high risk	As for MIPSS70-Plus *U2AF1* Q157 included as an HMR mutation
GIPSS	Yes	-	-	-	-	Yes – very high risk and unfavorable karyotype	Absence of *CALR* type 1/-like mutation Presence of HMR mutations[d]
MYSEC-PM	-	-	Yes	Age Constitutional symptoms	Hb <11 Circulating blasts ≥3% Platelets <150	-	*CALR*-unmutated

[a]Hemoglobin concentration, g/dL; white blood cell and platelet counts, x10^9/L. [b]Complex karyotype or one or two abnormalities that include +8, −7/7q-, i(17q), −5/5q-, 12p-, inv(3), or 11q23 rearrangement. [c]*ASXL1*, *EZH2*, *SRSF2*, and *IDH1/2*. [d]*ASXL1*, *SRSF2*, or *U2AF1* Q157. Dx: diagnosis; SMF: secondary myelofibrosis; PB: peripheral blood; BM: bone marrow; IPSS: International Prognostic Scoring System; Hb: hemoglobin; WBC: white blood cells; DIPSS: Dynamic International Prognostic Scoring System; DIPSS-Plus: DIPSS incorporating additional risk factors; RBC: red blood cell; MIPSS70: Mutation-enhanced International Prognostic Scoring System for transplantation-age patients with primary myelofibrosis; MF-2: fibrotic stage of myelofibrosis; HMR: high molecular risk; MIPSS70-Plus: MIPSS70 incorporating cytogenetic data; MIPSS70-Plus v2.0: Mutation and karyotype-enhanced International Prognostic Scoring System for primary myelofibrosis in adults 70 and younger; GIPSS: Genetically inspired International Prognostic Scoring System; MYSEC-PM: Myelofibrosis Secondary-Prognostic Model.

tologic factors, the *TP53*-mutated and high-risk mutation group independently maintained higher risks of death and progression to leukemia. The study thus provided evidence of additional mutations that could be considered 'HMR', including *TP53*, *CBL*, *NRAS*, *KRAS* and all *U2AF1* variants and highlighted a context-specific qualifier for *ASXL1* mutations, which were not independently adversely prognostic when occurring without high-risk mutations. Notably, *RAS*-pathway mutations have been independently associated with inferior overall survival in multiple independent cohorts of MF patients.[76,77]

Influence of high molecular risk on response to therapy

Various groups have examined molecular predictors of response to ruxolitinib. Predictive factors differ among groups but overall suggest that high-risk mutations do not preclude responses to ruxolitinib, but may shorten the durability of response. The efficacy of ruxolitinib compared to best available therapy in IPSS intermediate-2 or high-risk MF was established in the phase III COMFORT-I and II trials.[78,79] Molecular profiling of 14 myeloid genes was performed and analyzed in a representative subset of the COMFORT-II patients to determine molecular predictors of response.[80] High-risk mutations in this study (*ASXL1*, *EZH2*, *SRSF2* and *IDH1/2*) conferred inferior survival compared to that of patients without these mutations within the best available therapy arm. Among the patients treated with ruxolitinib, those with high-risk mutations still demonstrated equivalent benefits of ruxolitinib with no statistical differences between spleen response, constitutional symptoms or survival. In contrast, recent studies on 95 ruxolitinib-treated patients using a 28-gene NGS panel (notably not including *SRSF2*),[81] revealed that mutations in *ASXL1*, *EZH2* or *IDH1/2*, as well as the presence of ≥3 mutations were associated with lower rates of spleen response, time to treatment discontinuation and shorter overall survival (in contrast to the COMFORT-II analyses[80]). *ASXL1* or *EZH2* mutations along with other clinical factors of transfusion dependence prior to JAK-inhibitors and high DIPSS score were also identified as predictive factors for failure of treatment with JAK-inhibitors, ruxolitinib or momelotinib.[82] Lower frequency mutations, including those in *NRAS*, *KRAS* and *CBL* were shown to associate with reduced symptom and spleen response to ruxolitinib.[76]

Clonal evolution during therapy has also been associated with poor responses to ruxolitinib treatment. In 46 ruxolitinib- and 25 hydroxyurea-treated patients with MF, sequential samples demonstrated acquisition of new mutations in eight of the ruxolitinib-treated patients (17.4%) compared to six of the hydroxyurea-treated patients (24%).[83] The presence of HMR mutations (*ASXL1*, *EZH2*, *SRSF2*, *IDH1*, IDH2) at baseline did not alter spleen and symptomatic responses; however HMR, as well as acquisition of new clones, was associated with loss of spleen responses at 3 years. Notably, similar patterns and rates of clonal evolution were seen in both ruxolitinib and hydroxyurea-treated patients, suggesting that clonal evolution was associated with disease rather than treatment received. Outcomes of 107 patients with MF who discontinued ruxolitinib were also reported.[84] At the time of discontinuation, 14 (33%) patients had acquired at least one additional mutation during treatment, with the majority (64%) being variants in *ASXL1*, which was associated with shorter overall survival after ruxolitinib discontinuation.

The benefit of interferon-α in MF can be pronounced in early and pre-fibrotic phases.[42,85] However, recent reports indicate an adverse prognostic influence of specific concomitant mutations in patients treated with interferon-α.[86,87] Further analyses should continue to identify the impact of the clonal landscape on interferon-α response to improve the selection of patients for this therapy.

Several studies have identified molecular associations with inferior outcome of allogeneic HSCT. Number of mutations (≥3 additional to driver mutations)[88] as well as specific mutations in *ASXL1*, *CBL*, *DNMT3A*, *IDH2* and *U2AF1*[89-92] have been associated with inferior overall survival following transplant, although the prognostic influence of the individual genes has not been supported in all studies. The Myelofibrosis Transplant Scoring System (MTSS) aimed to determine prognosis (from relapse and non-relapse-related mortality) after transplantation in both primary and secondary MF.[90] Here, molecular features of *ASXL1* mutation and non-*CALR/MPL* driver mutation genotype were independent predictors of outcome. Other genetic factors considered, but not found to be significant in multivariate analyses, included mutations in *U2AF1*, *DNMT3A* and *TP53*, >3 concomitant mutations and cytogenetic risk category, suggesting that these did not continue to portend adverse survival in MF patients who undergo allogeneic HSCT.

Implications of high molecular risk on the management of primary myelofibrosis

Taken together, the clinical and molecular heterogeneity of MPN support a molecularly-informed risk stratification system, but should be ideally matched with risk-stratified management approaches. Prior to the ruxolitinib era, patients with intermediate-2 or high-risk DIPSS scores were shown to have improved survival after transplantation, whereas for those with intermediate-1-risk, there was no difference between a transplant and non-transplant approach and those with low-risk disease benefited from a non-transplant approach,[93] forming the basis of transplant referral guidelines for MF.[94] The addition of molecular data refines risk stratification; however the principles based on the DIPSS still hold and remain valid in the ruxolitinib era. In clinical practice, along with the molecular insights described above, clinical predictors remain relevant in predicting long-term response to ruxolitinib. The 'Response to Ruxolitinib after 6 months' (RR6) score is a prognostic model incorporating ruxolitinib dose, spleen response and

red blood cell transfusions and is used to predict survival in patients with MF treated with ruxolitinib. This model helps to identify patients who may need a shift to second-line treatments or allogeneic HSCT.[95] Other high-risk molecular features not captured in standard prognostic scores include mutations in *TP53*, *CBL*, *NRAS*, *KRAS* and all *U2AF1* mutations.[79] Clonal evolution, especially within a *TP53*-mutated context, also predicts poor response to medical therapies and is associated with leukemic transformation.[11]

Future considerations

While significant advances have been made in the understanding of HMR MPN and its clinical relevance, several questions remain regarding the clinical management of these patients. Should patients be monitored for development or clonal evolution of HMR lesions prior to clinical progression? Should patients with HMR MPN be treated differently from those without high-risk aberrations in chronic phase? What are relevant endpoints for clinical trials? Hematologic responses? Improvement of cytopenias? Progression-free, event-free and overall survival? Which therapies are considered disease-modifying?

Molecular profiling is not performed routinely in clinical practice.[96] Recently, NGS is becoming more widely available, and several studies have shown largely concordant results between NGS testing for mutations in peripheral blood compared to bone marrow.[97,98] Extracting clinical utility from this knowledge requires sufficient evidence that changes in genetic profiles constitute actionable information. Studies of serial sampling demonstrate that additional mutations will not be detected over time in the majority of patients.[53,12] An exception may be in the context of *TP53* mutations, which are an important driver of leukemia transformation.[5] Likewise, clonal diversification and evolution with loss of the respective driver mutations may indicate acceleration and progression to blastic phase MPN. Specific work delineating clonal evolution of *TP53* mutations has demonstrated that some low variant allele frequency mutations can remain stable for long periods of time prior to expansion, causing late AML transformation[5,12] following loss of the remaining wild-type allele.[37,12,99] The optimal role of serial monitoring for *TP53* variant allele frequency and acquisition of new genetic lesions, including structural variants or copy number variations is unknown. Methods to stratify a serial monitoring approach in MPN is required in order to define and detect high-risk clonal evolution. In high-risk patients, prospective evaluation during cytoreductive therapies (e.g., clonal evolution) or failure to achieve molecular responses could help to identify patients who require escalation of treatment or evaluation for allogeneic HSCT.

The design of clinical trials can potentially assist in exploring knowledge gaps. In 2015, the European LeukemiaNET (ELN) and International Working Group-MPN Research and Treatment (IWG-MRT) groups provided guidelines regarding acceptable clinical endpoints for drug treatment trials in *BCR::ABL*-negative MPN.[100] The group distinguished clinically relevant time-to-event endpoints (e.g., overall survival and progression-free survival) and surrogate endpoints. While time-to-event endpoints, such as overall and leukemia-free survival, are arguably gold standards in phase III clinical trials, they require large sample sizes and long-term follow-up, which may be unachievable in PV or ET where events accrue slowly over decades. Surrogate endpoints, such as molecular response, overall response and reductions in spleen size occur earlier, are only appropriate as surrogates for overall and leukemia-free survival if they reliably predict these endpoints. Regarding molecular responses, the Working Group concluded that there were insufficient data to validate these as reliable surrogates for survival endpoints. Other endpoints of clinical significance are those that indicate 'disease modification' and are being increasingly incorporated into trials of newer agents (especially trials investigating non-JAK-inhibitors).[101] In MF, in which clinical trial endpoints historically focused on symptom and spleen responses, driven by the striking improvements seen with JAK-inhibitors, progression-free survival, overall survival and improvement of cytopenias represent relevant readouts for future trials.[101-103]

Finally, focus on the very high molecular risk groups should be prioritized for research. The molecular heterogeneity of MPN creates multiple subgroups with differential responses to therapy, leaving increasingly smaller subgroups of patients for study when focusing on a single mutation or combination of mutations and masking treatment trends within these subgroups. In a disease type in which the majority of patients will have good clinical outcomes with the standard of care, clinical trials dedicated to high-risk groups, such as those with HMR MPN, should be undertaken, enriching for events and increasing the likelihood of a statistically significant outcomes and thus ability to progress treatments from trials to routine clinical practice. No relevant prognostic differences were seen between the clinical phenotype of ET *versus* PV in equivalent molecular subtypes,[2] which suggests a molecular classification in chronic phase MPN could be applicable, rather than traditional morphological and clinical diagnostic criteria for inclusion in clinical trials. The challenge of better treatments for HMR MPN needs to be overcome through international efforts to catalogue patients' genetics and treatment outcomes to facilitate large-scale meta-analyses assisted by artificial intelligence/machine-learning approaches[104] and supported by evidence from robust preclinical models.

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VYL, FHH and MJB wrote and edited the manuscript.

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