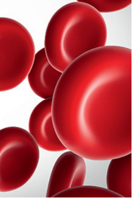

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CLASSIC MYELOPROLIFERATIVE NEOPLASMS

Genetic basis and molecular profiling in myeloproliferative neoplasms

Damien Luque Paz,[1] Robert Kralovics,[2] and Radek C. Skoda[3]

[1]Univ Angers, Nantes Université, CHU Angers, Inserm, CNRS, CRCI2NA, Angers, France; [2]Department of Laboratory Medicine, Medical University of Vienna, Vienna, Austria; and [3]Department of Biomedicine, Experimental Hematology, University Hospital Basel and University of Basel, Basel, Switzerland

BCR::ABL1-negative myeloproliferative neoplasms (MPNs) are clonal diseases originating from a single hematopoietic stem cell that cause excessive production of mature blood cells. The 3 subtypes, that is, polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), are diagnosed according to the World Health Organization (WHO) and international consensus classification (ICC) criteria. Acquired gain-of-function mutations in 1 of 3 disease driver genes (_JAK2, CALR,_ and _MPL_) are the causative events that can alone initiate and promote MPN disease without requiring additional cooperating mutations. _JAK2_-p.V617F is present in >95% of PV patients, and also in about half of the patients with ET or PMF. ET and PMF are also caused by mutations in _CALR_ or _MPL_. In ~10% of MPN patients, those referred to as being "triple negative," none of the known driver gene mutations can be detected. The common theme between the 3 driver gene mutations and triple-negative MPN is that the Janus kinase–signal transducer and activator of transcription (JAK/STAT) signaling pathway is constitutively activated. We review the recent advances in our understanding of the early events after the acquisition of a driver gene mutation. The limiting factor that determines the frequency at which MPN disease develops with a long latency is not the acquisition of driver gene mutations, but rather the expansion of the clone. Factors that control the conversion from clonal hematopoiesis to MPN disease include inherited predisposition, presence of additional mutations, and inflammation. The full extent of knowledge of the mutational landscape in individual MPN patients is now increasingly being used to predict outcome and chose the optimal therapy.

Introduction

The clonal origin of myeloproliferative neoplasms (MPNs) had already been recognized in 1976 using X-chromosome inactivation patterns in the peripheral blood of female MPN patients,[1] and it was later firmly established using somatic gene mutations as the clonal markers.[2] _JAK2_-p.V617F is detectable in purified human hematopoietic stem cells (HSCs),[3] and the stem cell origin of MPNs was demonstrated experimentally in mouse models of MPN disease. by transplanting a single HSC that expresses _JAK2_-p.V617F into recipient mice, which then developed MPN disease.[4] Recent studies using whole-genome sequencing (WGS) of DNA from single hematopoietic colonies confirmed the single-HSC origin in human MPN disease and established a timeline for the preclinical initiation and expansion of the _JAK2_-mutant clone (Figure 1).[5,6] With the use of next-generation sequencing (NGS), our knowledge of the mutational landscape in MPN disease has greatly improved, and we now have an advanced understanding of the genetic basis of MPNs. Until recently, the risk factors used to predict prognosis in MPN disease were mostly based on clinical parameters, but information on the mutational landscape of MPNs is now increasingly being added to improve and individualize the prognostic scores.

Genetic basis and clonal evolution of MPNs

A large number of genes carrying mutations in hematopoietic cells from MPN patients have been identified by targeted NGS and whole exome sequencing (WES; Table 1). No general consensus has been reached regarding the nomenclature that should be used to classify these mutations, although the distinction between "driver" vs "passenger" mutations is widely used. In the context of MPNs, we propose to further distinguish between "disease driver" mutations and "clonal driver" mutations.

"Disease driver" mutations recapitulate the human MPN phenotype when introduced into model organisms such as mice. A large proportion of MPN patients carry mutations solely in 1 of the 3 disease driver genes _JAK2_, _CALR_, or _MPL_,[7-15] without additional clonal driver mutations detectable by targeted NGS.[16,17] This finding was also confirmed by WES and

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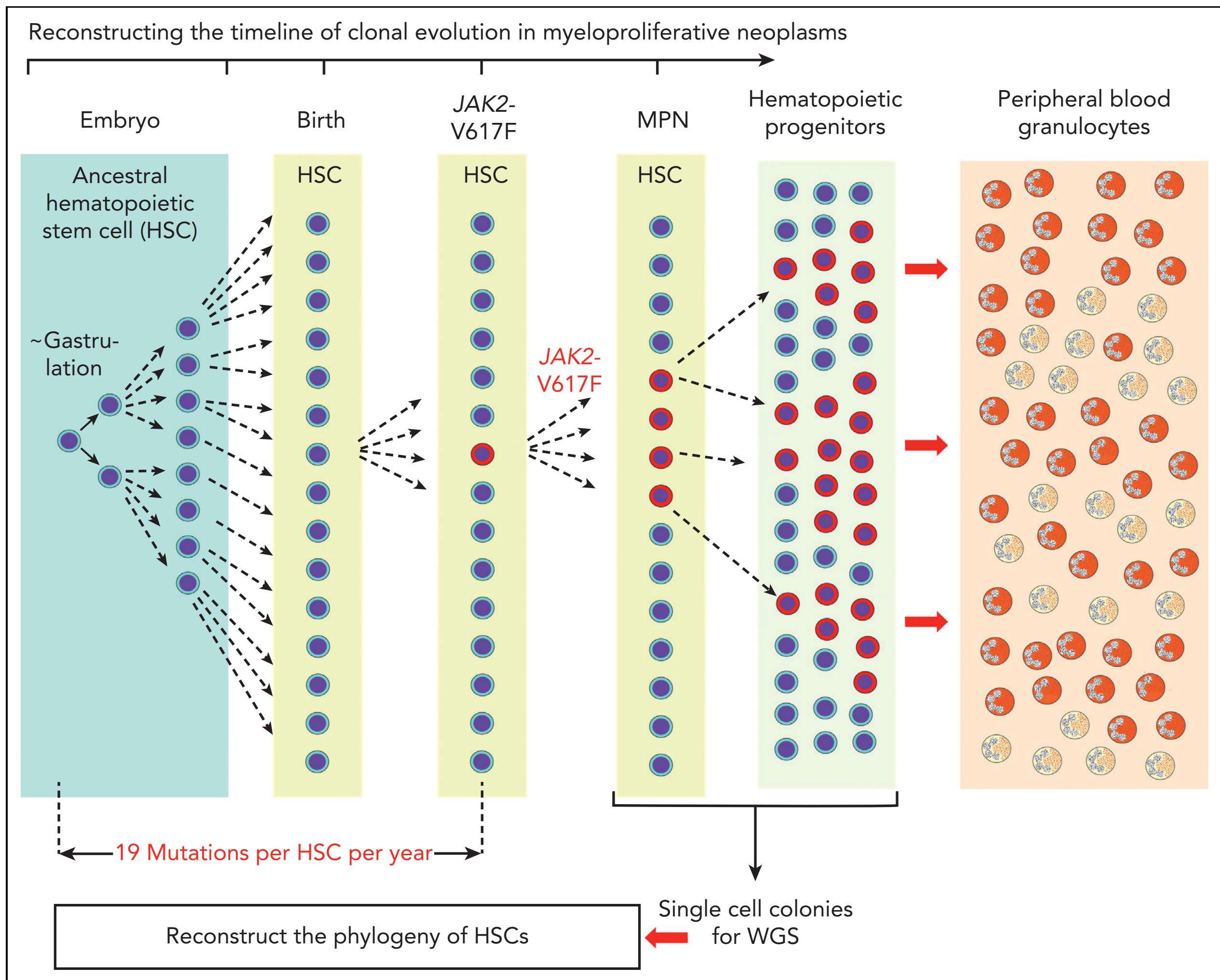

Figure 1. Reconstructing the timeline of clonal evolution in myeloproliferative neoplasms. Schematic drawing of the timeline (not to scale) and the different hematopoietic compartments during the clonal evolution of MPNs caused by *JAK2*-p.V617F. Starting in embryogenesis with the first division of an ancestral HSC, the daughter cells acquire a number of mutations in their genomes that can be used as markers to distinguish them from all other cells that underwent cell division. New mutations are added during each of the next cell divisions, and by comparing the sequence similarities and differences of individual HSCs, a phylogenetic tree can be reconstructed. This analysis relies on taking bone marrow cells after MPN has been diagnosed and depositing single HSCs or progenitor cells into wells where they can be expanded in liquid culture to obtain single cell–derived colonies. DNA from each of these colonies is then analyzed by WGS. By comparing the sequence similarities and differences of individual HSCs, a phylogenetic tree can be reconstructed that originates in a common ancestor HSC that first divided at the time of gastrulation. The estimate of the time when *JAK2*-p.V617F mutation was acquired is calculated by assuming a constant mutational rate of 19 mutations per HSC per year. This estimate is not confounded by the increased cell division rate of *JAK2*-p.V617F mutant HSCs, because only sequence alterations that occurred before *JAK2*-p.V617F was acquired were used for deriving the estimate.

WGS, which found that some sequence alterations can be detected, but these were not in any of the genes known to have a functional impact on hematopoiesis.[14,15]

"Clonal driver" mutations do not cause the MPN phenotype when expressed in mouse models, but they modify the phenotype when they are combined with one of the "disease driver" mutations. Some of these "clonal driver" mutations alter blood counts, increase HSC fitness or proliferation rate, and induce pre-malignant changes in hematopoiesis not directly related to MPN. The "clonal driver" genes that are most frequently mutated in MPNs include *TET2*, *DNMT3A*, *ASXL1*, and *EZH2*. These gene mutations are not MPN-specific and can be found across all myeloid malignancies. The MPN "disease driver" mutations, in particular *JAK2*-p.V617F, can also be "clonal drivers" in the context of clonal hematopoiesis, without resulting in MPNs.

Finally, "passenger" mutations are sequence alterations without a functional consequence. They can be useful as markers to determine and follow the subclonal structure in MPN. In many cases, whether a gene mutation alters function is difficult to predict, because many mutations have not yet been analyzed functionally. Such mutations are considered "variants of unknown significance". Recurrence of such mutations can be used as a criterion in favor of a clonal advantage, but functional assays, such as those in cell lines, will be required in order to assign variants of unknown significance to either the "passenger" or "clonal driver" category.

Given that MPNs originate from a single HSC, early events after the acquisition of a driver gene mutation that lead to the expansion of the clone have become the focus of intense research. This focus is particularly relevant for *JAK2*-p.V617F, because *JAK2* is also one of the genes most frequently mutated in

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Table 1. Recurrent somatic gene mutations in myeloproliferative neoplasms (MPNs) and secondary acute myeloid leukemia (AML)

Gene	Function	Location and type of mutation	Frequency, % PV	ET	PMF	Post-MPN AML	Clinical impact
Disease driver mutations							
JAK2	JAK-STAT signaling	V617F or exon 12	98%	55%	60%	—	WHO/ICC criterion for MPN diagnosis
MPL	TPO receptor JAK-STAT signaling	Exon 10	0%	5%-7%	7%-10%	—	WHO/ICC criterion for MPN diagnosis
CALR	Chaperone protein CALR-mut binds and activates MPL	Frameshift in exon 9	0%	25%-30%	20%-30%	—	WHO/ICC criterion for MPN diagnosis
Clonal driver mutations							
TET2	Epigenetic regulation	All exons	10%-20%	3%-10%	10%-20%	19%-25%	No prognostic impact reported
DNMT3A	Epigenetic regulation	R882 and all exons	5%-10%	1%-5%	8%-12%	3%-17%	No prognostic impact reported
IDH1	Epigenetic regulation	R132	1%-2%	1%-2%	5%-6%	13%	HMR in PMF and adverse prognostic impact in all MPN subtypes
IDH2	Epigenetic regulation	R140 or R172	1%-2%	1%-2%	5%-6%	7%-15%	HMR in PMF and adverse prognostic impact in all MPN subtypes
ASXL1	Epigenetic regulation	Mostly nonsense / frameshift in the last exon	2%-7%	5%-10%	15%-35%	17%-47%	HMR in PMF and adverse prognostic impact in all MPN subtypes
EZH2	Epigenetic regulation	All exons	1%-2%	1%-2%	7%-10%	7%-13%	HMR in PMF and adverse prognostic impact in all MPN subtypes
NRAS	ERK/MAPK signaling	G12, G13, or Q61	<2%	<2%	2%-4%	11%	Adverse prognostic impact in all MPN subtypes
KRAS	ERK/MAPK signaling	G12, G13, or Q61	<2%	<2%	2%	4%-7%	Adverse prognostic impact in all MPN subtypes
SH2B3	JAK signaling regulation	Exon 2	2%-9%	1%-3%	2%-4%	6%-11%	Rare mutations in JAK2-negative MPNs
CBL	JAK signaling regulation	Exons 8 and 9	<2%	<2%	4%	4%	Adverse prognostic impact in all MPN subtypes
SRSF2	mRNA splicing	P95	<2%	<2%	6%-14%	7%-22%	HMR in PMF and adverse prognostic impact in all MPN subtypes
SF3B1	mRNA splicing	Exon 14-16	2%-3%	2%-5%	5%-7%	7%-11%	Adverse prognostic impact in ET
U2AF1	mRNA splicing	S34 or Q157	<2%	<2%	7%-10%	5%-12%	Adverse prognostic impact in all MPN subtypes
NFE2	Transcriptional factor	All exons	3%,6%	1%-7%	3%-5%	?	Increased risk of leukemic transformation
RUNX1	Transcriptional factor	All exons	<2%	<2%	2%-3%	20%	Adverse prognostic impact in all MPN subtypes
TP53	Transcriptional factor	All exons	<2%		4%-5%	16%-50%	Adverse prognostic impact in all MPN subtypes

Only gene mutations occurring with a frequency of >2% are listed.

CALR-mut, calreticulin mutant; ERK, extracellular signal-regulated kinase; ET, essential thrombocythemia; HMR, high molecular risk category for PMF; ICC, international consensus classification; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MPL, thrombopoeitin receptor; PMF, primary myelofibrosis; PV, polycythemia vera; TPO, *thrombopoietin;* WHO, World Health Organization.

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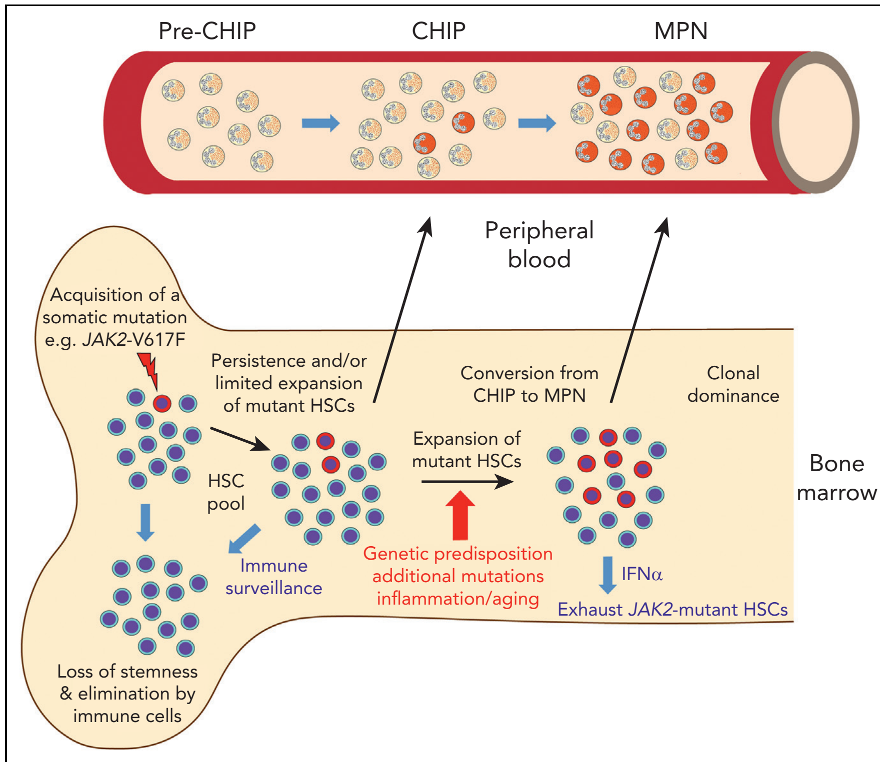

Figure 2. Evolution of MPNs originating from a single HSC that acquired a disease driver mutation. Model summarizing the events from the acquisition of *JAK2*-p.V617F until the development of MPN disease. The early events occur inside the bone marrow. A single HSC with *JAK2*-p.V617F can divide to yield 2 HSC daughter cells that carry the mutation, which leads to persistence, and later limited expansion of the mutated HSCs. Alternatively, the mutated HSC can differentiate into committed progenitors that produce a wave of mutant hematopoietic cells, but eventually are exhausted, due to loss of stemness. The mutant HSCs can also be eliminated at this early stage by cells of the immune system. During this phase, cells carrying *JAK2*-p.V617F are not yet detectable in peripheral blood ("pre-CHIP phase"). After expansion of the mutated HSCs and with a latency of years or decades, mature hematopoietic cells carrying *JAK2*-p.V617F are produced that become detectable in peripheral blood as "clonal hematopoiesis of undetermined potential (CHIP)." In only a minority of cases, the *JAK2*-p.V617F mutant HSC clone expands and produces committed progenitors that become dominant in bone marrow and can be diagnosed as MPN with elevated blood counts in peripheral blood. The factors favoring this conversion from CHIP to MPN are listed under the red arrow. The size of the *JAK2*-p.V617F mutant HSC clone can be reduced by interferon-α (IFNα), which acts by pushing the mutant HSCs into the cell cycle and thereby exhausting them,

healthy individuals with clonal hematopoiesis. Clonal hematopoiesis in the context of MPNs can be defined as the over-proportional presence of blood cells that originate from a single HSC. Detecting clonality relies on finding markers that allow these clonal cells to be distinguished from the progeny of other HSCs.

In a seminal study, DNA from single hematopoietic colonies derived from bone marrow of a healthy donor was analyzed by WGS to reconstruct the phylogeny of HSCs.[18] The method relies on the fact that during each cell division, the 2 daughter cells acquire a number of mutations in their genomes, owing to the imperfect fidelity of DNA replication and DNA repair. The vast majority of these sequence alterations are functionally silent, but they can be used as markers that distinguish the daughter cells from all other cells that underwent cell division. With a sufficient number of sequence alterations, markers can be found that distinguish very early HSC ancestors, from which a large proportion of adult HSCs are derived. By WGS of 140 single cell–derived colonies, all blood cells were mapped to originate from a common ancestor at the time of gastrulation (Figure 1).[18] Markers that identify very early cell divisions, when applied to adult hematopoiesis, would define large "clones" in normal hematopoiesis, and would falsely suggest clonality, because the markers are shared by a large number of adult HSCs. The most useful markers of clonality in hematopoiesis are gene alterations that distinguish between HSCs at the time when the clonal driver gene mutation was acquired—that is, the best clonality marker is the actual "clonal driver" mutation itself. A second aspect to consider when assessing clonality is the threshold percentage of contribution of a single HSC to peripheral blood that is sufficient to be considered "over-proportional." Unfortunately, the number of different HSCs that at any given time are contributing to peripheral blood cells under healthy steady state conditions is not known. Age-dependent constriction of the HSC pool was recently described, and this could decrease the threshold for "clonality."[19]

Clonal hematopoiesis of indeterminate potential and the evolution of MPNs

The term "clonal hematopoiesis of indeterminate potential" (CHIP) was created to denote a potentially "pre-malignant" state in individuals without a hematologic malignancy, and the most frequently found markers in CHIP, not surprisingly, also represent mutations in known cancer-associated genes, in particular the epigenetic regulators *DNMT3A*, *TET2*, and *ASXL1*, and at lower frequencies also *JAK2*. Recurrent somatic mutations in *TET2* were the first to be described in elderly individuals with clonal hematopoies.[20] The threshold for CHIP in most studies was set at variant allele fraction (VAF) >2% (ie, >4% of peripheral blood cells carry this heterozygous mutation). The measured %VAF is also influenced by the source of cells used for the analysis. DNA from unfractionated total white blood cells, which represent a mixture of B and T-cells with a long half-life, will decrease the VAF of most CHIP-markers, compared with granulocytes that have a short half-life and high turnover.

In the context of *JAK2*-p.V617F, several studies used highly sensitive polymerase chain reaction (PCR) methods and expanded the definition of CHIP to include individuals with *JAK2*-p.V617F VAF as low as VAF 0.01%,[21] which means that 1 in 5000 cells was positive for the *JAK2*-p.V617F. With this expanded definition of CHIP, the authors characterized a cohort of 19 958 probands and found 613 that carried *JAK2*-p.V617F

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(3.1%) and 32 that carried *CALR* mutations (0.2%). With a threshold of 1% VAF, the prevalence of *JAK2*-p.V617F was still 0.46%.[21] Within these 613 patients, 16 previously unrecognized cases of MPN were found, but the vast majority of individuals with *JAK2*-p.V617F CHIP did not fulfill the diagnostic criteria for MPN. The cumulative incidence of evolution from *JAK2*-p.V617F CHIP to MPN remains to be determined, but VAF *JAK2*-p.V617F >2%, or an increase in VAF during follow-up, was associated with a high rate of conversion from CHIP to MPN.[22,23] Some patients with very low VAF in purified granulocytes can still display MPN phenotypes, due to selective expansion of the *JAK2*-mutant clone in late stages of erythropoiesis and thrombopoiesis.[24]

The time required for the conversion from CHIP to MPN was estimated, in a study that analyzed blood donors who progressed to MPN, and from whom earlier blood samples were available for analysis, to be 5 to 15 years.[25] WGS of single cell colonies was also applied to reconstruct the phylogeny and timing of acquisition of the *JAK2*-p.V617F mutation in MPNs.[5,6] Both studies concluded that the *JAK2*-p.V617F mutation occurred several decades before the diagnosis of MPN. Thus, the conversion from acquisition of *JAK2*-p.V617F to manifestation of MPN appears to take several decades, and from CHIP to MPN takes 5 to 15 years. Therefore, the time from acquisition of *JAK2*-p.V617F until it is detectable as CHIP is also likely to be in the range of decades (Figure 1).[26]

In the context of MPN, a noteworthy point is that the prevalence of *JAK2*-p.V617F CHIP is 15-fold higher than that of mutated *CALR*. This difference could be due to more frequent acquisition of *JAK2*-p.V617F. Alternatively, *CALR* mutations, by conferring a higher rate of clonal expansion compared to *JAK2*-p.V617F, may shorten the transition from acquisition of the mutation through the CHIP phase to MPN, resulting in a lower prevalence of CHIP.[27] Reconstructing the phylogeny of *CALR* mutations by WGS will likely clarify these issues. The likelihood that an HSC carrying a driver gene mutation will escape immune surveillance, expand, and produce peripheral blood cells detectable as CHIP could also contribute to the observed differences. As many healthy individuals have been found to carry memory T-cells that recognize the mutated CALR-neoantigen without any evidence for *CALR*-mutated CHIP,[28] the lower prevalence of *CALR*-mutated CHIP could be due to a more effective elimination of *CALR*-mutated cells at the pre-CHIP phase by the immune system in an HLA-restricted manner. In contrast, mutant CALR protein was also shown to decrease major histocompatibility complex-I expression and cause defects in peptide loading,[29,30] providing a possible explanation for why immune checkpoint blockade and mutant CALR-derived peptide vaccination failed to generate clinical responses in MPN patients.[31,32] Peptides containing the *JAK2*-p.V617F mutation also are unlikely to form high-affinity interactions with any HLA allotype,[33] and activation of JAK/STAT signaling by *JAK2*-p.V617F increases programmed death ligand 1 (PD-L1) expression.[34,35] Therefore, cells expressing *JAK2*-p.V617F are expected to evade immune surveillance, which could explain why *JAK2*-p.V617F CHIP is so frequent. Conversely, *JAK2*-p.V617F CHIP only rarely progresses to MPN, suggesting that some mechanisms are active mainly at this later conversion stage (Figure 2).

Factors influencing the appearance of CHIP and conversion to MPNs

Genetic predisposition to MPNs has been documented by many studies and could play an important role in increasing the likelihood of conversion from CHIP to MPNs. On a population scale, relatives of MPN patients have an approximately 6- to 8-fold higher risk of developing MPNs.[36] The "strength" (penetrance) with which germline genetic factors exert their effect on developing MPNs inversely correlates with their prevalence in the general population (Figure 3). Rare families with autosomal dominant inheritance and a massively increased odds ratio (~500×) for acquiring MPN disease have been described,[37,38] but the mutations responsible for the predisposition have been identified in only very few cases. The best-studied cases are the duplication on chromosome 14q32,[39] and mutations in *RBBP6*.[40] The identification of mutations in other families with MPNs has been hampered by the low penetrance and also by the genetic heterogeneity among the different pedigrees. A mutation in *EPOR* (*EPOR*-p.P488S) that increases the risk of acquiring MPNs in one family was recently described.[41]

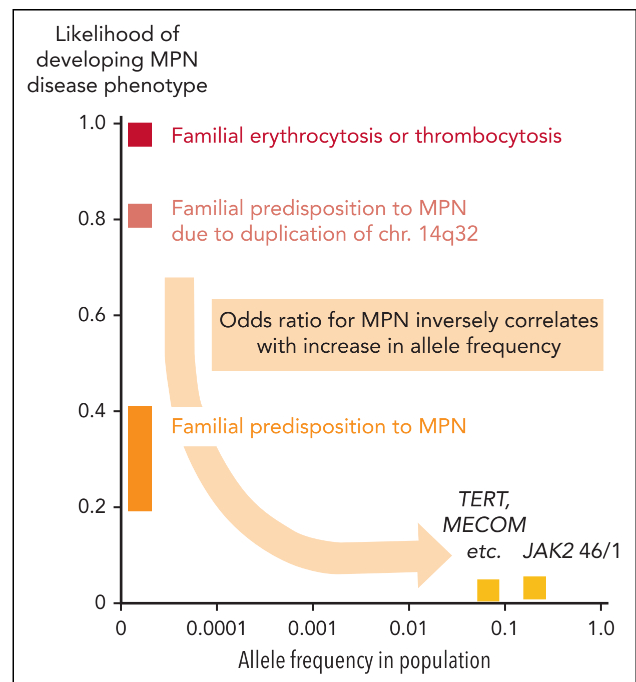

Figure 3. Frequencies of germline variants and the associated likelihood for developing a phenotype. Graph depicting the frequencies of germline gene mutations in the general population (x-axis) and the likelihood that they will promote the manifestation of MPN or MPN-like disease (y-axis). The highest penetrance (close to 100%) is observed in rare families with erythrocytosis or thrombocytosis caused by mutations in a single gene that are most frequently inherited as a Mendelian trait with autosomal dominant transmission (*EPOR*, *EPO*, *THPO*, and *MPL*, etc), or autosomal recessive transmission (Chuvash polycythemia due to mutations in *VHL*). Rare familial cases with inherited predisposition to MPN require the acquisition of somatic disease driver mutations for disease manifestation, most frequently *JAK2*-p.V617F, or mutations in *CALR* or *MPL*. The predisposing germline mutations are inherited as autosomal dominant traits with reduced penetrance, in most cases ~20% to 40%. Inherited predisposition to MPN due duplication of chr.14q32 is an exception, reaching higher penetrance around 80%. Finally, common polymorphisms found at high frequencies in the general populations located in, for example, the *JAK2*, *MECOM*, and *TERT* genes, are associated with a much weaker predisposing effect and also require the acquisition of somatic disease driver mutations for MPN.

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A growing number of single-nucleotide polymorphisms (SNPs) or haplotypes (genetically linked sets of SNPs) have been associated with an increased likelihood of acquiring MPNs. A haplotype located in the *JAK2* gene (46/1 or GGCC) contributes to a 2 to 6 times increased risk of developing MPNs.[42-44] Large genome-wide studies also identified SNPs in *TERT*, *MECOM*, *CHEK2*, and several additional loci that weakly increase the risk of acquiring MPNs on the population scale.[45-48] The mechanisms of how these SNPs influence the likelihood of developing MPNs is unknown, but we suspect that they increase the conversion rate by synergizing with the driver gene mutations and facilitating the expansion of the mutant HSC clone. In most familial and population-based cases with germline predisposition to MPNs, the predominant acquired disease driver mutation is *JAK2*-p.V617F, and less frequently *CALR* or *MPL*.[49] Interestingly, some patients acquired the *JAK2*-p.V617F mutation twice, located on the 2 homologous chromosomes.[42]

The fitness of *JAK2*-p.V617F CHIP clones showed interindividual variability that did not correlate with the presence or absence of known predisposition polymorphisms.[19,26] The number of additional somatic mutations was among nonhereditary factors that increased the fitness of *JAK2*-p.V617F clones in human MPNs (Figure 2).[6] Increased efficiency of MPN disease initiation of HSCs carrying *JAK2*-p.V617F and *Ezh2* loss-of-function mutations was also shown in limiting dilution transplantations in mouse models of MPNs.[50] Furthermore, the promoting effects of inflammation on the early steps of CHIP and MPN evolution are increasingly recognized. Loss of interleukin-1β (IL-1β) decreased the frequency of MPN disease initiation in limiting dilution transplantations in mouse models of MPN.[51] The presence of CHIP was found to be associated with an increased risk for hematologic malignancies and unexpectedly also for cardiovascular disease,[52] resulting in reduced survival.[53-55] The cardiovascular risk was particularly high for *JAK2*-p.V617F CHIP (hazard ratio ~12), and it was lower for other frequent CHIP mutations (~2).[52] Whether CHIP is a cause of the increased cardiovascular risk or just an associated symptom remains unclear. Inflammation could promote atherosclerosis and at the same time favor the expansion of mutated HSCs resulting in clonal hematopoiesis.[56] In some patients with polycythemia vera (PV), complete molecular remission has been achieved by treatment with pegylated interferon-α (pegIFNα). A recent meta-analysis found a lower rate of thromboembolic events in patients treated by pegIFNα.[57] Type I IFN was found to inhibit IL-1β production and inflammasome activation,[58] providing a possible link between the 2 observations. When pegIFNα was discontinued in patients with complete molecular response, blood counts remained in the normal range, but *JAK2*-p.V617F remained detectable at low VAFs (<1%-5%), reminiscent of CHIP. The thrombotic risk in these patients is currently unknown, but an elevated basal level of inflammation could continue to increase the risk for cardiovascular disease, despite very low *JAK2*-p.V617F VAFs.

Determining the mutational landscape of MPN at diagnosis and during follow-up

Detecting disease driver gene mutations is a mandatory step in the diagnostic workup according to the World Health Organization and International Consensus Classification guidelines.[59,60] NGS techniques allow detection of a large number of additional gene mutations in MPNs that occur in lower frequencies than they do in other hematologic malignancies.[15,61,62] The median number of somatic mutations detected per patient by WES was 6.5 for essential thrombocythemia (ET) and PV, and 13 for primary myelofibrosis (PMF),[15] but most of these were passenger mutations.[17] Sequential analysis revealed that most mutations were already present at MPN diagnosis, and very few were acquired during follow-up.[16,63] This finding is unexpected, given that *JAK2*-p.V617F has been reported to induce genetic instability.[64,65] Therefore, a useful approach is to perform in-depth mutational profiling by NGS at diagnosis. Targeted NGS panels of 30 to 50 genes are now commonly used because they provide high coverage at low cost with rapid turnaround time.[66] However, WES and WGS are becoming more widely available with decreasing costs, higher throughput, and improved sensitivities due to proofreading. These genome-wide techniques will likely replace targeted NGS panels in the future.

Using and interpreting NGS data presents 2 major challenges—clarifying the somatic vs germline origin of the mutations and defining whether the mutations have functional consequences or represent functionally silent variants. A reliable distinction between somatic and germ-line mutations would be an important first step in filtering NGS results. Mutations with low VAF are likely to be somatic, but mutations with VAF closer to 50% require sequencing of DNA from non-hematopoietic tissues, such as hair roots, nails, or fibroblasts, to exclude germline origin. DNA from buccal swabs is frequently contaminated by leukocytes and is therefore less reliable as a non-hematopoietic control,[66] unless strict optimization procedures are applied.[67] Functional assays testing the consequences of gene mutations are ultimately required, but these constitute a bottleneck, as they are difficult to perform with high throughput. Eliminating nonfunctional variants and distinguishing between somatic and germline mutations would improve the prognostic value of mutational profiles in MPNs.

PMF is characterized by a complex molecular landscape, with enrichment of mutations in chromatin regulator and spliceosome genes.[16,17,68,69] However, mutations in spliceosome genes were rarely found together with *CALR* mutations (Figure 4),[15,16,70] due to reduced HSC fitness when co-expressed.[71] Although *JAK2*-p.V617F and *CALR* mutations rarely occur in the same patients and usually represent separate clones, no negative impact on HSC function was observed when *JAK2*-p.V617F and *CALR*-del52 were co-expressed in the same cells.[72,73] Post-PV and post-ET myelofibrosis had molecular profiles similar to those of PMF, but mutations in *ASLX1* and *SRSF2* were less frequent.[74,75] Similar to gene mutations, chromosomal aberrations are more frequently found in PMF than in PV and ET.[76,77] The most frequent karyotypic abnormalities involve chromosomes 9, 8, 20, 14, and 13. NGS technologies allow detection of large chromosomal abnormalities by monitoring of changes in the coverage of the affected regions. Similarly, uniparental disomy on chromosome 9p (9pUPD), which renders cells homozygous for *JAK2*-p.V617F,[8,78] can be detected, whereas NGS currently cannot detect balanced translocations or minor subclonal

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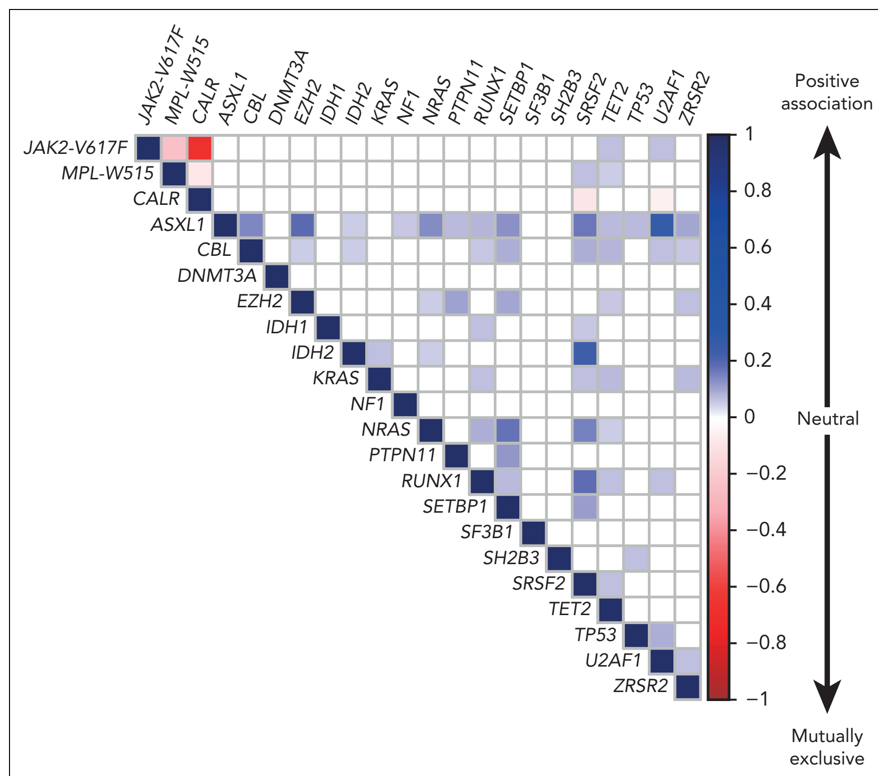

Figure 4. Correlation plot showing the likelihood of co-occurrence of gene mutations in the same patient. The association between gene mutations is based on the compilation of data from 5 published cohorts comprising a total of 3002 MPN patients, including 1497 ET, 535 PV, and 970 PMF or secondary MF patients.[16,17,79,80] The color code and numbers on the y-axis represent the Pearson R-coefficient, which indicates the strength of positive or negative associations between 2 gene mutations. Only the significant associations are depicted (*P*-value < .01).

chromosomal aberrations on DNA, but transcriptome sequencing can be used instead.[33]

In triple-negative ET patients, NGS covering the entire coding regions of *JAK2* or *MPL* genes detected mutations located outside of mutational hot-spots in 20% to 30% of cases.[17,81-84] These variants can be germline or acquired.[85-88] Only a few of them have been tested functionally (Table 2), often showing either no or only weak oncogenic potential, indicating that other mutations must drive the phenotype.[81,82] Sensitive functional tests for weak gain-of-function *MPL* mutations in cell lines are needed.[81,82,89] Additional genes should be sequenced in suspected cases of hereditary erythrocytosis and thrombocytosis.[90]

During follow-up of MPN patients, monitoring of VAF of mutations detected at diagnosis can be useful, but it is currently applied in only observational studies. An increase in VAF for *JAK2*-p.V617F was associated with higher risk of progression to myelofibrosis in ET and PV patients.[91] Determining the VAF for *JAK2*-p.V617F and *CALR* mutations can be applied to monitor molecular response to treatment. Currently, only pegIFNα induces complete molecular responses in some patients.[92-94] IFN is thought to act by exhausting HSCs that carry disease driver mutations,[95-97] but not all patients respond similarly. Polymorphisms in the *IFNL4* gene are correlated with the molecular response to pegIFNα.[98] Overall, a higher rate of molecular responses to pegIFNα is observed in patients with *JAK2*-p.V617F than in patients with *CALR* mutations.[99,100] Also, clones harboring additional mutations in *TET2* or *DNMT3A* are less responsive to pegIFNα,[101,102] and pegIFNα even promoted the expansion of subclones carrying both *JAK2*-p.V617F and *DNMT3A* mutations.[103,104]

Relevance of gene mutations for prognostic stratification

The type of disease driver mutations has been shown to influence prognosis in respect to thrombo-hemorrhagic complications and overall survival, with *CALR* mutations having a better prognosis than mutations in *JAK2* or *MPL*, and triple-negative PMF having the least favorable outcome.[105] The presence of additional mutations has not been reported to be associated with thrombosis. In contrast, the number of additional mutations per patient represents a risk factor for leukemic transformation and is inversely correlated with overall survival in MPN.[16] This correlation was confirmed in PMF.[106] Using a global pan-MPN approach based on clinical and molecular data in a cohort of 2035 patients, a personalized prognostic algorithm was developed that is accessible online.[17] The presence of a mutation in one of 18 genes involved in chromatin or splicing regulation was associated in ET and PV with reduced overall survival and higher risk of progression to myelofibrosis or acute myeloid leukemia (AML). Some gene mutations often occur simultaneously in the same patient, whereas other combinations are mutually exclusive (Figure 4).

The impact of additional somatic mutations has been studied in great detail in patients with PMF or secondary myelofibrosis who were considered as candidates for allogeneic stem cell transplantation. Predicting prognosis in these patients primarily depends on estimating the risk of leukemic transformation and bone marrow failure with the associated complications due to cytopenia. Presence of mutations in *ASXL1*, *EZH2*, *IDH1*, *IDH2*, and *SRSF2* has a stronger negative effect on prognosis, and these mutations were therefore designated to confer "high molecular risk" (HMR).[107] HMR mutations have been

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Table 2. Mutations in *JAK2* and *MPL* genes described in myeloproliferative neoplasm (MPN) and MPN-like diseases

Mutation	Protein domain	Somatic or germline	Disease phenotype	Found alone or together with another mutation	Mean allele frequency	Functional effect (gain-of-function)
***JAK2* mutations (ENST00000381652 transcript)**						
p.T108A	FERM	germ	PV	*JAK2* p.V617F	0.0007%	Weak
p.Y317H	FERM	NT	PMF	*CALR*	0.002%	Weak
p.H345L	FERM	som	PMF	*CALR*	0%	Weak
Exon 12 mutations between M535 to F547	JH2	som	PV	Alone	0%	Strong driver gene mutations
p.F556V	JH2	germ	ET	Alone	0.001%	Weak
p.R564Q	JH2	germ	HT	Alone	0.003%	Intermediate
p.L583_A586delins	JH2	som	PV	Alone	0%	Intermediate
p.H608N	JH2	germ	HT	Alone	0%	Weak
p.L611S	JH2	germ or NT	HT or PV	Alone or with *JAK2* p.V617F	0%	Weak
p.V617F	JH2	som	PV, ET or PMF	Alone	0.034%	Strong driver gene mutation
p.V617I	JH2	germ or som	HT or ET	Alone	0%	Weak
p.V625F	JH2	germ	ET, PMF	Alone	0%	Weak
p.E846D	JH1	germ	HE	Alone	0.045%	Need cooperating mutation
p.R867Q	JH1	germ	HT	Alone	0.0005%	Intermediate
p.T875N	JH1	germ or som	HT or PMF	Alone	0%	Strong
p.R755S/R938Q	JH2/1	germ	HT	Alone	0.003%	Intermediate
p.R1063H	JH1	germ	HT	Alone	0.47%	Need cooperating mutation
***MPL* mutations (ENST00000372470 transcript)**						
p.K39N	EC	germ	HT	Alone or with other *MPL* mutations	0.46% and 4.6% in Africans	Reduced cell surface MPL
p.P106L	EC	germ	HT for hom	Alone	0.038%	Reduced cell surface MPL
p.T119I	EC	som	ET or PMF	Alone	0%	Minimal
p.S204P/F	EC	som	ET or PMF	Alone	0%	Weak
p.E230G	EC	som	ET or PMF	Alone	0%	Minimal
p.V285E	EC	germ	HT	Alone	0%	Minimal
p.R321W	EC	germ	HT	Alone	0%	Minimal
p.L498W	TM	som	ET	*MPL* p.S505N	0%	Intermediate
p.L498_H499insVIAL	TM	som	ET	Alone	0%	Intermediate
p.V501A	TM	som or NT	ET or PMF	*MPL* p.W515L/K or p.S505N	0%	Intermediate
p.S505N	TM	som or germ	HT and ET, PMF	Alone	0%	Strong driver gene mutation
p.W515K/L/A/R	TM	som	ET or PMF	Alone	0%	Strong driver gene mutation
p.Y591D/N	IC	som	ET or PMF	Alone	0%	Weak

Mutations of *JAK2* and *MPL* genes in MPN and MPN-like diseases were screened in PubMed publications and the Catalogue Of Somatic Mutations In Cancer (COSMIC) database. Only mutations with a functional impact on Janus kinase–signal transducer and activator of transcription (JAK-STAT) signaling and/or cell proliferation were retained. Functional effect was classified as being minimal to strong, as follows: minimal if only a constitutive STAT signaling was found; weak if a hypersensitivity to cytokine was found in transfected cell lines; intermediate if the mutation induced cytokine-independent growth; and strong if a mouse model had an MPN-like phenotype. *MPL* mutations inducing a reduction of the cell surface expression of thrombopoeitin receptor MPL lead to an increased level of plasmatic *thrombopoietin* (TPO) that stimulates megakaryopoiesis. The allele frequencies in the general population were extracted from the gnomAD v2.1 database. EC, extracellular; ET, essential thrombocythemia; FERM, protein 4.1R/ezrin/radixin/moesin; germ, germline; HE, hereditary erythropoiesis; hom, homozygous; HT, hereditary thrombocytosis; IC, intracellular; NT, not tested; PMF, primary myelofibrosis; PV, polycythemia vera; som, somatic; TM, transmembrane.

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incorporated into the International Prognostic Scoring System (IPSS)/Dynamic IPSS prognostic scores, resulting in MIPSS70.[108] Recently, the *U2AF1* gene has been added to the list of HMR genes (MIPSS70v2, GIPSS).[109,110] Chromosomal aberrations are also associated with adverse prognosis and have been integrated into prognostic scores for PMF (MIPSS70+).[108] Mutations in *NRAS*, *KRAS*, *CBL*, and *TP53* are also associated with poor prognosis and were proposed to be added to the HMR list.[17,75,111] The adverse impact of *ASXL1* mutations alone was suggested to be weak, and the HMR effect of *ASXL1* was shown to be dependent on the presence of other mutations.[75,112]

The order of acquisition of mutations may matter, and *JAK2*-p.V617F can be acquired as the first event, or after the acquisition of other mutations such *TET2*, *ASXL1*, and *DNMT3A*.[16,113,114] The order of acquisition between *JAK2*-p.V617F and *TET2* mutations was reported to influence the disease phenotype and the thrombotic risk.[115] Clonal architecture can be determined by genotyping hematopoietic colonies derived from a single HSC or progenitor, but recently genotyping of individual single cells (single-cell DNA sequencing [scDNA-seq]) was described as an alternative method suited for high-throughput applications.[116-118] A recent study defined the clonal architecture of 22 MPN patients that carried *JAK2*-p.V617F and at least one additional somatic mutation and found good agreement between the 2 methods that were used to analyze the same patient samples side-by-side.[119] Unsupervised classification allowed defining clusters based only on clonal architecture data. One of the 4 clusters represented a risk factor for decreased survival independent of the MPN subtype or the age at diagnosis.[119] These results suggest that deciphering the clonal architecture in patients with MPN can further improve and refine the molecular prognostic stratification.

Leukemic transformation

Transformation to secondary AML is a rare but devastating complication associated with a median survival of less than 6 months.[120] The cumulative incidence of leukemic transformation at 10 years of follow-up was around 20% for PMF and 2.5% for both ET and PV.[121-123] The mutational profile of post-MPN AML differs from *de novo* AML by the frequent occurrence of mutations in the *ASXL1*, *SRSF2*, *IDH1/2*, *SH2B3*, *NRAS*, *RUNX1*, and *TP53* genes,[124-132] In some patients, *JAK2*-p.V617F was present at MPN diagnosis, but absent in the leukemic blasts,[133] indicating that AML either arose from a common mutated HSC, representing 2 branches of the same ancestral clone, or alternatively, represented a true bi-clonal disease originating from 2 different HSCs.[134] Epigenetic dysregulation of gene expression also favors leukemic transformation and provides potential therapeutic targets.[135,136]

TP53 is the most frequently mutated gene in post-MPN AML.[16,127] The frequency is particularly high (~50%) when AML develops from PV or ET, typically following a long latency of >8 years after MPN diagnosis.[137] In contrast, *TP53* mutations are less frequent in post-PMF AML.[130] Leukemic transformation with a short latency after MPN diagnosis often involves other oncogenic mechanisms characterized by a more complex molecular landscape and mutations in *IDH1/2*, *EZH2*, or *DNMT3A* genes, or acquisition of *de novo* mutations in *RUNX1* that were not detectable during the chronic phase.[137] Subclones carrying *TP53* mutations are often detectable with low VAFs at MPN diagnosis. Using ultra-deep NGS, *TP53* mutations were detected in 16% of MPNs, but the vast majority of these patients remained stable during follow-up.[138] Leukemic transformation is frequently associated with an increase in *TP53* VAF >50%.[16,129,137,139] These results suggest that transformation of MPNs with *TP53* mutations is a slow process requiring loss of the second *TP53* allele or other oncogenic events. Indeed, in mouse models of *Jak2*-p.V617F, homozygous inactivation of *Tp53* was required for leukemic transformation.[129,136] Despite the known dominant negative and/or gain-of-function effect of some *Tp53* missense mutations,[140] no difference in phenotype was found for the heterozygous *Tp53*-p.R172H mutation, compared to that for the heterozygous *Tp53*$^{+/-}$.[136] These conclusions are supported by a recent study using single-cell genomics in post-MPN AML patients, which found that AML clones often displayed loss or another mutation in the second *TP53* allele.[141,142]

Outlook and conclusions

MPN is a genetically heterogeneous disease in which germline and somatic mutations contribute to disease initiation and the course of disease. NGS-based techniques capture this heterogeneity, and we are at the beginning of understanding how to utilize this information for improving clinical management. To reach this goal, large international registries of MPN patients fully characterized by NGS for somatic and germline mutations will be needed to allow correlation of the disease course, complications, and response to therapies with patients' mutational profiles.

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ORCID profiles: D.L.P., 0000-0002-4523-675X; R.K., 0000-0002-6997-8539; R.C.S., 0000-0002-3626-9496.

Correspondence: Radek C. Skoda, Department of Biomedicine, Experimental Hematology, University Hospital Basel and University of Basel, Hebelstrasse 2, 4054 Basel, Switzerland; email: radek.skoda@unibas.ch; Robert Kralovics, Department of Laboratory Medicine, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria; email: robert.kralovics@meduniwien.ac.at; and Damien Luque Paz, Laboratory of Hematology, Angers University Hospital, 4 Rue Larrey, 49100 Angers, France; email: damien.luquepaz@chu-angers.fr.

Footnote

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