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REVIEW

An overview on *CALR* and *CSF3R* mutations and a proposal for revision of WHO diagnostic criteria for myeloproliferative neoplasms

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Disease-specific mutations facilitate diagnostic precision and drug target discovery. In myeloproliferative neoplasms (MPN), this is best exemplified by the chronic myeloid leukemia-associated *BCR-ABL1*. No other mutation in MPN has thus far shown a similar degree of diagnostic accuracy or therapeutic relevance. However, *JAK2* and *KIT* mutations are detected in more than 90% of patients with polycythemia vera and systemic mastocytosis, respectively, and are therefore used as highly sensitive clonal markers in these diseases. *JAK2* and *MPL* mutations also occur in essential thrombocythemia (ET) and primary myelofibrosis (PMF), but their diagnostic value is limited by suboptimal sensitivity and specificity. The molecular diagnostic gap in *JAK2/MPL*-unmutated ET/PMF is now partially addressed by the recent discovery of calreticulin (*CALR*) mutations in the majority of such cases. However, bone marrow morphology remains the central diagnostic platform and is essential for distinguishing ET from prefibrotic PMF and diagnosing patients those do not express *JAK2*, *MPL* or *CALR* (triple-negative). The year 2013 was also marked by the description of *CSF3R* mutations in the majority of patients with chronic neutrophilic leukemia (CNL). Herein, we argue for the inclusion of *CALR* and *CSF3R* mutations in the World Health Organization classification system for ET/PMF and CNL, respectively.

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INTRODUCTION

The World Health Organization (WHO) system uses peripheral blood (PB) counts and smear analysis, bone marrow (BM) morphology, karyotype and molecular genetic tests to classify myeloid malignancies into five major categories (Table 1).[1,2] Myeloproliferative neoplasms (MPN) constitute one of these five categories and are further classified into eight subcategories, including the *BCR-ABL1*-defined chronic myeloid leukemia (CML) and the so-called '*BCR-ABL1*-negative MPN': polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) (Table 1). Also included under the umbrella of MPN are chronic neutrophilic leukemia (CNL), systemic mastocytosis (SM), chronic eosinophilic leukemia, not otherwise specified (CEL-NOS) and MPN, unclassifiable (MPN-U).[2] In the current perspective, we focus on two classes of mutations (*CALR* and *CSF3R*) and their potential role in refining the classification and diagnostic criteria for ET, PMF and CNL.

CURRENT DIAGNOSTIC CRITERIA FOR ET, PMF AND CNL

In the context of MPN, the WHO classification system considers the presence of *BCR-ABL1* as being diagnostic for CML and its absence for all other MPN. However, it has become increasingly evident that a small fraction of patients with ET or PMF might harbor low-allelic burden *BCR-ABL1* subclones that are of undetermined significance.[3,4] Such a scenario does not necessarily imply a diagnosis of CML, which should be confirmed by BM examination showing CML-characteristic megakaryocyte proliferation.[3,5] The morphologic denominator in *BCR-ABL1*-negative MPN includes megakaryocytic proliferation associated with variable amounts of granulocytic and erythroid proliferation. In addition, MPN diagnosis requires absence of dysgranulopoiesis or dyserythropoiesis. Table 2 summarizes the 2008 WHO diagnostic criteria for PV, ET and PMF and highlights the role of *JAK2* mutations and BM morphology.[1]

CNL is considered in the presence of marked PB leukocytosis ($\geqslant 25 \times 10(9)/l$), $>80\%$ segmented/band neutrophils, $<10\%$ immature myeloid cells, $<1\%$ circulating blasts and absence of dysgranulopoiesis or monocytosis. CNL should be distinguished from other MPN, including CML, PV, ET and PMF, as well as entities currently classified under MDS/MPN overlap (Table 3). The latter include atypical CML, *BCR-ABL1*-negative (aCML), chronic myelomonocytic leukemia (CMML), juvenile myelomonocytic leukemia (JMML) and MDS/MPN, unclassifiable (MDS/MPN-U). CNL diagnosis also requires exclusion of other causes of nonclonal neutrophilia, including plasma cell neoplasms, solid tumor, infections and inflammatory processes.[6] BM in CNL is hypercellular and displays increased number and percentage of neutrophils with very high myeloid to erythroid ratio and minimal left shift, myeloid dysplasia or reticulin fibrosis.[1]

THE *CALR* STORY

Although the molecular diagnostic gap in *JAK2*V617F-negative PV was adequately addressed by the discovery of other *JAK2*

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Table 1. World Health Organization classification of myeloid malignancies

1. Acute myeloid leukemia (AML) and related precursor neoplasms[a]
2. Myeloproliferative neoplasms (MPN)
   2.1. Chronic myelogenous leukemia, *BCR-ABL1* positive (CML)
   2.2. Polycythemia vera (PV)
   2.3. Primary myelofibrosis (PMF)
   2.4. Essential thrombocythemia (ET)
   2.5. Chronic neutrophilic leukemia (CNL)
   2.6. Chronic eosinophilic leukemia, not otherwise specified (CEL-NOS)
   2.7. Mastocytosis
   2.8. Myeloproliferative neoplasm, unclassifiable (MPN-U)
3. Myelodysplastic syndromes (MDS)
   3.1. Refractory cytopenia[b] with unilineage dysplasia (RCUD)
      3.1.1. Refractory anemia (ring sideroblasts $<15\%$ of erythroid precursors)
      3.1.2. Refractory neutropenia
      3.1.3. Refractory thrombocytopenia
   3.2. Refractory anemia with ring sideroblasts (RARS; dysplasia limited to erythroid lineage and ring sideroblasts $\geqslant 15\%$ of bone marrow erythroid precursors)
   3.3. Refractory cytopenia with multi-lineage dysplasia (RCMD; ring sideroblast count does not matter)
   3.4. Refractory anemia with excess blasts (RAEB)
      3.4.1. RAEB-1 (2–4% circulating or 5–9% marrow blasts)
      3.4.2. RAEB-2 (5–19% circulating or 10–19% marrow blasts or Auer rods present)
   3.5. MDS associated with isolated del(5q)
   3.6. MDS, unclassifiable (MDS-U)
4. MDS/MPN overlap
   4.1. Chronic myelomonocytic leukemia (CMML)
   4.2. Atypical chronic myeloid leukemia, *BCR-ABL1* negative (aCML)
   4.3. Juvenile myelomonocytic leukemia (JMML)
   4.4. MDS/MPN, unclassifiable (MDS/MPN-U)
      4.4.1. Provisional entity: Refractory anemia with ring sideroblasts associated with marked thrombocytosis (RARS-T)
5. Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA, PDGFRB* or *FGFR1*[c]
   5.1. Myeloid and lymphoid neoplasms with *PDGFRA* rearrangement
   5.2. Myeloid neoplasms with *PDGFRB* rearrangement
   5.3. Myeloid and lymphoid neoplasms with *FGFR1* abnormalities

[a]AML-related precursor neoplasms include 'therapy-related MDS' and 'myeloid sarcoma'. [b]Either mono- or bi-cytopenia: hemoglobin level <10 g/dl, absolute neutrophil count $<1.8 \times 10^9$/l or platelet count $<100 \times 10^9$/l. However, higher blood counts do not exclude the diagnosis in the presence of unequivocal histological/cytogenetic evidence for MDS. [c]Genetic rearrangements involving platelet-derived growth factor receptor α/β (*PDGFRA/PDGFRB*) or fibroblast growth factor receptor 1 (*FGFR1*).

Table 2. 2008 World Health Organization diagnostic criteria for myeloproliferative neoplasms

	Polycythemia vera (PV)[a]	*Essential thrombocythemia (ET)*[a]	*Primary myelofibrosis (PMF)*[a]
Major criteria			
1	Hemoglobin (Hgb) >18.5 g/dl (men) >16.5 g/dl (women) or[b]	Platelet count $\geqslant 450 \times 10^9$/l	Megakaryocyte proliferation and atypia,[c] accompanied by either reticulin and/or collagen fibrosis, or[d]
2	Presence of *JAK2*V617F or *JAK2* exon 12 mutation	Megakaryocyte proliferation with large and mature morphology.	Not meeting WHO criteria for CML, PV, MDS, or other myeloid neoplasm
3		Not meeting WHO criteria for CML, PV, PMF, MDS or other myeloid neoplasm	Demonstration of *JAK2*V617F or other clonal marker or no evidence of reactive BM fibrosis
4		Demonstration of *JAK2*V617F or other clonal marker or no evidence of reactive thrombocytosis	
Minor criteria			
1	BM trilineage myeloproliferation	—	Leukoerythroblastosis
2	Subnormal serum erythropoietin level	—	Increased serum LDH level
3	Endogenous erythroid colony growth	—	Anemia
4		—	Palpable splenomegaly

Abbreviations: BM, bone marrow; CML, chronic myelogenous leukemia; LDH, lactate dehydrogenase; MDS, myelodysplastic syndrome; WHO, World Health Organization. [a]PV diagnosis requires meeting either both major criteria and one minor criterion or the first major criterion and two minor criteria. ET diagnosis requires meeting all four major criteria. PMF diagnosis requires meeting all three major criteria and two minor criteria. [b]Hgb or hematocrit >99th percentile of reference range for age, sex or altitude of residence or red cell mass $>25\%$ above mean normal predicted or Hgb >17 g/dl (men) and >15 g/dl (women) if associated with a sustained increase of $\geqslant 2$ g/dl from baseline that cannot be attributed to correction of iron deficiency [c]Small to large megakaryocytes with aberrant nuclear/cytoplasmic ratio and hyperchromatic and irregularly folded nuclei and dense clustering. [d]In the absence of reticulin fibrosis, the megakaryocyte changes must be accompanied by increased marrow cellularity, granulocytic proliferation and often decreased erythropoiesis (that is, prefibrotic PMF).

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Table 3. World Health Organization (WHO) diagnostic criteria for CNL, aCML and CMML

Chronic neutrophilic leukemia (CNL)	*Atypical chronic myeloid leukemia (aCML)*	*Chronic myelomonocytic leukemia (CMML)*
PB leukocytosis $\geqslant 25 \times 10^9$/l $>$80% Segmented neutrophils/bands $<$10% PB immature granulocytes[a] $<$1% PB myeloblasts	PB leukocytosis $\geqslant 13 \times 10^9$/l ↑Neutrophils/precursors with prominent dysgranulopoiesis $\geqslant$10% Immature granulocytes[a] $<$20% PB myeloblasts	Persistent PB monocytosis $> 1 \times 10^9$/l No *BCR-ABL1* No *PDGFRA or PDGFRB*mutations
BM hypercellular Neutrophils increased in % and number $<$5% myeloblasts Normal neutrophilic maturation Megakaryocytes normal/left shifted	No *BCR-ABL1* No *PDGFRA or PDGFRB*mutations $<$2% PB basophilia	$<$20% Blasts or promonocytes in BM or PB Dysplasia in one or more myeloid lineages or Clonal cytogenetic or molecular abnormality Monocytosis has lasted for $\geqslant$3 months AND all other causes of monocytosis excluded
Hepatosplenomegaly	No monocytosis and $<$10% PB monocytes	
Absence of reactive neutrophilia[b] Or presence of cytogenetic/molecular abnormality	BM hypercellular ↑Granulocyte proliferation and granulocytic dysplasia with or without dysplasia in erythroid or megakaryocytic lineages	
No *BCR-ABL1*		
No *PDGFRA, PDGFRB* or *FGRF1*mutations	$<$20% Myeloblasts	
No evidence PV, PMF or ET		
No evidence of MDS or MDS/MPN overlap No granulocytic dysplasia No MDS-like changes PB monocytes $< 1 \times 10^9$/l		

Abbreviations: BM, bone marrow; ET, essential thrombocythemia; MDS, myelodysplastic syndromes; MPN, myeloproliferative neoplasms; PB, peripheral blood; PMF, primary myelofibrosis; PV, polycythemia vera. [a]Immature granulocytes include myeloblasts, promyelocytes, myelocytes and metamyelocytes [b]Causes of reactive neutrophilia include plasma cell neoplasms, solid tumor, infections and inflammatory processes.

mutations,[7] the same had not been realized for the 35–40% of patients with ET or PMF who do not express *JAK2* mutations.[8–11] The discovery of *MPL* mutations in 2006[12] only partially addressed the problem because of its low mutational frequency, estimated at 10–20% in *JAK2*-unmutated ET or PMF.[10,11,13] In December 2013, two groups reported the occurrence of novel calreticulin (*CALR*) mutations in *JAK2/MPL*-unmutated PMF or ET.[14,15] Both groups found mutual exclusivity between *CALR*, *JAK2* and *MPL* mutations. CALR is a multi-functional Ca^{2+}-binding protein chaperone mostly localized in the endoplasmic reticulum (ER). Located on chromosome 19p13.2, *CALR* contains nine exons and its protein three domains: N-domain (residues 1–180), P-domain (residues 181–290) and C-domain (residues 291–400). Knocking out *CALR* in mice is lethal and causes impaired cardiac development.[16]

In the study by Klampfl *et al.*,[15] *CALR* mutations were not seen in 382 cases of PV but were detected in 25% of patients with ET ($n = 311$) and 35% of those with PMF ($n = 203$). The authors subsequently enriched their *JAK2/MPL*-unmutated patient population by adding 211 such cases with ET or PMF and reported *CALR* mutational frequencies of 67% and 88% in *JAK2/MPL*-unmutated ET ($n = 289$) and PMF ($n = 120$), respectively. They also found *CALR* mutations in 3 (13%) of 24 patients with refractory anemia with ring sideroblasts and marked thrombocytosis (RARS-T). In this particular study,[15] *CALR* mutations were not seen in acute myeloid leukemia (AML; $n = 254$), MDS ($n = 73$), CMML ($n = 64$) or CML ($n = 45$). In regards to clinical and laboratory correlative studies, *CALR*, compared with *JAK2*, mutations were associated with lower hemoglobin level, lower leukocyte count, higher platelet count, lower risk of thrombosis and better survival in ET and lower leukocyte count, higher platelet count and better survival in PMF.[15]

In the second study by Nangalia *et al*,[14] *CALR* mutations were not seen in 258 patients with PV/post-PV MF or 253 patients with *JAK2/MPL*-mutated ET/PMF/ post-ET MF. *CALR* mutational frequencies were 71%, 56% and 86% in *JAK2/MPL*-unmutated ET ($n = 112$), PMF ($n = 32$) or post-ET MF ($n = 14$), respectively. In this study by Nangalia *et al.*,[14] *CALR* mutations were infrequently seen in MDS (8.3%; $n = 120$) including RARS (11%; $n = 27$), CMML (3%; $n = 33$) and atypical CML (3.4%; $n = 29$) but not in RARS-T ($n = 6$), CML ($n = 28$), AML ($n = 48$), systemic mastocytosis ($n = 114$), eosinophilic disorders ($n = 2$), 'idiopathic' erythrocytosis ($n = 5$), transient abnormal myelopoiesis ($n = 10$), lymphoid malignancies ($n = 287$), solid tumors ($n = 502$) or control samples ($n = 550$). Similar to the observations by Klampf *et al.*,[15] Nangalia *et al.*[14] also found an association between *CALR* mutations and higher platelet count and lower hemoglobin level in ET; in addition, their study suggested an increased incidence of fibrotic transformation in *CALR*-mutated ET without apparent survival difference.[14]

All *CALR* mutations seen in the above two studies[14,15] were exon 9 frame-shift mutations with somatic insertions or deletions. Two variants constituted more than 80% of the *CALR* mutations seen: type 1 variant (p.L367fs*46) resulted from 52 bp deletion and was more frequent in PMF, and type 2 variant (p.K385fs*47) resulted from 5-bp TTGTC insertion. In the study by Klampfl *et al.*,[15] these two common variants, respectively, accounted for 53% and 32% of all mutant *CALR*; the corresponding frequencies in the study by Nangalia *et al.*[14] were 45% and 41%. Only three cases of homozygous *CALR* mutations were reported and all three displayed p.K385fs*47. Several other distinct variants were seen infrequently. All *CALR* mutations resulted in one base pair reading frame shift and an altered C-terminal that is missing the KDEL (lysine, aspartic acid, glutamic acid and leucine) endoplasmic reticulum retention motif and is positively rather than negatively

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charged. *CALR* mutations were found in hematopoietic progenitors and did not appear to affect the intracellular localization of the mutant protein.[14] Overexpression of the most frequent *CALR* deletion in Ba/F3 cell lines caused cytokine-independent growth, and activation of signal transducer and activator of transcription 5 that was sensitive to pharmacologic JAK2 inhibition.[15]

Several other papers on *CALR* mutations in MPN have since been published.[10,11,17,18] Rotunno *et al.*[10] studied 576 patients with WHO-defined ET and reported mutational frequencies of *JAK2*, *CALR*, *MPL* and 'triple-negative' at 64%, 15%, 4% and 16%, respectively. In *JAK2/MPL*-unmutated cases, *CALR* mutational frequency was 49%, which was significantly lower than those reported above by Nangalia *et al.*[14] (71%) and Klampfl *et al.*[15](67%). This discrepancy probably reflects differences in patient selection in terms of the methodology used in diagnosing ET; the study by Rotunno *et al.*[10] used strict WHO criteria, whereas the other studies did not and might have therefore included patients with early/prefibrotic PMF. *CALR* vs *JAK2* mutations were associated with male sex, younger age, lower leukocyte count, lower hemoglobin level and higher platelet count. The association with male sex but not young age, leukocyte count or hemoglobin level was sustained when *CALR* mutations were compared with *MPL* mutations or triple-negative cases. Platelet counts were similar between *CALR*- and *MPL*-mutated cases. *CALR*-mutated and triple-negative cases displayed superior thrombosis-free survival.[10]

In another ET study, Rumi *et al.* [17] reported mutational frequencies of 62, 24, 4 and 10% for *JAK2*, *CALR*, *MPL* and triple-negative cases. Compared with *JAK2*-mutated cases, *CALR*-mutated patients were younger and displayed lower leukocyte count, lower hemoglobin level and higher platelet count. This study also found no difference in overall survival, risk of leukemic or fibrotic transformation but better thrombosis-free survival in *CALR*- vs *JAK2*-mutated patients. In a third study of 289 patients referred for evaluation of persistent thrombocytosis but not confirmed ET,[18] mutational frequencies were 65 *JAK2*, 9 *CALR*, 3 *MPL* and 23% triple-negative. The latter group had lower hemoglobin and platelet levels compared with *JAK2*-mutated cases and lower platelet count compared with *CALR*-mutated cases.[18]

In PMF, Tefferi *et al.* studied 254 patients who were cytogenetically characterized and were also screened for several MPN-characteristic mutations including *ASXL1*, *EZH2*, *IDH* and spliceosome mutations (*SF3B1*, *SRSF2* and *U2AF1*). Mutational frequencies were 58 *JAK2*, 25 *CALR*, 8 *MPL* and 9% triple-negative. Mutational frequency in JAK2/MPL-unmutated cases was 74%. Interestingly, one patient expressed both *JAK2* and *CALR* mutations. *CALR* mutations were associated with younger age, higher platelet count and lower DIPSS-plus score. *CALR*-mutated patients were also less likely to be anemic, require transfusions or display leukocytosis. Spliceosome mutations were infrequent in *CALR*-mutated patients, but no other molecular or cytogenetic associations were evident. In multivariable analysis, *CALR* mutations had a favorable impact on survival that was independent of both DIPSS-plus risk and *ASXL1* mutation status. Compared with CALR-mutated cases, triple-negative patients displayed inferior leukemia-free survival. The study by Tefferi *et al* identified '$CALR^{-}ASXL1^{+}$' and 'triple-negative' mutation profiles to be prognostically detrimental.

THE *CSF3R* STORY

Granulocyte colony-stimulating factor (G-CSF), also known as colony-stimulating factor 3 (CSF3), contributes to the proliferation and granulocyte differentiation of myeloid progenitor cells. Recombinant CSF3 has been used for the treatment of severe neutropenia associated with a variety of conditions, including severe congenital neutropenia (SCN).[19] More than a third of patients with SCN transform into AML after 15 years of treatment with CSF3 and close to 80% of such patients harbor acquired nonsense mutations of the gene encoding the receptor for CSF3 (*CSF3R*); mutational frequency in SCN patients without AML is estimated at 30%.[19] SCN-associated *CSF3R* mutations occur in the region of the gene (nucleotides 2300–2600) coding for the cytoplasmic domain of CSF3R and result in truncation of the C-terminal-negative regulatory domain.[20]

In 2009, Plo *et al.* [21] described a germline *CSF3R* transmembrane mutation (C-to-A substitution at nucleotide 2088; T617N) in autosomal dominant hereditary neutrophilia. The same exon 16 mutation is now identified as T640N and was previously described as an acquired event in AML.[22]*CSF3R*T617I was shown to induce CSF3R-independent granulocyte proliferation and differentiation and JAK2 inhibitor-sensitive constitutive phosphorylation of JAK2, STAT3, AKT and ERK.[21] In mice, the mutation produced neutrophilia and splenomegaly.[21] In 2012, Beekman *et al.*[23] described a novel *CSF3R* autoactivating mutation (*CSF3R*T595I, which is the same mutation as *CSF3R*T618I) in a patient with SCN/AML that co-existed with a truncating *CSF3R* mutation. The same mutation was noted in a single patient among 199 AML cases from a separate database.

In 2013, Maxson *et al.*[24] established the connection between CNL and activating *CSF3R* mutations. The authors detected *CSF3R* mutations in 16 (59%) of 27 patients with CNL ($n=9$) or aCML ($n=18$) and 1 of 92 patients with AML; mutational frequency was 89% in CNL and 44% in aCML. The main mutations were membrane proximal and included T618I ($n=12$; exon 14 C-to-T substitution at nucleotide 1853) and T615A ($n=2$) but other truncating mutations, often in association with T618I/T615A, were also seen; 9 of 12 T618I mutations occurred without associated truncating mutations. *In vitro* drug sensitivity assays suggested JAK inhibitor sensitivity for membrane proximal but not truncating mutations, which were instead sensitive to dasatinib. These authors subsequently reported *CSF3R*T618I-induced lethal myeloproliferative disorder in a murine bone marrow transplant model.[25] Treatment with ruxolitinib was reported to have a salutary effect both in this mouse model and a patient with *CSF3R*T618I mutation.[24]

Subsequent to the report by Maxson *et al.*,[24] Pardanani *et al.*[26] performed *CSF3R* mutation analysis on 54 patients with clinically suspected CNL ($n=35$) or aCML ($n=19$). After central pathology review, 12 cases were confirmed to have WHO-defined CNL and 9 WHO-defined aCML. *CSF3R* mutations were detected in 13 patients and included *CSF3R*T618I in 10 cases. The latter occurred exclusively in WHO-defined CNL for a mutational frequency of 83% and not seen in WHO-defined aCML, PMF ($n=76$) or CMML ($n=94$). Moreover, the two T618I-negative patients with WHO-defined CNL harbored other *CSF3R* mutations (M696T and I598I). Interestingly, 4 of the 10 T618I-mutated cases also expressed *SETBP1* mutations. In a subsequent study of a patient harboring both *CSF3R*T618I and *SETBP1*D868N, the authors demonstrated the presence of both mutations in granulocytes, mononuclear cells and CD34+ myeloid progenitors but not in T cells. Additional observations in the particular study included the sighting of homozygous *CSF3R*T618I single colonies, relative resistance to *in vitro* treatment with JAK1 and JAK2 inhibitors and lack of clinical response to ruxolitinib. Other studies have subsequently reported the rare occurrence of T618I ($n=5$) or T617I ($n=2$) *CSF3R* mutations among 1446 consecutive patients with *de novo* AML[27] and no exon 14 mutations among 354 patients with CMML; approximately 4% of the latter displayed other *CSF3R* mutations including E808K and M696T.[28]

PROPOSALS FOR REVISION OF THE DIAGNOSTIC CRITERIA FOR PV, ET AND PMF

The discovery of *JAK2*V617F in 2004 led to a sharp turn in our diagnostic approach and therapeutic direction in *BCR-ABL1*-

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negative MPN.[29] Soon thereafter, other *JAK2*[7] and *MPL*[12] mutations were described and further enriched the pool of clonal markers for the distinction of MPN from reactive myeloproliferation. *JAK2*V617F is present in more than 95% of patients with PV, and other *JAK2* mutations are detected in the majority of the remaining *JAK2*V617F-negative cases.[30,31] Therefore, in its 2008 revision of the WHO document, it was appropriate for the WHO MPN subcommittee to include the presence of *JAK2* mutations as a major criterion for the diagnosis of PV (Table 2).[2] Furthermore, because *JAK2* mutations also occur in 60–65% of patients with ET or PMF,[10,11] they were also listed as useful clonal markers in the diagnosis of ET and PMF (Table 2). The 2008 WHO diagnostic criteria for *BCR-ABL1*-negative MPN also allowed the use of other clonal markers, such as cytogenetic abnormalities or presence of *MPL* mutations, in distinguishing between clonal and reactive thrombocytosis or bone marrow fibrosis (Table 2). The committee also recognized the fact that neither *JAK2* nor *MPL* mutations were disease-specific and able to distinguish between the three variants of *BCR-ABL1*-negative MPN. Accordingly, BM morphology was included as a minor criterion in PV and major criterion in both ET and PMF (Table 2).

Where do *CALR* mutations fit in the above scheme? *CALR* mutations are frequent in *JAK2/MPL*-unmutated ET/PMF (estimated at 49% in strictly WHO-defined ET and 74% in WHO-confirmed PMF)[10,11] and thus provide a much needed clonal marker in such cases. Therefore, they should be included as named clonal markers, along with *JAK2* and *MPL* mutations, in the list of major diagnostic criteria for ET and PMF (Table 4). Furthermore, in the context of consistent morphology, *CALR*, as well as *JAK2* and *MPL* mutations, is relatively specific to MPN and should therefore be separated from 'other' clonal markers (for example, abnormal karyotype) and bundled together as a distinct category in the criteria list (Table 4). However, *CALR* mutations do not fully address the molecular gap in *JAK2/MPL*-unmutated disease or distinguish between ET and early/prefibrotic PMF. As such, their availability does by no means undercut the necessity of BM morphology as a major diagnostic criterion in both ET and PMF (Table 4). Accordingly, ET diagnosis still requires meeting the four major diagnostic criteria listed in Table 4, or, in the absence of *JAK2/MPL/CALR* (16% of cases),[10] the first three major criteria and the one minor criterion (Table 4).

In PMF, about 90% of patients harbor *JAK2*, *CALR* or *MPL* mutations.[11] Furthermore, in the presence of a typical BM morphology and absence of evidence for other myeloid malignancies, these clonal markers are specific enough to confirm the diagnosis. Therefore, it is reasonable to make the diagnosis of PMF in the presence of three major criteria: (i) typical BM morphology, (ii) absence of evidence for another myeloid malignancy including CML, PV and ET, and (iii) presence of *JAK2*, *CALR* or *MPL* mutations (Table 4). Such a strategy excludes the possibility of nonclonal causes of bone marrow fibrosis, and inter-MPN diagnostic confusion is minimized by strict adherence to the second major criterion (Table 4). In the absence of *JAK2*, *CALR* or *MPL* mutations (that is, triple-negative cases), diagnosis of PMF requires not only meeting the first two major criteria but also (i) exclusion of reactive bone marrow fibrosis and (ii) presence of clinical and laboratory features that are typical of PMF. These additional criteria are now listed as minor criteria in the proposed revised scheme (Table 4). In other words, in the absence of *JAK2/CALR/MPL* mutations, the first minor criterion helps exclude the possibility of non-clonal bone marrow fibrosis, whereas the second and third minor criteria reinforce the morphologic impression of PMF.

In PV, according to the 2008 WHO system, the first major diagnostic criterion requires one of the following four components: (i) hemoglobin level >18.5 g/dl in men and >16.5 g/dl in women, or (ii) hemoglobin or hematocrit level that is >99th percentile of reference range for age, sex, or altitude of residence, or (iii) red cell mass that is >25% above mean normal predicted, or (iv) hemoglobin level >17 g/dl (>15 g/dl in women) associated with a sustained increase of ⩾2 g/dl from baseline that cannot be attributed to correction of iron deficiency. The latter three components were designed to capture PV patients with borderline increased hemoglobin that is >18.5 g/dl in men and >16.6 g/dl in women. In a somewhat similar gesture, we have recently re-introduced[32] the term 'masked PV' for *JAK2*-mutated patients who display PV-characteristic BM morphology but display hemoglobin levels between 16 and 18.5 g/dl for men and 15 and 16.5 g/dl for women.[33,34] We subsequently determined a

Table 4. 2014 proposed revision for World Health Organization (WHO) diagnostic criteria for *BCR-ABL1*-negative myeloproliferative neoplasms

	Polycythemia vera (PV)[a]	*Essential thrombocythemia (ET)*[b]	*Primary myelofibrosis (PMF)*[c]
Major criteria			
1	Hemoglobin >16.5 g/dl (men) >16 g/dl (women) or hematocrit >49% (men) >48% (women)	Platelet count ⩾450 × 10^9/l	Megakaryocyte proliferation and atypia[d], accompanied by either reticulin and/or collagen fibrosis or[e]
2	BM trilineage myeloproliferation with pleomorphic megakaryocytes	Megakaryocyte proliferation with large and mature morphology	Not meeting WHO criteria for CML, PV, ET, MDS or other myeloid neoplasm
3	Presence of *JAK2* mutation	Not meeting WHO criteria for CML, PV, PMF, MDS or other myeloid neoplasm	Presence of *JAK2*, *CALR* or *MPL* mutation
4		Presence of *JAK2*, *CALR* or *MPL* mutation	
Minor criteria			
1	Subnormal serum erythropoietin level	Presence of a clonal marker (e.g. abnormal karyotype) or absence of evidence for reactive thrombocytosis	Presence of a clonal marker (e.g. abnormal karyotype) or absence of evidence for reactive bone marrow fibrosis
2			Presence of anemia or palpable splenomegaly
3			Presence of leukoerythroblastosis[f], or increased lactate dehydrogenase[f]

Abbreviations: BM, bone marrow; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome. [a]PV diagnosis requires meeting either all three major criteria or the first two major criteria and one minor criterion. [b]ET diagnosis requires meeting all four major criteria or first three major criteria and one minor criterion. [c]PMF diagnosis requires meeting all three major criteria or the first two major criteria and all three minor criteria. [d]Small-to-large megakaryocytes with aberrant nuclear/cytoplasmic ratio and hyperchromatic and irregularly folded nuclei and dense clustering. [e]In the absence of reticulin fibrosis, the megakaryocyte changes must be accompanied by increased marrow cellularity, granulocytic proliferation and often decreased erythropoiesis (that is, prefibrotic PMF). [f]Degree of abnormality can be borderline or marked and institutional reference range should be used for lactate dehydrogenase level.

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Table 5. Proposal for revision of World Health Organization (WHO) criteria for diagnosis of chronic neutrophilic leukemia

Major criteria	1	Peripheral blood (PB) leukocytosis $\geqslant 13 \times 10^9$/l
	2	>80% Neutrophils or bands
	3	Presence of *CSF3R*T618I or other membrane-proximal *CSF3R* mutations
Minor criteria	1	Bone marrow: Hypercellular Increased granulocytic proliferation without significant left shift No dysgranulopoiesis
	2	Peripheral blood: <10% immature granulocytes <2% myeloblasts $\leqslant 1 \times 10^9$ absolute monocyte count or <10% monocyte percentage No dysgranulopoiesis
	3	Presence of a clonal marker or absence of evidence for reactive/secondary granulocytosis, including plasma cell neoplasm
	4	Absence of *BCR-ABL1*
	5	Not meeting WHO diagnostic criteria for any other myeloid neoplasm

Diagnosis requires presence of all three major criteria or the first two major criteria and all minor criteria.

hemoglobin level of 16.5 g/dl in men and 16 g/dl for women or a hematocrit level of 49% in men and 48% in women to be the optimal cutoff levels for distinguishing *JAK2*-mutated ET from masked PV (manuscript in preparation).

Strict adherence to all four components of the first major criterion for diagnosis of PV might capture a substantial number of patients with masked PV. In contrast, lowering the diagnostic hemoglobin/hematocrit level to 16.5 g/dl (49%) in men and 16 g/dl (48%) in women, as suggested by the aforementioned masked PV study, might simplify the first major diagnostic criterion, but would require morphologic confirmation. Furthermore, regardless of whether or not one buys into the concept of masked PV, such a measure conjures therapeutic relevance because of the recent associations between increased thrombotic complications and borderline increased hematocrit (45–50%) in PV and *JAK2* mutation in ET.[35,36] We therefore propose consolidation of the currently listed four components of the first major diagnostic criterion into one component based on a lower threshold hemoglobin level and hematocrit reinforced by the inclusion of BM morphology as a major criterion (Table 4). The inclusion of BM morphology as a major criterion also allows the diagnosis of PV based on major criteria alone without the need for additional minor criteria. However, in order to address the rare possibility of a *JAK2*-unmutated PV, we have included 'subnormal erythropoietin level' as a minor criterion. Endogenous erythroid colony growth is now removed from the list of minor criteria because of redundancy in value and limited practical use.

In terms of routine clinical practice, *JAK2* mutation screening should start with *JAK2*V617F because it represents more than 95% of all *JAK2* mutations. Other *JAK2* mutations, including *JAK2* exon 12 mutations, should be studied only in the absence of *JAK2*V617F and presence of subnormal serum erythropoietin level. Mutation screening in ET or PMF should also start with *JAK2*V617F, followed by *CALR* mutations in *JAK2*-unmutated case, whereas *MPL* mutation screening, because of its low mutational frequency, is best reserved for *JAK2* and *CALR* unmutated cases. In regards to bone marrow examination, whereas it is mandatory for clinical trial and research study purposes, it might not be necessary for clinically overt cases of PV. These include *JAK2*-mutated patients with hemoglobin (hematocrit) levels of >18.5 g/dl (>52%) in men and >16.5 g/dl (>48%) in women.

PROPOSALS FOR REVISION OF THE DIAGNOSTIC CRITERIA FOR CNL

The current WHO diagnostic criteria for CNL are primarily driven by the absence of a clonal marker and disease-specific BM morphologic traits.[37] Accordingly, they require a relatively high leukocyte count threshold ($\geqslant 25 \times 10(9)$/l) and a list of exclusion criteria that focus on reactive granulocytosis, plasma cell neoplasm-associated neutrophilia, CML, aCML and CMML (Table 3). In clinical practice, only a fraction of clinically suspected cases of CNL meets strict WHO criteria and this might also be true for some published cases.[26,38] Conversely, it is equally possible to misdiagnose some cases of CNL as aCML or CMML (Table 3).

The recent discovery of *CSF3R* mutations and their almost invariable association with WHO-defined CNL presents the opportunity to make significant changes in our diagnostic approach (Table 5).[24,26] Such availability of a clonal marker for the majority of patients with CNL should allow lowering of the diagnostic leukocyte count threshold level from 25×10^9/l to 13×10^9/l because the latter represents a level above 3 s.d. from the normal mean and would be consistent with what is currently being used for the diagnosis of WHO-defined aCML.[1]

In addition to the above, we propose separate sets of major and minor criteria to accommodate the diagnostic possibility in both *CSF3R*-mutated and unmutated CNL (Table 5). The presence of a membrane proximal *CSF3R* mutation in a patient with predominantly neutrophilic granulocytosis should be sufficient for the diagnosis of CNL.[24,26] This is now signified in the form of three major diagnostic criteria including (i) a PB leukocyte level of $\geqslant 13 \times 10^9$/l, (ii) a PB neutrophil/band percent distribution of >80% and (iii) presence of a *CSF3R*T618I or other membrane proximal *CSF3R* mutations. We realize that one can argue for including the absence of dysgranulopoiesis as a major criterion based on the discrepancy in current literature regarding the occurrence of *CSF3R* mutations in aCML.[24,26] However, careful analysis of Mayo Clinic cases[26] and additional evidence from other centers have not confirmed the occurrence of *CSF3R* mutations in strictly WHO-defined aCML. Regardless, we believe that the inclusion of '*CSF3R*-mutated aCML' in the molecularly similarly defined CNL category makes better scientific sense.

Unfortunately, several exclusionary criteria still need to be met for diagnosing CNL in the absence of *CSF3R* mutations, and these are now listed as minor criteria (Table 5). Finally, before embarking on a testing spree for *CSF3R* mutations, one should be aware of the fact that CNL is an extremely rare disorder and that it is very unlikely to be the cause of neutrophilia that is encountered in routine clinical practice.[37,38]

CONCLUSIONS

The last 10 years have been marked by remarkable genetic discoveries in MPN, which should in time lead to equally impressive therapeutic advances. The identification of disease-specific mutations justifies, in certain cases, the use of peripheral blood mutation analysis for diagnosis, prognosis and monitoring of treatment response. It is inevitable that more mutations will be discovered in the near future, which is welcome news not only for patients but also for the highly profitable *in vitro* diagnostics market. However, new rules on reimbursement for molecular tests dictate a joint effort between clinicians and pathologists in order to formulate affordable testing schedules with measurable clinical benefit.[39]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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