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by Victoria Y. Ling, Florian H. Heidel, and Megan J. Bywater

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Pathogenesis and management of high molecular risk myeloproliferative neoplasms

Victoria Y. Ling[1-4], Florian H. Heidel[5,6,7] and Megan J. Bywater[1,4]

[1]QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia.
[2]Department of Haematology, Princess Alexandra Hospital, Brisbane, Queensland, Australia
[3]Pathology Queensland, Brisbane, Queensland, Australia
[4]The University of Queensland, Brisbane, QLD, Australia.
[5]Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Hannover Medical School (MHH), Hannover, Germany
[6]Leibniz Institute on Aging, Jena, Germany
[7]Cellular Therapy Center (CTC), Hannover Medical School (MHH), Hannover, Germany

Author Contributions

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Competing interests

MJB has received reagents for research use from PharmaEssentia and AstraZeneca, reagents and research funding from Bristol Myers Squibb and Cylene Pharmaceuticals and holds a patent on the use of MYC in regenerative therapy (PCT/GB2020/050350).
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Abstract
Classical myeloproliferative neoplasms (MPNs) are clonal stem cell disorders characterised by driver mutations that affect the constitutive activation of JAK-signalling. Additional mutations 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 will 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 will discuss the definitions of high molecular risk MPN, 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 utilised to improve clinical outcomes.

Main Text
The classical *BCR*::*ABL*-negative myeloproliferative neoplasms (MPNs), 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 (HSCs); thus MPNs are considered clonal stem cell disorders.

Most patients present in a 'chronic-phase' (CP) of the disease (i.e. PV, ET, pre-fibrotic MF), with elevated peripheral blood (PB) parameters and accompanying systemic inflammation. Relevant clinical challenges include (i) symptom control, (ii) prevention of thromboembolic (TE) 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 TE events achieved using acetylsalicylic acid or anticoagulants, and cytoreductive measures, such as phlebotomy or pharmacologic 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 pharmacologic and supportive interventions. Up to 40% of MPN patients experience disease progression during their lifetimes, either from CP to fibrotic MF, or CP/MF to an accelerated phase (AP) (10-20% blasts) or overt acute myeloid leukemia (AML) (≥20% blasts), also referred to as blast-phase (BP) MPN. Progression is frequently associated with clinical deterioration and shortened overall survival. Allogeneic hematopoietic stem cell transplantation (alloHSCT) is the only potentially curative therapy currently available for MPN, however due to high morbidity and mortality rates, is only indicated for younger/fitter patients at higher risk for disease progression.

Next generation sequencing (NGS) technology has facilitated increased resolution of the mutational landscape that underpins the process of malignant transformation in humans. This is exemplified by AML, where particular patterns of mutational co-occurrence define prognostically relevant sub-classes [1]. Somatic co-mutations of relevance in myeloid cancers, in addition to MPN-drivers are present in approximately 50% of patients with CP 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 AP/BP MPN[2,4]. The concept and definition of high molecular risk (HMR) mutations has facilitated their predictive use in addition to clinical disease parameters for patient outcomes and informed treatment decisions.

In this review, we will 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 will 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

CP MPN are a neoplastic state consequent to a single oncogenic driver, like *JAK2* V617F, necessary but insufficient for secondary transformation. However, the complexity and phenotypic diversity in the pathogenesis of MPNs 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 (CH)).

MPN-driver mediated

In HSCs, 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 CP MPN, this state of DNA damage may provide a selective pressure for the loss of key regulators of DNA-damage checkpoints, like 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], that 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 1 mutation per 66 patient years[12], arguing against a strong hypermutable state in MPN. Consistently, individuals exclusively harbouring *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 first evidence that driver mutations can be acquired decades before clinical manifestation of MPN[13,14], as in some cases, *JAK2* mutations could already be detected in cord blood. However, data investigating the behaviour 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.

Age-related clonal hematopoiesis (ARCH)-mediated

Somatic mutations acquired prior to the driver (e.g. a *JAK2* mutation) may provide a 'fertile ground' for malignant transformation[8,12]. CH is where the maturing cell progeny derived from a single HSC ancestor, or "clone", dominates the hematopoietic compartment of an individual. CH occurs as a consequence declining clonal diversity during aging and may not be associated with a neoplastic state, leading to the terminology of ARCH or CH of indeterminate potential (CHIP). Mutations with relevance in myeloid malignancies include *DNMT3A, TET2* and *ASXL1 (DTA* mutations*)* account for over 90% of cases of ARCH. Due to its association with increased risk of future development of myeloid malignancies, including MPNs, 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 HSCs show progressive loss in clonal diversity leading to increased variant allele frequency (VAF) 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 MPN

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 AP/BP without a prior diagnosis of MF. Furthermore, disease progression to MF or AP/BP is not an inevitable outcome in PV or ET with chronic phases lasting sometimes 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 either be 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:

(i) 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 wildtype progeny from a *JAK2* V617F heterozygous founder. However, single nucleotide polymorphisms (SNPs) 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* wildtype loss of heterozygosity (LOH) progeny do not expand appreciably in MPN patients[19]. Furthermore, in *JAK2*-wildtype post-MPN AML, LOH, determined by SNP genotyping, was not detected in leukemic blasts[19,20], providing evidence against loss of *JAK2* V617F during blast transformation.

(ii) Can AML evolve in the context of MPN, from an independent clonal precursor?

Combined analysis of *JAK2* V617F granulocytes and *JAK2* wildtype leukemic blasts from the same patient have demonstrated the inactivation of the same parental X-chromosome[20].

Also, shared somatic mutations in *JAK2* V617F cells and *JAK2*-wildtype leukemic blasts (like *DTA*), 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 rather a consequence of branching or parallel evolution (Figure 1). Pathways of branching and parallel evolution in MPN, 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].

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 HSCs [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 the increased expression of stemness-related genes[22,23]. However, additional *DTA* mutations occur more frequently in patients diagnosed with MF, compared to 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 myelofibrosis 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 *Jak2V617F* allele is also associated with increased expression of genes involved in TNFα 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 BM 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 HSCs. *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 *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 patient 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 the functional studies in mice demonstrating the exacerbation of the disease phenotype with co-existing *Tet2*-loss compared to *Jak2V167F* alone[23]. 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. Thus surprisingly, co-expression of *Srsf2* P95H reduces MPN severity in *Jak2* V617F knock-in mice, evident from reduced splenomegaly and blood cell counts[33]. Furthermore, *Srsf2* P95H co-expression can attenuate MF and reduce the expansion and repopulation capacity of HSCs[33]. However, serial transplantation studies demonstrate the capacity of *Srsf2* P95H co-expression to prevent *Jak2* V617F stem cell exhaustion by extending their long-term replicative capacity[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 mRNA splicing and processing related molecules 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 pharmacologic 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 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 appear 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 (MEPs) are the leukemia initiating cell (LIC) population able to generate AML directly in secondary recipients. In contrast, transplant of more primitive LSK (lineage negative, $Sca1^+$, kit^+) cells only generate leukemia after an MPN disease phase[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 their survival or proliferation[7]. It has also been shown that BM from *Jak2* V617F/*Trp53*-null leukemic mice harbors recurrent chromosomal copy number variations, that are absent in the MPN phase[37]. Furthermore, MEPs isolated from *Jak2* V617F/*Trp53*-null leukemic mice are transcriptionally distinct from MEPs 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.

Continued 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 CP, AP and BP MPN. Therefore, the functional consequences of 3- and 4-way genetic interactions with all MPN drivers on MPN disease progression will be valuable to expanding understanding of the pathogenesis of HMR MPN.

Influence of mutational heterogeneity on current clinical management of MPN

We now discuss how molecular risk is defined clinically and how it influences clinical management of MPN. Current clinical guidelines and consensus documents[39–41] provide algorithms for the management of patients with MPNs, separated into the clinical phenotypes of PV, ET and MF. The canonical driver mutation (*JAK2*, *MPL* and *CALR*) occurs as a sole genetic abnormality in 45% of MPNs, but more frequently co-occurs with concomitant (passenger) mutations, especially in myelofibrosis[2]. Approximately 5-10% of patients with ET or MF lack a canonical driver and are termed 'triple-negative' [2,42]. Triple negative MF patients may harbor non-driver mutations, especially within spliceosome and chromatin modifying genes and have inferior survival compared to MF 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 high-risk mutations or 'HMR' differs for PV, ET and MF. It derives from NGS-profiling studies of patient cohorts, that have identified genetic mutations associated with inferior overall survival (OS), leukemia-free survival (LFS) and (for PV and ET) myelofibrosis free survival (MFFS). A large study incorporating 2035 patients (including 1321 ET, 356 PV and 309 MF and 49 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, sixty-three clinical and genomic variables were identified as significant to 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 and complexity of integration into prognostication and selection of therapeutic approaches in clinical practice.

PV

Box 1. PV management in a snapshot: In PV, hyperproliferation of erythropoiesis is predominant resulting in high hematocrit (Hct) levels, and the therapeutic focus is on reducing the risk of venous and arterial thromboembolic complications and symptom reduction. 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 Hct of <0.45[45]. Indication for medical cytoreductive therapies is predominantly based on stratification of thrombosis risk, integrating risk factors such as age ≥60 years, prior TE event, cardiovascular risk factors and leucocytosis[46], although other factors including platelet >1000 x 10^9/L, symptomatic splenomegaly, microcirculatory disturbances and persistent high phlebotomy frequency (>2x/month) also represent indications. Hydroxyurea (HU) 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. Ropeginterferon alfa-2b has shown superiority in clinical trials[47] and was recently approved for PV treatment in several countries. For second-line treatment after HU refractoriness or intolerance, Ruxolitinib is effective for cytoreduction and symptom control compared with best available therapies[48,49]. Progression to secondary myelofibrosis occurs in 10-15% of cases and to secondary leukemia in up to 15% of PV and 25% of post PV-MF patients[43] and thus represent significant causes of morbidity and mortality in PV.

HMR influence on Prognostication of PV

Concomitant mutations in PV can predict higher risk of reduced survival and disease progression, as well as thrombosis. Mutations in *ASXL1*, *SRSF2* and *IDH2* were associated reduced OS and LFS[50], with *SRSF2* integrated into the weighted prognostic score, MIPSS-PV that stratifies patients into low, intermediate and high risk for survival[51]. The MFPS (Multiple Factor-Based Prognostic Score) 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[52].

HMR influence 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 [53]. In this study, 17% of patients achieved a complete molecular response (CMR) (defined as undetectable *JAK2* V617F based on an assay with sensitivity of 5%) over a median of 42 months follow-up. In those failing to achieve CMR, 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 CMR). In patients with CMR, concomitant mutations were also cleared. Genomic predictors of response were investigated in the DALIAH trial (low dose interferon alpha (IFNa) vs hydroxyurea (HU)) including 202 patients with PV, ET and MF[54]. Patients had

routine re-sequencing at 24 months (n=135). Treatment emergent mutations were detected in 32 patients (24%) at 24 months. In IFNa treated patients, these were most commonly *DTA* mutations. Interestingly, *TP53* and *PPM1D* were commonly acquired mutations in HU treated patients, paralleling findings of *TP53* and *PPM1D* clonal hematopoiesis emerging post cytotoxic therapy in solid cancer patients[55,56]. The emergence of *DNMT3A* mutations was the only factor described in the study to be associated with failure to achieve complete hematologic response to IFNa. The authors speculate that *DNMT3A* mutated clones were likely pre-existing at baseline and were selected for with IFNa therapy, perhaps due to aberrant self-renewal described as a consequence of cooperation between *JAK2* V617F and *DNMT3A* mutation[22]. Recent pre-clinical studies in *JAK2* V617F and *Dnmt3a* mutant murine hematopoiesis provided first evidence that combined treatment with IFNa and hypomethylating agents (HMA, 5-Aza) enhances clonal regression overcoming the adverse effect of *Dnmt3a* co-mutation[57]. In a randomized Phase 3 study comparing the effects of Ropeg-Interferon alpha 2b against Best Available Therapy (BAT), PV patients harboring *ASXL1* mutations showed relevant responses when treated with IFNa[58]. In the MAJIC-PV study, Ruxolitinib showed a higher rate of molecular response (defined as a 50% reduction in *JAK2* V617F allele frequency) which was associated with improved progression-free survival (PFS), event-free survival (EFS), and OS. Here, the presence of *ASXL1* mutations was associated with worse EFS (adjusted hazard ratio (HR) of 3.02 compared to those without these mutations)[49]. An additional study supported the negative impact of additional baseline mutations on PFS 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[59].

Implications of HMR on management of PV

Currently, the presence of HMR mutations does not alter management recommendations or target thresholds for cytoreductive therapies. Whilst there is increasing information about predictors of response to therapies like 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. AlloHSCT is currently not recommended for 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 CP to AP or BP[2] and MIPSS-PV high risk patients have a predicted median OS of 4.6 years[60]. 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 alloHSCT.

ET

Box 2. ET management in a snapshot: ET phenotypically affects the megakaryocytic cell line, 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 TE and bleeding events (due to extreme thrombocytosis-related secondary von Willebrand syndrome), as well as to reduce disease-associated symptoms[61]. 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[46]. Numerous studies have shown that the presence of a *JAK2* mutation is associated with a significantly higher thrombosis risk compared to the presence of *CALR* mutations. *JAK2* mutation is incorporated as a risk factor into scores such as the IPSET-thrombosis score[62]. 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 *CALR* mutation do not benefit from anti-platelet therapy[63]. Cytoreductive medication is specifically indicated for high-risk patients and options include HU, anagrelide and peg-IFN (where approved)[64]. Ropeg-Interferon is being evaluated in an international multicenter trial for ET[65]. Data on Ruxolitinib from two studies in relatively small patient populations with HU-refractory or -intolerant ET showed a reduction in platelets and leukocytes and an improvement in ET-associated symptoms, but no significant improvement in the hematological complete remission rate or the rate of thrombosis, hemorrhages, or leukemic transformation[66,67].

HMR influence on Prognostication of ET

In ET, mutations in *SH2B3*, *SF3B1*, *U2AF1*, *TP53*, *IDH2*, and *EZH2* were associated with inferior OS[50]. In a further analysis, *SF3B1* and *SRSF2* predicted reduced OS, while mutations in *SF3B1* and *U2AF1* predicted reduced MFFS and those in *TP53* predicted inferior LFS. These genes have been integrated into the MIPSS-ET score that significantly stratifies patients into low, intermediate and high risk for survival[51].

Implications of high molecular risk on management of ET

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

Myelofibrosis

Box 3. PMF management in a snapshot: The early phase of PMF 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) myelofibrosis. 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). Especially for younger patients with PMF and those without relevant comorbidities, the curative option of alloHSCT is recommended for intermediate-2 or high-risk patients. Here, pretreatment with JAK inhibitors (JAKi) is beneficial, especially for patients with splenomegaly and symptom burden. Symptom-oriented treatment with JAKi or experimental therapies in clinical trials are available, if alloHSCT is not indicated or possible.

HMR Influence on Prognostication of PMF

Risk stratification tools for myelofibrosis (Table 1) predict OS and LFS and are used to determine treatment approaches for patients. Newer risk classifications such as the mutation-enhanced international prognostic scoring system (MIPSS70)[68] and its iterations[69] and the genetically inspired prognostic scoring system (GIPSS) incorporate molecular and other genetic data in risk stratification scores that help to identify patients at high risk of disease progression that were previously not identified by clinical markers. These represent evolution from more traditional prognostic scores which solely assess clinical and hematological factors, such as the International prognostic scoring system (IPSS)[70] and dynamic IPSS (DIPSS)[71] and with the additional prognostic influence of karyotype, DIPSS-plus[72].

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 [73]. *ASXL1*, *EZH2*, *SRSF2*, *IDH1* and *IDH2* were associated with high risk for death or leukemic transformation. The number of mutations was also predictive, with ≥2 associated with inferior LFS[74]. The mutation status of these 5 genes (coined 'high molecular risk' (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)[68] and adding *U2AF1* Q157 hotspot variant as an additional HMR variant (MIPSS70-plus Version 2.0)[75], 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[76].

Additional high-risk mutations have been identified with expanded molecular characterization. A study using a 77-gene NGS panel to molecularly profile patients with myelofibrosis (n=479, incorporating 305 PMF and 174 SMF (70 post-PV and 104 post-ET) patients) was performed by the French Intergroup of Myeloproliferative Neoplasms (FIM)[77]. Within this cohort, 4 prognostic groups with significantly different rates of OS and LFS were identified including 1) *TP53*-mutated (median OS 20 months), 2) presence of ≥1 high-risk mutation (*EZH2*, *CBL*, *U2AF1*, *SRSF2*, *IDH1*, *IDH2*, *NRAS* or *KRAS* (median OS 49 months), 3) *ASXL1* without *TP53* or other high-risk mutation as per group 2) (*ASXL1*^{mut}-only) (median OS 90 months) and 4) other mutational profiles (median OS 116 months). When assessed in multivariate analyses with clinical and hematological factors, the *TP53*-mutated and high-risk mutation group independently maintained higher risk 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* mutations have been independently associated with inferior overall survival in multiple independent MF patient cohorts[78,79].

HMR influence on response to therapy

Various groups have examined molecular predictors of response to ruxolitinib. Predictive factors differ amongst groups but overall suggest 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 (BAT) in IPSS intermediate-2 or high-risk myelofibrosis was established in the Phase III COMFORT-I and II trials[80,81]. 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[82]. High-risk mutations in this study (*ASXL1*, *EZH2*, *SRSF2* and *IDH1/2*) conferred inferior survival compared to patients without these mutations within the BAT arm. In patients treated with ruxolitinib, patients 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*)[83], revealed mutations in *ASXL1*, *EZH2* or *IDH1/2*, as well as those with ≥3 mutations were associated with lower rates of spleen response, time to treatment discontinuation and shorter OS (in contrast to the COMFORT-II analyses[82]). *ASXL1* or *EZH2* mutations along with other clinical factors of pre-JAKi transfusion dependence and high DIPSS score were also identified as predictive factors for treatment failure of JAKi, ruxolitinib or momelotinib [84]. Lower frequency mutations, including those in NRAS, KRAS and CBL were shown to associate with reduced symptom and spleen response to ruxolitinib[85].

Clonal evolution during therapy has also been associated with poor outcomes to ruxolitinib therapy. In 46 ruxolitinib and 25 HU treated patients with MF, sequential samples demonstrated acquisition of new mutations in 8 of ruxolitinib treated patients (17.4%) compared to 6 HU-treated patients (24%) [86]. The presence of HMR mutation (*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 was seen in both ruxolitinib and HU-treated patients suggesting clonal evolution was associated with disease rather than treatment received. Outcomes from 107 patients with MF who discontinued ruxolitinib were also reported [87]. At time of discontinuation, 14 (33%) patients had acquired at least 1 additional mutation during treatment, with the majority (64%) being variants in *ASXL1*, which was associated with shorter OS after ruxolitinib discontinuation.

The benefit of IFNa in myelofibrosis can be pronounced in early and pre-fibrotic phases[42,88]. However, recent reports indicate adverse prognostic influence of specific concomitant mutations in patients treated with IFNa[89,90]. Further analyses should continue to identify the impact of the clonal landscape on IFNa response to enhance patient selection for this therapy.

Several studies have identified molecular associations with inferior outcome alloHSCT. Number of mutations (≥3 additional to driver mutations)[91] as well as specific mutations in *ASXL1*, *CBL*, *DNMT3A*, *IDH2* and *U2AF1*[92–95] have been associated with inferior OS following transplant, although the prognostic influence of the individual genes have 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 PMF and SMF[93]. 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 these did not continue to portend adverse survival in MF patients who undergo alloHCT.

Implications of high molecular risk on management of PMF

Taken together, the clinical and molecular heterogeneity of MPNs support a molecularly-informed risk stratification system, but should be ideally matched with risk-stratified management approaches. Prior to ruxolitinib era, patients with intermediate-2 or high risk DIPSS scores were shown to have improved survival after transplantation, with intermediate-1 showing no difference between a transplant and non-transplant approach and low risk benefitting from a non-transplant approach[96], forming the basis of transplant referral guidelines for MF[97]. The addition of molecular data refines risk stratification, however the principles based on DIPSS still hold and remain valid in the ruxolitinib era. In clinical practice, along with molecular insights described above, clinical predictors remain relevant in predicting long-term response to ruxolitinib. The 'Response to Ruxolitinib after 6 months score' (RR6) is a prognostic model incorporating ruxolitinib dose, spleen response and red blood cell transfusions and used to predict survival in patients with MF treated with ruxolitinib. This model helps identify patients who may need a shift to second-line treatments or allo-SCT[98]. Other high-risk molecular features not captured within standard prognostic scores include mutations in *TP53*, *CBL*, *NRAS*, *KRAS* and all *U2AF1* mutations[81]. Clonal evolution, especially within a *TP53*-mutated context, also predict poor response to medical therapies and is associated with leukemic transformation[11].

Future considerations

While the field has made significant advances in the understanding of molecular high-risk MPN and its clinical relevance, several questions remain in the clinical management of these patients:

1) Should patients be monitored for development or clonal evolution of molecularly-high risk lesions prior to clinical progression?
2) Should patients with high-molecular risk MPN be treated differently to those without high-risk aberrations in chronic phase?
3) What are relevant endpoints for clinical trials? Hematologic responses? Improvement of cytopenias? Progression-, Event-Free and Overall Survival?
4) Which therapies are considered disease modifying?

Molecular profiling in clinical practice is not routinely performed[99]. 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 [100,101]. Providing clinical utility of this knowledge requires sufficient evidence that changes in genetic profiles constitute actionable information. Studies of serial sampling demonstrate the majority of patients will not have additional mutations detected over time[54,102]. 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 MPN-BP. Specific work delineating clonal evolution of *TP53* mutations demonstrate that some low VAF mutations can remain stable for long periods of time prior to expansion, causing late AML transformations[5,102] following loss of the remaining wild-type allele[37,102,103]. The optimal role

of serial monitoring for *TP53* VAF and acquisition of new genetic lesions, including structural variants or copy number variations is unknown. Methods to stratify a serial monitoring approach in MPNs is required 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 of alloHSCT.
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 as to acceptable clinical endpoints for drug treatment trials in BCR::ABL negative MPNs[104]. The group distinguished clinically relevant time-to-event endpoints (eg. OS or progression free survival) vs surrogate endpoints. While time-to-event endpoints like OS or LFS are arguably the gold standard 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 OS or LFS if they reliably predict these endpoints. Regarding molecular responses, the working group concluded there was 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-JAKi)[105]. In MF, where clinical trial endpoints historically focused on symptom and spleen responses, driven by the striking improvements seen with JAKi, PFS, OS and improvement of cytopenias represent relevant readouts for future trials[105–107].

Finally, focus on the very high molecular risk groups should be prioritized for research. The molecular heterogeneity of MPNs 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 where the majority of patients will have good clinical outcomes with standard of care, clinical trials dedicated to high-risk groups, like high-molecular risk MPNs should be undertaken, enriching for events and increasing the likelihood of a statistically significant outcome and thus ability to progress treatments from trials to routine clinical practice. No relevant prognostic differences were seen between the clinical phenotype of ET vs PV in equivalent molecular subtypes[2], which suggests a molecular classification in chronic-phase MPNs could be applicable, rather than traditional morphologic and clinical diagnostic criteria for clinical trial inclusion criteria. The challenge of better treatments for HMR MPNs needs to be overcome via international effort to catalogue patient genetics and treatment outcomes to facilitate large-scale meta-analyses assisted by AI/machine-learning approaches[108] and supported by evidence from robust pre-clinical models.

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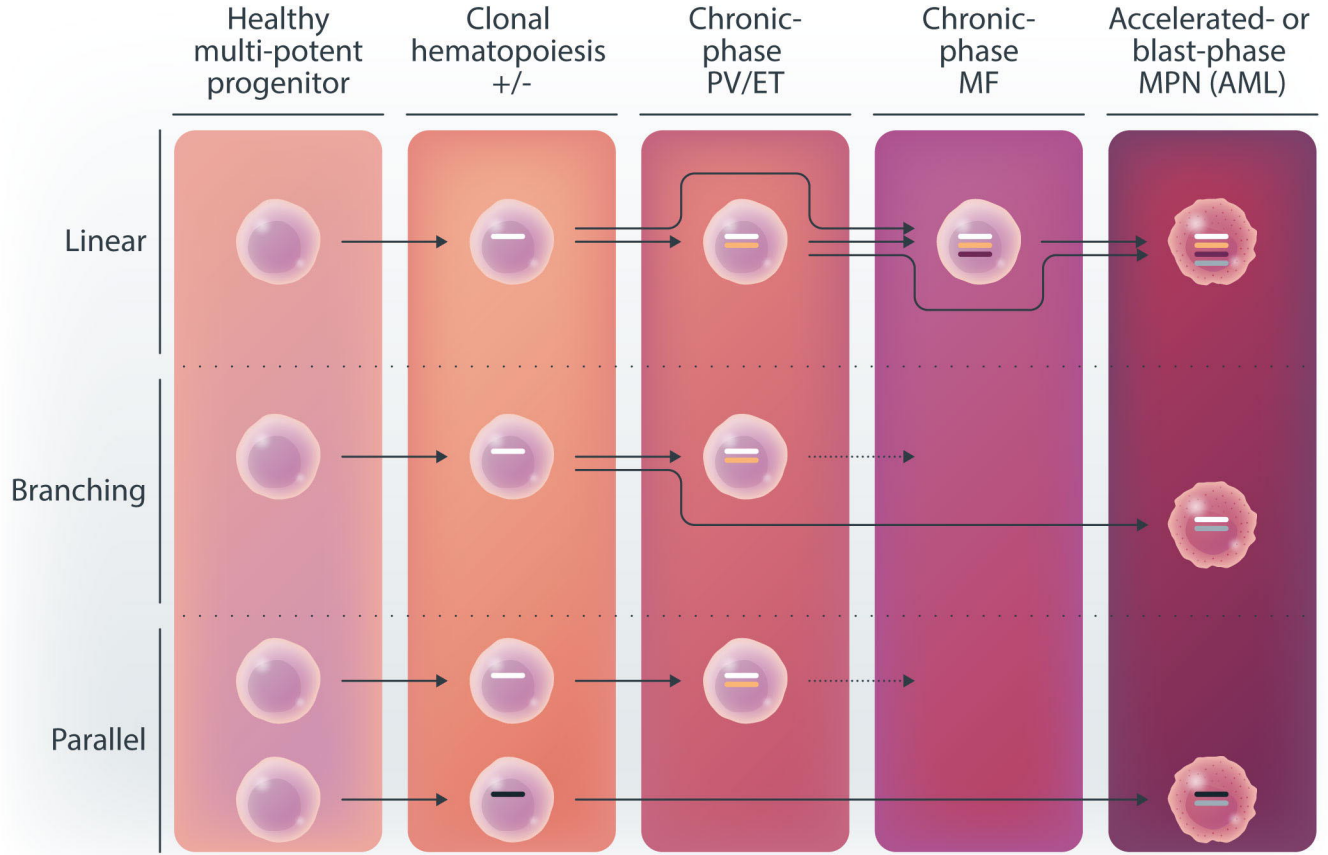
Table 1. Prognostic stratification scores in myelofibrosis.

Prognostic score	For use			Patient characteristics	Peripheral blood / BM parameters	Karyotype	Molecular
	Diagnosis	After diagnosis	Secondary MF				
IPSS	Yes	-	-	Age >65 years Constitutional symptoms	Hb < 10 Leuk >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[1]	-
MIPSS70	Yes	-	-	(validated in age ≤70) Constitutional symptoms	Hb <10 Leuk >25 Platelets <100 Circulating blasts ≥2% BM fibrosis ≥MF-2	No	Absence of CALR Type 1 mutation Presence of ≥2 HMR mutations[2]
MIPSS70-Plus	Yes	-	-	(validated in age ≤70) Constitutional symptoms	Hb <10 Circulating blasts ≥2%	Yes - high risk	Absence of CALR Type 1 mutation Presence of ≥2 HMR mutations[2]
MIPSS70-Plus v2.0	Yes	-	-	-	As for MIPSS70-Plus with adjusted Hb thresholds	Yes - high and very high risk	As for MIPSS70-Plus Included U2AF1 Q157 as an HMR mutation
GIPSS	Yes	-	-	-	-	Yes – very high risk and unfavo	Absence of CALR Type 1/like mutation Presence of

						urable karyotype	HMR mutations[3]
MYSEC-PM	-	-	Yes	Age Constitutional symptoms	Hb <11 Circulating blasts ≥3% Platelets <150		CALR mutation

[1]complex karyotype or one or two abnormalities that include trisomy 8, del 7/7q, i(17q), del5/5q, del12p, inv(3), or 11q23 rearrangement
[2]ASXL1, EZH2, SRSF2, and IDH1/2
[3]ASXL1, SRSF2, or U2AF1 Q157
Hb, haemoglobin, g/dL; leuk, leukocytes, $x10^9$/L, RBC, red blood cell; BM, bone marrow; MF, myelofibrosis; HMR, high molecular risk.

Figure 1: Potential paths of clonal evolution in MPN. The combinations of mutations that occur in chronic-phase MPN and disease progression to accelerated or blast-phase MPN (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 PV or ET is not required for the emergence of MF, and prior presentation of MF is not required for the emergence of AML.


Healthy multi-potent progenitor
Clonal hematopoiesis +/-
Chronic-phase PV/ET
Chronic-phase MF
Accelerated- or blast-phase MPN (AML)
Linear
Branching
Parallel