2014 WHO Clinical Molecular and Pathological (WHO-CMP) Diagnostic Criteria for the Classification and Staging of Five Distinct JAK2, MPL and CALR Mutated Myeloproliferative Neoplasms

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Abstract

Somatic mutations in the JAK2, MPL and calreticulin (CALR) genes are the driver causes of clonal myeloproliferative neoplasms (MPN). Applying the WHO Clinical, Moleculare and Pathologic (WHO-CMP) classification of MPN, the JAK2V617F positive ET patients comprise three phenotypes of ET: normocellular ET, hypercellular ET due to increased erythropoiesis (prodomal PV) and ET with hypercellular megakaryocytic-granulocytic myeloproliferation (ET-MGM) or PV. The percentage of JAK2V617F mutation load is low and stable in heterozygous normocellular ET and increased in hypercellular ET. The JAK2V617F allele burden is related to MPN disease burden in terms of splenomegaly, constitutional symptoms and myelofibrosis. Five distinct clonal MPNs can be distinguished: JAK2V617F mutated ET and PV; JAK2 exon 12 and the JAK2 wild type ET and MF caused by the somatic mutations MPL515 or CALR. JAK2 mutated trilinear MPN reflects a broad spectrum of ET, prodomal or masked PV, but the JAK2 wild type MPL or CALR positive ET and MF lack features of PV at diagnosis and during follow-up. Bone marrow features in JAK2V617F mutated ET and PV are similar and featured by medium sized to large (pleomorphic) megakaryocytes with only a few giant forms. Bone marrow histology in MPL515-mutated ET and MF is featured by clustered small and giant megakaryocytes with hyperlobulated stag-horn-like nuclei, in a normocellular bone marrow with no features of PV. Bone marrow histology in CALR mutated ET and MF is featured by dense clustered large immature dysmorphic megakaryocytes and bulky (cloude-like) hyperchromatic nuclei similar as described in primary megakaryocytic granulocytic myeloproliferation (PMGM), which are never seen in JAK2V617F.

Keywords: Myeloproliferative neoplasms; Essential thrombocythemia; Polycythemia vera; Primary megakaryocytic granulocytic myeloproliferation; Myelofibrosis; JAK2V617F mutation; MPL515 mutation; Calreticulin; CALR mutation; Bone marrow pathology

Introduction

Polycythemia vera (PV) is described by Vaquez [1], Osler [2,3] and essential thrombocythemia (ET) and PV has been delineated as distinct clinical disease entities. In 1915, the World Health Organization (WHO) defined PV disease, but for ET, the original diagnostic criteria used a minimal platelet count of 1000 x 10^9/L for the diagnosis of ET [10]. The 1975 PVSG diagnostic criteria for PV did not use bone marrow histology and excluded by definition the erythrocythemic stage I PV (idiopathic erythrocythemia: IE) with normal platelets, leukocytes and spleen size [11-13]. The 1980 WHO criteria for PV used bone marrow smears and bone marrow histology in the late 1970s (Table 1) [5,14]. The PVSG reduced in 1986 the minimum platelet count for the diagnosis of ET from 1000 x 10^9/L to 600 x 10^9/L [15] as the consequence of two evidence-based studies by Michiels et al. [5] and Van De Pette et al. [16]. Lengfelder et al. [17] demonstrated in 1998...
that PVSG defined ET at platelet count above 600 x 10^9/L overlooks 30% of early stage ET as compared to the cut-off level of 400 x 10^9/L (Table 1). Platelets in excess of 400 x 10^9/L, and increase of clustered enlarged megakaryocytes in a bone marrow biopsy material were found to be diagnostic for ET and excluded reactive thrombocytosis [5,17].

Between 1975 and 1980, we routinely used bone marrow histopathology and erythrocyte count above 6 x 10^12/L according to Dameshek in 1940 [9] as specific clues to the diagnosis of PV to clearly differentiate PV from all variant of primary and secondary erythrocytosis (Table 1). The RCP modifications of PVSG criteria for PV include 4 changes (Table 1). The major criterion O2-saturation of >92% is deleted and replaced by bone biopsy as a major criterion (A3) to differentiate between PV and secondary erythrocytosis. Splenomegaly is used as a minor criterion (Table 1). Raised B12 (>900 ng/L) or raised B12 binding capacity (>2200 ng/L) is skipped as completely irrelevant for the diagnosis of PV (Table 1). Bone marrow histology has a specificity and sensitivity near to 100% to differentiate between the MPDs ET and PV from reactive thrombocytosis and primary or secondary erythrocytoses [14,17].

Idiopathic erythrocythemia (IE) is featured by increased red cell mass, normal spleen size, normal leukocyte and platelet counts and no clinical or laboratory evidence of primary or secondary erythrocytosis and a typical PV bone marrow histology (Table 1). The PV experts in the UK and France did not use bone marrow biopsy for the diagnostic differentiation between PV and primary or secondary erythrocytosis and therefore overlooked stage 1 erythremic PV by definition [18,19]. IE represent a significant number of early stage erythremic PV of about 10% to 15% at time of PV presentation [18,19]. The diagnostic difficulties regarding the original PVSG criteria without the use of bone marrow pathology were solved by Tom Pearson by applying on top of PVSG criteria low serum erythropoietine (EPO) levels and

Table 1: The Rotterdam Clinical and Pathological (RCP) criteria for Essential Thrombocythemia (ET) and Polycythemia Vera (PV) 1975-1980.

<table>
<thead>
<tr>
<th>A. The 1980 RCP major (A) and confirmative (B) criteria for prefibrotic ET</th>
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<tbody>
<tr>
<td>A1 Persistent platelet count in excess of 400 x 10^9/L [4,5].</td>
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<tr>
<td>A2 Increase and clustering of enlarged megakaryocytes in bone marrow biopsy.</td>
</tr>
<tr>
<td>A3 No or slight increase of reticulin fibers (RF 0 or RF 1)</td>
</tr>
<tr>
<td>B1 Presence of large platelets in a peripheral blood smear</td>
</tr>
<tr>
<td>B2 Absence of any underlying disease for reactive thrombocytosis and normal ESR.</td>
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<tr>
<td>B3 No splenomegaly (&lt;12 cm) or slight splenomegaly on palpation or scan (&lt;15 cm)</td>
</tr>
<tr>
<td>B4 Increase of LAP-score and no signs of fever or inflammation</td>
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Exclusion criterion
Pv+ chromosome and any other cytogenetic abnormality in blood or bone marrow nucleated cells

B. The 1980 RCP major (A) and minor (B) criteria for prefibrotic PV

| A1 Increased erythrocyte count above 6 x 10^12/L: Dameshek 1940 [9]. |
| A3 Increase in bone marrow biopsy of clustered, large pleomorphic megakaryocytes with hyperlobulated nuclei and increased cellularity due to increased megakaryopoiesis erythropoiesis or typically trilinear mega-erythro-granulopoiesis. Typical PV bone marrow excludes erythrocytosis. |
| B1 Thrombocythemia, persistent increase of platelet >400 x 10^9/L [4,5]. |
| B2 Leukocytosis, leucocyte count >10^9/L and low erythrocyte sedimentation rate (ESR) |
| B3 Raised leukocyte alkaline phosphatase (LAP) score >100, absence of fever or infection |
| B4 Spleenomegaly on palpation or on isotope/ultrasound scanning |

A1 or A2 plus A3 and none of B establishes erythrocythemic PV |
A1 or A2 plus A3 plus one of B establishes PV and excludes erythrocytosis

Table 2: The Dameshek one cause hypothesis of trilinear PV [33] in 1950 and Vainchenker’s discovery of heterozygous and homozygous JAK2V617F somatic mutations [36] in 2005 as the driver cause of trilinear MPN with clinical and pathological manifestations of Essential Thrombocythemia (ET), Polycythemia Vera (PV) and Primary Megakaryocytic Granulocytic Myeloproliferation (PMGM) and secondary Myelofibrosis (MF) [27,31] Designed by Michiels in 2005 [31].

2005 Molecular Etiology of Platelet-Mediated Microvascular Thrombosis, Increased Red Cell Mass, and Secondary Myelofibrosis in JAK2 V617F-Positive MPDs (ET, PV, and PMGM: JAK2 V617F Gain of Function Mutation in Trilinear Hematopoietic Cells of MPD Patients is Detectable in Platelets, Erythroblasts, and Granulocytes

| Molecular Step 1 V617F+ |
| Spontaneous CFU-MK/ECG |
| BM: ET picture |
| Increase of enlarged hypersensitive platelets |
| Platelet > 400 x 10^9/L |
| Clinical step 1 |
| Microvascular |
| Thrombosis |
| Treatment |
| Aspirin sensitive |

| Molecular Step 1 → 2 V617F++ 9p LOH |
| Spontaneous EEC/CFU-MK ET-PV picture |
| Increase of hematocrit to above 0.45–0.50: PV Platelets > 400 x 10^9/L |
| Clinical step 2 |
| Macrovascular |
| Thrombosis |
| Treatment |
| Aspirin/phlebotomy |

| Molecular Step 1 → 2 V617F++ 9p LOH |
| PVR-1 [= LAP score] |
| Granulocytes growth/activation |
| Myeloid metaplasia: PMGM |
| Leukocytosis/lycopines |
| Fatigue, splenomegaly, masked PV, MF, MF |
| Clinical step 3 |
| Secondary MF: 30% Constitutional symptoms |

MPD, myeloproliferative disorder; ET, essential thrombocythemia; PV, polycythemia vera; PMGM chronic secondary myelofibrosis; LOH, loss of heterogeneity; CFU-MK, colony-forming units megakaryocytes; EEC, endogenous erythroid colony; LAP, leukocyte alkaline phosphatase; BM, bone marrow; IFN, interferon.

Table 2: The Dameshek one cause hypothesis of trilinear PV [33] in 1950 and Vainchenker’s discovery of heterozygous and homozygous JAK2V617F somatic mutations [36] in 2005 as the driver cause of trilinear MPN with clinical and pathological manifestations of Essential Thrombocythemia (ET), Polycythemia Vera (PV) and Primary Megakaryocytic Granulocytic Myeloproliferation (PMGM) and secondary Myelofibrosis (MF) [27,31] Designed by Michiels in 2005 [31].


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spontaneous endogenous erythrocyte colony formation (EEC) as specific clues to PV [20]. About half of ET patients are EEC positive and have decreased or low serum EPO levels and are in fact prodomal phases (forme frustae) of PV. Standardized and easy-to-perform commercial serum EPO assays are used for the differential diagnosis of either erythrocytosis or PV [21,22]. In a multicenter study on 241 patients, Mossuz et al. identified two thresholds of serum EPO levels, allowing a specific and correct diagnosis in 65.6% (65 out of 99 PV patients) of PVSG-defined PV patients with serum EPO levels below 1.4 U/L and in 19.7% (13 of 66 SE patients) of secondary erythrocytosis (SE) with serum EPO levels above 13.7 U/L [21]. Consequently, about 50% of patients with increased RCM could not be diagnosed as PV or erythrocytosis indicating the need to perform a bone marrow biopsy to distinguish PV from SE (Table 1) [5,17].

Bone Classification and WHO criteria for ET, PV and PMGM

Georgii et al. discovered in 1980 prefibrotic chronic megakaryocytic granulocytic myeloproliferation (CMGM) as the third distinct entity of primary MPD in the absence of reticulin or collagen fibrosis in bone marrow biopsy material [23]. In 1987 Michiels et al. defined strict morphological, biochemical, and cytogenetic criteria for BCR/ABL-positive ET and chronic myeloid leukemia (CML) as a separate malignant and individual entity, whereas ET, PV and CMGM form a chronic proliferation of three hematopoietic cell lines [24]. Michiels et al. [24] and Georgii et al. (Hannover Bone Marrow Classification of CML and MPD, Table 1) [25,26] separated the Ph-positive or BCR/ABL-positive CML and ET from the Ph- or BCR/ABL-negative MPDs ET, PV and CMGM based on distinct bone marrow histology findings for each of the three MPDs ET, PV and CMGM [23-28]. The difference in size and morphology of small mononucleated megakaryocytes in Ph-positive CML and ET from the large pleomorphic megakaryocytes in the Ph-negative MPDs ET and PV is so obvious that cytologists and pathologists can easily distinguish [24,25]. The 1990 Hannover Bone Marrow Classification distinguished three primary prefibrotic MPDs, ET, PV and chronic megakaryocytic granulocytic myeloproliferation (CMGM) from advanced fibrotic stages of MPD (Table 2) [25,26]. As myelofibrosis (MF) is a secondary event in all variants of MPD and the terms chronic idiopathic myelofibrosis (CIMF) and primary myelofibrosis (PMF) are a misconception, Georgii consequently replaced the terms CIMF and PMF by CMGM and used grading of reticulin fibrosis (RF) and increase of reticulin and collagen myelofibrosis (MF) for staging of prefibrotic, early fibrotic and overt and advanced fibrotic MPDs ET, PV and CMGM [8,9]. Prefibrotic CMGM is the third MPD entity without features of ET, PV or CML and its diagnosis is based on the presence of loose to dense clustering of large megakaryocytes with immature cytoplasm and cloud-like nuclei not seen in ET, PV and CML [25-28]. The term CMGM of the Hannover Bone Marrow Classification has illogically replaced again by Thiele and Vardiman by chronic idiopathic myelofibrosis (CIMF) in the 2001 WHO classification [29], and as PMF by Tefferi and Thiele in the 2008 WHO classification (Figure 1) [30]. The diagnosis of prefibrotic CMGM is based on the association of hypercellular ET with the presence of large immature megakaryocytes with immature cytoplasm and cloud-like nuclei not seen in ET and PV (Table 2) [31]. The WHO Clinical, Molecular and Pathological (2014-CMP) classification of Michiels et al. of the myeloproliferative neoplasms extended the CMGM concept of Georgii et al. and replaced the term CMGM by primary megakaryocytic granulocytic myeloproliferation (PMGM, Figure 1) [32,33].
JAK2V617F mutation leading to constitutively activated megakaryocytes concept according to Vainchenker and Michiels is that heterozygous bone marrow transformation into myelofibrosis (MF) [42]. The 2005 high risk for myeloid metaplasia of the spleen with splenomegaly and with increased sensitivity to TPO and EPO is enough to induce ET with JAK2V617F mutation load in grumulocytes is usually low MF [31]. The JAK2V617F mutation as the driver JAK2 a trilinear MPD has been proven to be correct by Vainchenker's activity of an inhibitory factor [34,35]. This original observation of PV as stimulation by an unknown factor, or the lack or diminution of thrombocythemia, granulocythemia: either excessive bone marrow possibilities as the cause of trilinear PV (erythrocythemia, megakaryocytic granuloctic myeloproliferation and (relative) reduction erythropoiesis and granulopoiesis in the bone marrow as the cause of constitutively activation and genetic instability of megakaryopoiesis, megakaryocytic granuloctic myeloproliferation and (relative) reduction erythropoiesis is consistent with hypercellular ET associated with relative) reduction erythropoiesis and granulopoiesis in the bone marrow as the cause of hypercellular trilinear PV with a high risk of myelofibrosis (Table 2). The JAK2V617F mutation is associated with pronounced erythropoiesis and granulopoiesis in the bone marrow as the cause of hypercellular trilinear PV with a high risk of myelofibrosis (Table 2). The JAK2V617F mutation is associated with pronounced erythropoiesis and granulopoiesis in the bone marrow as the cause of hypercellular trilinear PV with a high risk of myelofibrosis (Table 2).


JAK2V617F mutated trilinear MPNs in ET and PV: Dameshek-Vainchenker’s Disease

In 1950, Dameshek (1900-1969) proposed two highly speculative possibilities as the cause of trilinear PV (erythrocythemia, thrombocythemia, granulocythemia: either excessive bone marrow stimulation by an unknown factor, or the lack or diminution of inhibitory factor [34,35]. This original observation of PV as a trilinear MPD has been proven to be correct by Vainchenker’s discovery in 2005 of the somatic JAK2V617F mutation as the driver cause of the trilinear MPNs ET, PV and MF [36]. This has been rapidly confirmed by Green UK, Kravalics Europe and Levine USA [37-39]. On position 617 of the JAK2 JH2 domain Valine (V) is replaced by Fenylalanine (F) in the JAK2V617F mutation and induces a loss of inhibitory activity of the JH2 pseudokinase part on the JH1 kinase part of JAK2, leading to enhanced activity of the normal JH1 kinase activity of JAK2 [36]. The JAK2V617F makes the mutated hematopoietic stem cells hypersensitive to hematopoietic growth factors TPO, IGF1, SCF and GCSF, resulting in PV as a trilinear MPN (Table 2) [31]. Detection of JAK2V617F has become the first intention diagnostic test for ET and PV (Tables 3 and 4) [31]. The prevalence of the JAK2V617F mutation in PVSG defined PV is 95% and about 50% in ET and MF [31]. The JAK2V617F mutation load in granulocytes is usually low in heterozygous ET, less that 10 to maximal 50% and either low with less than 50% (heterozygous homozygous) or high between 50 to 100% (homozygous) in PV [40,41]. Patients with hypercellular ET, masked PV and PV homozygous for the JAK2V617F mutation patients are at high risk for myeloid metaplasia of the spleen with splenomegaly and bone marrow transformation into myelofibrosis (MF) [42]. The 2005 concept according to Vainchenker and Michiels is that heterozygous JAK2V617F mutation leading to constitutively activated megakaryocytes with increased sensitivity to TPO and EPO is enough to induce ET with the production of constitutively activated (hypersensitive) platelets (Table 2) [31]. So-called heterozygous PV with allele load less than 50% appeared to be hetero/homozygous for the JAK2V617F mutation at the EEC level in blood and bone marrow for the JAK2V617F mutation, whereas ET patients are heterozygous for the JAK2V617F mutation at the EEC level with a maximal JAK2V617F mutation load ranging from low to maximal 50% [43,44]. The change from heterozygous ET into homozygous masked or overt PV is due to the loss of 9p heterogeneity (9p LOH) of the JAK2V617F locus through mitotic amplification resulting in homozygosity of JAK2V617F somatic mutation on chromosome 9p (Table 2) [25]. Godfrey et al. studied the JAK2 mutation status of BFU-E grown in low erythropoietin conditions in 77 patients with PV or ET [45]. Using microsatellite PCR to map loss-of-heterozygosity breakpoints within individual colonies, homozygous JAK2V617F mutant colonies were absent or present in low percentages in heterozygous ET, but prevalent and common in patients with JAK2V617F-positive PV [46]. PV was distinguished from ET by expansion of a dominant homozygous JAK2V617F subclone, the selective advantage of which is likely to reflect additional genetic or epigenetic lesions. Hetero/homozygous or homozygous JAK2V617F mutation is associated with pronounced constitutively activation and genetic instability of megakaryopoiesis, erythropoiesis and granulopoiesis in the bone marrow as the cause of hypercellular trilinear PV with a high risk of myelofibrosis (Table 2).

According to WHO [30] and WHO-CMP criteria (Tables 3 and 4) [32,33], heterozygous JAK2V617F positive ET is defined by a normocellular bone marrow histology with slight increase of erythropoiesis (Figure 2) or with a hypercellular bone marrow histology due to increased erythropoiesis (prodromal PV or masked PV). JAK2V617F positive hypercellular ET associated with prefibrotic megakaryocytic granulocytic myeloproliferation and (relative) reduction of erythropoiesis is consistent with hypercellular ET associated with...
Clinical and molecular criteria | Bone marrow pathology criteria (WHO)
--- | ---
**ET** | Normocellular ET
1. Platelet count of >350 x 10^9/L and the presence of large platelets in a blood smear
2. Heterozygous JAK2V617F mutation, low mutation load
3. Normal erythrocytes <5.8 x 10^12/L, females, <5.8 x 10^11/L, males, Hemoglobin (Hb) and hematocrit (Ht) normal or upper range of normal range of normal

**Prodomal PV** | ET with bone marrow features of PV
1. Platelet count of >350 x 10^9/L Hb and Ht normal or in the upper range of normal range of normal erythrocytes <5.8 x 10^12/L, males, <5.6 x 10^10/L, females.
2. Presence of JAK2V617F mutation
3. Low serum EPO level, increased LAP score and spontaneous EEC.

**Prefibrotic hyperetietic ET** | EMGM
1. Platelet count of >350 x 10^9/L
2. Slight or moderate splenomegaly on ultrasound or on palpation
3. No preceding or allied CML, PV, PMGM, RARS-T or MDS.

**Clinical stage 1:** No anemia with Hb and Ht in the normal or low normal range: 
   hbg >12 g/dL, normal LDH and CD34.
**Clinical stage 2:** slight anemia Hbg <12 to >10 g/dL, LDH1, and splenomegaly
**Clinical stage 3:** anemia, Hbg <10 g/dL, LDH1, CD34, leukoerythroblastoid and, tear drop erythrocytes

**Post-PV MF:** RF 4=MF-3

**Clinical and molecular criteria | Bone marrow pathology criteria (WHO)
--- | ---
**Major criteria for PV** | P1. Bone marrow pathology: increased cellularity (60-100%) due to trilineal increase of erythropoiesis, megakaryopoiesis and granulopoiesis and clustering of small to giant (pleomorphic) megakaryocytes with hyperplulated nuclei. Absence of stainable iron. No pronounced inflammatory reaction
**P2.** Erythrocytosis. Normal erythropoiesis, normal granulopoiesis and megakaryopoiesis of normal size, morphology and no clustering
**P3.** Grading of reticulin fibrosis (RF) and reticulin collagen myelofibrosis (MF)

**Minor** | A2 + B1 + P1 establish early PV (mimicking ET) prodomal PV CMP stage 0
A1 + A2 + A3 + P1 and none of B establish idiopathic erythrocythemia (IE) or stage 1 PV
A1 + A2 + A3 + P1 and one or more of B establish classic stages of PV stage 2 and 3
A2 + B3 + P1 detect masked cases of PV with splenomegaly and hypersplenism to be labelled as Inapparent PV (IPV) frequently seen Budd-Chiari syndrome or splanchic vein thrombosis

**Clinical and Pathobiological (WHO-CMP) criteria for the diagnosis of JAK2V617F mutated essential thrombocythemia (ET) [32,33].**

<table>
<thead>
<tr>
<th>Clinical and molecular criteria</th>
<th>Bone marrow pathology criteria (WHO)</th>
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<tr>
<td>JAK2V617F mutated WHO defined PV typically shows a hypercellular bone marrow histology due to increased trilineal hematopoiesis of megakaryopoiesis, erthropoiesis and granulopoiesis (pamylnoysis of Dameshek [34]) and no or slight increase of reticuline fibers (Figure 3 and Table 4).</td>
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<td>The UK MPN Study Group assessed the clinical features in the cohort of 806 PVSG defined ET patients subdivided in 414 JAK2V617F positive and 362 JAK2 wild type ET and evaluated the bone marrow features in 393 ET patients [46,47]. JAK2V617F positive ET patients had multiple features of PV such as a significantly higher hemoglobin, lower serum EPO and ferritin, higher neutrophils, bone marrow erythrocytosis and granulocytosis, more venous thrombosis and a higher rate of polyclastemic transformation. PVSG defined JAK2 wild type ET had significantly higher platelet counts (962, range 668-1535x10^9/L) than JAK2V617F-positive ET (846, range 632-1222x10^9/L) [46]. In the UK MPN (Primary Thrombocythemia 1 (PT-1) study, bone marrow trephine of 209 JAK2V617F positive and 184 JAK2 wild type ET was independently assessed by 3 hematopathologists who did not know the JAK2 mutation status [47]. The overall cellularity was significantly increased in JAK2V617F mutated ET as compared to JAK2 wild type ET, indicating that increased erythroid and granulocytic cellularity are features of prodromal PV or masked PV [47].</td>
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<td>Pich et al. prospectively analyzed histological changes in diagnostic bone marrow biopsy from 2006-2010 of 103 newly diagnosed WHO defined ET patients [48]. Bone marrow features in 44 JAK2 wild ET cases revealed prominent clusters of large megakaryocytes with staghorn nuclei, less micromegakaryocytes and no or minor erythroid hyperplasia as compared to JAK2V617F positive ET [48]. In contrast, 59 JAK2V617F positive ET patients revealed a typical PV bone marrow histology with higher hemoglobin, hematocrit, erythrocytoses and increased bone marrow due to hyperplasia of erythroid and myeloid lineages and the presence of pleomorphic megakaryocytes very similar as in WHO-CMP defined ET and PV (Tables 3 and 4). The mean and median JAK2V617F mutation burden in 2008 WHO defined ET was 14.4% and 8.7% respectively [48]. Interestingly LDH (604±132) and spleen size (15.4±4.9 cm on echogram) in 16 ET cases with a JAK2V617F mutation load above 12.5% were significantly increased as compared to normal LDH (386±94) and normal spleen size (11.2±2.1 cm on echogram) in 37 ET cases with a JAK2V617F mutation load below 12.5% [48].</td>
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JAK2 exon 12 mutations as cause of IE and PV

The finding of the JAK2 exon 12 mutations in PV patients negative for the JAK2 V617F mutation further confirms the strong association between the JAK2 mutations and MPN [49,50]. The 5% PV patients negative for JAK2 V617F are frequently heterozygous for exon 12 JAK2 mutations and usually present with early stage PV or idiopathic erythrocythemia (IE) with favourable outcome and normal life expectancy. The UK MPN Study Group identified JAK2 exon 12 mutations in 10 JAK2 V617F – negative PV patients with increased red cell mass, which according to PVSG criteria could diagnosed as PV in 6 and IE in 4 cases [49]. Pre-treatment bone marrow biopsies in 5 patients carrying one of the JAK2 exon 12 mutations showed characteristic erythroid hyperplasia...
Clinical and molecular JAK2 wild type ET

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Bone marrow pathology criteria (WHO)</th>
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<tbody>
<tr>
<td>1. Platelet count &gt;350 x 10^9/L and presence of large platelets in blood smear</td>
<td>P1. Proliferation of large to giant mature megakaryocyte with hyperlobulated, staghorn-like nuclei in a normocellular bone marrow (&lt;65%)</td>
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<tr>
<td>2. Hemoglobin, haematocrit and erythrocyte count in the normal range</td>
<td>No increase of erythropoiesis, and no increase or immaturity of granulopoiesis or erthropoiesis, No or slight increase in reticulinRF 0/1</td>
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<tr>
<td>3. Presence of MPN 355 mutation and JAK2 wild type</td>
<td>ET → MF</td>
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<tr>
<td>4. Normal serum EPO</td>
<td>Increased reticulin fibrosis around dense clustered megakaryocytes in a normocellular bone marrow and reduced erythropoiesis. Follow-up data of RF and MF related to splenomegaly in MPL515 ET transitional states to MF are lacking. Grading of reticulin fibrosis (RF) and myelofibrosis (MF) similar as described for PV</td>
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<td>5. Normal LAP score and CD11b expression</td>
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<td>6. No or slight splenomegaly</td>
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<td>7. No leukoerythroblastosis</td>
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<tr>
<td>8. No or slight splenomegaly</td>
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<tr>
<td>9. No or slight splenomegaly</td>
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Table 5: 2014 WHO Clinical Molecular and Pathological (WHO-CMP) criteria for the diagnosis of normocellular ET carrying one of the MPL515 mutations [32].

with some morphological abnormalities of the megakaryocyte and normal granulopoiesis in bone marrow biopsy specimens clearly different from primary or secondary erythrocytosis. In the Mayo Clinic study [50], 5 cases of JAK2V617F negative PV carrying JAK2 exon 12 mutation (F537-K539delins or N542-E543del) were diagnosed as IE with increased hemoglobin and hematocrit, low serum EPO, normal platelet and leukocyte counts, no or palpable spleen and a typical hypercellular bone histopathology predominantly due to erythroid hyperplasia and clusters enlarged megakaryocytes with hyperploid nuclei was observed in 2 cases [51]. The bone marrow histology by Lakey et al. in 7 JAK2 exon 12 mutated MPN patients (IE in 4, PV in 2, MF in 1) revealed hyperplasia of atypical small to medium-sized large megakaryocytes was present in all (Figure 4) [51], which differs from JAK2V617F mutated PV (Figure 3). The JAK2 exon 12 MPN cases lack the prominent clusters of large megakaryocytes with hyperlobulated nuclei that characterize JAK2V617F-positive prodomal and classical PV (Figure 3). A spectrum of small to medium sized megakaryocyte is seen in JAK2 exon 12 PV bone marrows with a predominance of smaller forms with atypical nuclei with various degrees of monolobulation to hyperlobation and abnormal chromatin distribution (Figure 4) [51]. Bone marrow reticulin fibrosis was normal or slightly increased in 6, and one case evolved 15 years after initial diagnosis into post-PV myelofibrosis with reticulin fibrosis grade 3 [51]. The JAK2 exon 12 IE or PV patients presented aquagenic pruritis and/or erythromelalgia in 3 and microvascularrevents including headache, dizziness, blurred vision and distal extremity numbness (aspirin responsive platelet thrombophilia or Sticky Platelet Syndrome) [4,5] in 4 at platelet counts between 152 and 790 x 10^9/L (of whom 5 below and 2 above 300 x 10^9/L). The hemoglobin ranged from 18.3 to 22 g/dL and leukocytes were below 10.5 x 10^9/L. The MPL515 mutations [32].

JAK2 wild type ET and MF carrying the MPL515 mutation

Congenital ET due to a gain of function mutation in the cMPL gene has been described in 2004 [52]. This led to the search and discovery of the MPLW515X and MPLW515K mutations as the driver cause of ET and myelofibrosis (MF) [53-55]. Within the JAK2 wild type MPN, there is a small subgroup who carry an acquired gain of function mutation of the MPL receptor as the cause of ET: 3% in the Italian study [55], and 8.5% in the UK studies [56,57]. In contrast to JAK2V617F mutated trilinear MPN, MPL515 mutated ET patients have no clinical, laboratory and bone marrow features of prodomal PV at diagnosis, do not evolve into overt PV during follow-up, have normal serum EPO and ferritin levels, and show pronounced megakaryocytic proliferation of small and large (giant) mature megakaryocytes and no increase of erythropoiesis in the bone marrow (Table 5) [33]. In 2014 we produced good evidence that JAK2 wild type ET carrying the MPL515 mutation typically present with normocellular ET histology with the presence of giant megakaryocyte with hyperlobulated staghoen-like nuclei and
no increase of erythropoiesis clearly differ from JAK2 V617F mutated ET (Table 5) [33]. As compared to bone marrow histopathology in JAK2 V617F mutated ET (Figure 2) there were significant differences on three points. First, the megakaryocytes in MPL mutations mutated PT are larger than in PV (Figure 5). In contrast, the megakaryocytes in JAK2 V617F mutated ET are not larger than in PV and show similar pleomorphic megakaryocytes morphology as in PV (compare Figures 2 and 3). Second, there was local increase of erythropoiesis in areas of loose clustered pleomorphic megakaryocytes in JAK2 V617F mutated ET (Figure 2), but not in MPL mutations mutated ET (Figure 5). Third, we observed increased reticulin fibers grade 2 in a rather normocellular bone marrow in areas of dense clustered megakaryocytes in MPL mutated ET, which is not seen in JAK2 V617F mutated normocellular ET, hypercellular prodomal PV and early stage PV [33]. Whether such differences of megakaryocyte morphology in bone marrow biopsies are characteristic enough to distinguish normocellular JAK2 V617F mutated ET with low JAK2 mutation load (Table 3) from MPL mutations mutated ET and MF (Table 5) respectively by expert hematopathologists remains to be evaluated in large prospective clinical and basic research studies of newly diagnosed and previously untreated MPN patients.

**Calreticulin (CALR) mutated ET and MF**

The molecular etiology of JAK2/MPL wild type ET and MF remained elusive until Kralovics in Vienna in Green and the UK independently discovered in 2013 the *calreticulin (CALR)* mutations in JAK2 wild type ET and MF patients [8,58]. Dr Kralovics and his team in Austria and Italy described the occurrence of CALR mutation in 78 of 311 (25%) ET patients and in 72 of 203 (35%) MF patients and in none of 382 PV patients [58]. CALR mutations are mutually exclusive with both JAK2 V617F and MPL mutations. All ET and MF patients with CALR in exon 9 are JAK2 and MPL wild type. Out of a cohort of 289 JAK2 wild type ET 195 (67%) carried one of the CALR mutations. Out of a cohort of 120 JAK2 wild type MF a CALR mutation was detected in 105 (80%). In the 150 patients with the CALR mutation for whom matched T-lymphocyte DNA was available, the CALR mutations were somatic. In none of 45 CML, 73 MDS, 64 CMML and 24 RARS-T (refractory anemia with increased ringed sideroblast and thrombocytosis) patients the CALR mutation was not found except that 3 SF3B1 positive RARS-T patients with myelodysplasia carried a CALR mutation. A total of 36 types of somatic mutations (insertions and deletions) were detected in exon 9 of the CALR gene encoding the C-terminal amino acids of CALR protein and only 3 patients were homozygous. In the total cohort of 1235 ET and MF patients 63.4% and 23.5% carried the JAK2 V617F, MPL mutations and CALR mutation respectively, and 8.8% were triple negative for these clonal driver mutation [58]. Green and his team in the UK independently found somatic CALR mutations in 70 to 84% of MPN samples with nonmutated JAK2 ET or MF [59]. CALR exon 9 mutations were found in 26 of 31 (84%) patients with ET or MF and nonmutated JAK2. CALR exon 9 mutations were absent in all 120 patients who had JAK2 or MPL mutations. CALR mutations were present in 110 of 158 MPN patients lacking JAK2 or MPL, including 80 of 112 (70%) ET patients, 18 of 32 (56%) MF patients and 12 of 13 patients with progression of ET to MF. CALR mutations were identified in 10 of 120 (8%) MDS patients (RA in 5 of 53, RARS in 3 of 27 and RAEB-T in 2 of 27), and in one patient each with chronic myelomonocytic leukemia (CML) and atypical CML. No CALR mutations were found in control samples, lymphoid cancers, solid tumors, or cell lines [59]. Overall, CALR exon 9 mutations were identified in 148 patients. All CALR mutations were indels with 19 distinct variant: 14 deletions, 2 insertions and 3 complex indels, which generated a +1 base-pair frameshift, which would result in a mutant with a novel C-terminal [59].

According to our experiences bone marrow histology in prefibrotic and early fibrotic MPN in JAK2 wild type hypercellular ET of subtype PMGM show dysmorphic megakaryocytes with definite abnormalities of maturation with bulky (boublous) hyperchromatic nuclei and some disturbances of the nuclear cytoplasmic ratio (Table 6), which are not seen in JAK2 wild type ET carrying the MPL S515 mutation and also not in prefibrotic JAK2 V617F mutated ET, ET/PP and PV (32,33). In six consecutive previously untreated CALR mutated ET and MF patients we found that the bone marrow histology findings in CALR mutated ET and early MF were consistent with hypercellular ET as the presenting feature of prefibrotic and early fibrotic stages of PMGM (Figures 6 and 7). The bone marrow histology findings in these 6 consecutive CALR mutated ET and MF patients showed a typical PMGM picture (Table 6), which were significantly different from giant megakaryocytes and hyperlobulated staghorn-like nuclei in MPL515 mutated ET (Figure 5), in JAK2 exon 12 mutated ET or PV (Figure 4) and in JAK2 V617F mutated ET and PV (Figures 2 and 3). The WHO-CMP features of a large group of CALR mutated ET and MF patients (N=50) as compared to a group of 50 cases with JAK2 V617F positive ET, prodomal PV and PV at time of first diagnosis are currently under investigation.

**Discussion**

With the advent of the JAK2 V617F mutation latent, masked, early and overt stages of PV will be picked up more than 5 to 10 years earlier by the WHO-CMP criteria as compared to the PVSG and WHO criteria. A broad spectrum of heterozygous JAK2 V617F mutated ET, hetero/
homozygous JAK2\(^{V617F}\) mutated masked PV, classical PV and post-ET MF or post-PV MF is very characteristic for JAK2\(^{V617F}\) mutated trilinear MPN as the main distinct and most frequent MPN disease entity (Tables 3 and 4). JAK2 wild type ET and MF carrying one of the MPL\(^{515}\) mutations is the second distinct MPN without features of PV at diagnosis and during follow-up (Table 5). The CALR mutated ET and MF category became in 2013/2014 the third distinct MPN entity without features of PV (Table 6). CALR mutations were found in a few MDS (RARS-T) patients, very rarely in atypical CML or CMML patients, but not in JAK2\(^{V617F}\) mutated ET and PV patients and also not in BCR/ABL positive CML patients [58,59]. CALR patients had no features of PV with lower hemoglobin and white blood cells counts but higher platelet counts [60,61]. The lower incidence of thrombotic complications in CALR mutated thrombocytopenia has been attributed to the fact that CALR-positive ET and MF patients lack PV features with significantly lower hemoglobin and WBC values than those in JAK2\(^{V617F}\) mutated ET
and prodromal PV patients [60,61]. Patients with JAK2\textsuperscript{V617F} mutated ET and PV had a similar high risk of major thrombosis, which was twice that of CALR mutated thrombocythemia patients [60,61]. CALR mutated ET patients presented with higher platelet counts and lower hemoglobin levels similar as observed in PMGM [31] as compared to hemoglobin levels in the upper range of normal in JAK2 mutated MPN patients (prodromal PV) [60,61]. WHO-CMP features of PV and polycythemia transformation has never been observed both in PMGM [26,31] and in CALR mutated ET and MF patients [60,61]. The evolution of ET to MF belong to the natural history of all molecular variants of the MPNs, which appeared to be related to the acquisition of epigenetics events on top of the driver mutation JAK2, MPL or CALR [62,63]. Life expectancy was significantly longer in CALR mutated MF patients as compared to those with a JAK2\textsuperscript{V617F}, which can be explained by the fact that CALR mutated MPN patients were about 10 years younger at time of diagnosis [60-62]. The overall survival of CALR mutated MF patients in the Italian studies was 23 years as compared to 14.4 years of MF patients with the JAK2\textsuperscript{V617F} mutated MF.

Retrospective analysis of 254 WHO-defined MF patients in the Mayo Clinics, Rochester showed that the JAK2\textsuperscript{, MPL-} and CALR-mutations were detected in 58%, 8.3 and 25% respectively and 8.7% were triple negative [62]. The median overall survival (OS) among 253 mutated ET and MF patients were younger, had higher platelet count, lower leukocyte count, higher hemoglobin (less anemic) and lower DIPSS-plus score. CALR-mutated MF patients had a favorable impact on median survival as compared to CALR-negative MF patients whether ASXL1-negative or positive. Among 181 WHO-defined CALR-negative MF patients, the median overall survival was 2.3 years in 55 CALR-negative/ASXL1-positive as compared to 5.6 years in 126 CALR-negative/ASXL1-negative MF patients. Among 146 CALR-positive MF cases the median survival was 7 years in 20 CALR-positive/ASXL1-positive MF patients as compared to 9.6 years in 126 CALR-positive/ASXL1-negative MF patients [62].

The etiology of triple JAK/MPL/CALR negative MF remains elusive whether they represent MPN or MDS. The awareness of the molecular heterogeneity of the MPNs including JAK, MPL and CALR mutations on top of epigenetic factors reflect the funeral of the term primary myelofibrosis (PMF) [64]. The terms CIMP and PMF according to the 2001 and 2008 WHO classifications should be replaced by 2014 WHO-CMP defined CALR-, MPL\textsuperscript{515}-mutated ET and MF (Tables 3 and 6) and JAK2\textsuperscript{V617}+-mutated ET, PV and MF (Tables 3 and 4) with variable degrees of anemia, splenomegaly, hypersplenism and myelofibrotic transformation of the bone marrow. Grading of reticulin fibrosis (RF) and reticulin collagen myelofibrosis (MF) is a second event in all variant of JAK2, MPL and CALR mutated MPNs (Tables 3-6 and Figures 8-10). In the congenital hereditary ET (HET) caused by gain of function mutation in the TPO and JAK2 gene (JAK2\textsuperscript{V617F} and JAK2\textsuperscript{B696C}) the responses of mutated CD33 and CD34+ cells to TPO are increased, but the responses to EPO were normal thereby explaining why HET caused by heterozygous germline TPO and JAK2 mutations are associated with the biological and clinical characteristics of ET without PV features (Figure 10) [64-66]. The clinical manifestations of autosomal dominant HET are typically complicated by Platelet Sticky Syndrome (ASPS), a novel disease entity of congenital aspirin responsive platelet thrombophilia [64-66].

We conclude that Bone Marrow Pathology (BMP) is a pathognomonic clue to each of the JAK2, MPL and CALR mutated MPN and clearly distinguishes all variants of MPN from CML, RARS-T, and MDS including the 5q syndrome mainly based on megakaryocyte morphology with a sensitivity and specificity of near

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**Figure 8:** Diagnostic algorithm for diagnosis and classification of essential thrombocythemia (ET) in MPN of various molecular etiology and diagnostic sensitivity and specificity of 1975. PVSG, the 2008 WHO, bone marrow biopsy and the 2014 WHO-CMP criteria for the diagnosis of ET in MPN of various molecular etiology.
Serum EPO and Granulocyte JAK2V617F mutation screening

- JAK2V617F positive (+) Serum EPO N / ↓
- JAK2V617F negative Serum EPO N / ↓
  - Bone Marrow Biopsy: BMB Grading RF, EEC (optional)
  - BMB (EEC optional) JAK2 exon 12 mutation
  - Search for congenital or acquired erythrocytosis
  - Normal Erythrocytosis unlikely
  - BMB: erythrocytosis JAK2 exon 12 negative → search for cause

Polycythemia:
- Early
- RCM
- Overt

Hematocrit: Normal ~ 0.45 ~ 0.50 ~ >0.70

| IE (15%) | PVSG (*65%/95%) |
| WHO (*80%/>99%) |
| Bone marrow biopsy (**/>99%/>99%) including grading of reticulin fibrosis: RF and MF |
| JAK2V617F (*95%/100%); JAK2 exon12 (3%) |

2014 WHO-CMP criteria for polycythemia vera versus erythrocytosis 99%/100%

*: sensitivity/specificity

Figure 9: Diagnostic algorithm for the diagnosis and staging of JAK2 mutated Polycythemia vera (PV) and diagnostic sensitivity and specificity of the 1975 PVSG, the 2008 WHO, bone marrow biopsy and the 2014 WHO-CMP criteria for the diagnosis of JAK2 mutated PV.

Figure 10: The Spectrum of Myeloproliferative Neoplasms ET, PV and MF according to 2014 WHO-CMP Classification, Staging and Translational states of congenital heterozygous JAK2 mutated essential thrombocythemia (ET) without features of polycythemia vera (PV) [64-66], acquired JAK2 mutated ET, PV and myelofibrosis (MF) [31,33,36] versus MPL mutated ET and MF [32,33] and CALR mutated ET and MF [58-62].

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to 100% (Figures 8-10) [67,68]. Within the JAK2 mutated ET and PV at time of diagnosis the megakaryocytes are similar large and pleomorphic with normocellular bone marrow in heterozygous JAK2 mutated ET and increased cellularity of 60-80% due to increased erythropoiesis=prodromal PV and strongly increased 90-100% cellularity in more advanced EMGM=masked PV and in classical prefibrotic PV due to increased trilinear hematopoiesis of increased erythropoiesis, megakaryoiesis and granulopoiesis (Tables 3 and 4) [14,27-35,67,68]. Staging of MPN disease burden in the trilinear MPN phenotypes of JAK2 mutated ET, masked PV and overt PV is based on grading of splenomegaly, LDH, leukocyte count and grading of reticulin fibrosis on top of JAK2 allele burden (Figures 8-10).
Exon 12 JAK2 mutated PV reflects a more favorable presentation of mainly erythrocythemia and and early stage PV with no or slight increase of platelet counts and leukocytes during long-term follow-up as compared to JAK2V671F mutated masked and classical PV. JAK2 wild type ET carrying the MPL or CALR mutation do not show PV features in blood and bone marrow during the prebiotic and early stages of reticulin fibrosis (myelofibrosis=MF) at diagnosis and during longterm follow-up. CALR mutated ET present with high platelet counts around 1000x10^9/L which after longterm follow-up tend to decline, which is related to progressive splenomegaly and increased MF (Figures 8-10). CALR mutated ET and MF is featured by hypercellular bone marrow due to dual primary megakaryocytic granulocytic myelofibroliferation (PMGM) with relative or absolute reduction of erythropoiesis and the presence of loose to dense clustered more or less immature large megakaryocytes with immature cloud-like nuclei, which are not seen in JAK2 mutated ET and PV and also not in MPL mutated ET (Figures 8-10). Bone Marrow Pathology (BMP) has a specificity and sensitivity near to 100% to differentiate between all molecular variants of the MPNs ET, PV and early stage MF from reactive thrombocytosis and primary or secondary erythrocytoses [67-70]. Bone marrow histology alone has a near to 100% accuracy to distinct MPN from CML and MDS (RARS-T and 5q-minus syndrome). The sensitivity and specificity to distinguish JAK2 on one hand vs MPL vs CALR mutated ET on the other hand just based on bone marrow histology alone surely will not always be possible [69,70]. The distinction of JAK2, versus MPL versus CALR mutated ET based on megakaryocyte morphology and background increase of cellularity is predicted to have an estimated accuracy of 70 to 80% in pretreatment diagnostic bone marrow biopsies at time of diagnosis [67-70]. The diagnostic differentation and staging of the early and fibrotic stages of JAK2, MPL and CALR mutated MPNs should be based on bone marrow morphology on top of quantitative measurement of JAK2, MPL and CALR mutation load related to the degree of anemia and splenomegaly to validate the natural history of each of the MPN disease burden prospectively during evolution of all molecular variants of ET into MF [25-28,67-70].

References


