Biology Diagnosis and Classification of MPD

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Abstract

According to strict morphological, biochemical, cytogenetic and molecular criteria including the Philadelphia (Ph) chromosome and bcr/abl fusion gene and protein, chronic myeloid leukemia is a malignant disease with an obligate transition into acute leukemia, whereas essential thrombocythemia (ET), polycythemia vera (PV) and agnogenic myeloid metaplasia (AMM) form the Ph-chromosome and brc/abl negative chronic myeloproliferative disorder (MPD) featured by a benign proliferation of the 3 hematopoietic cell lines. Increase and clustering of abnormal enlarged megakaryocytes together with no or various degrees of increased erythropoiesis and/or granulopoiesis in bone marrow biopsy specimens appears to be a pathognomonic clue to the diagnosis of prefibrotic MPD when WHO bone marrow features are applied. On top of established WHO bone marrow features in combination with $JAK2^{V617F}$ mutation screening, and laboratory features including endogenous elytroid colony (EEC) formation and serum erythropoietin (EPO) levels we propose updated European clinical, molecular and pathological (ECMP) criteria for the differential diagnosis of true ET, PV and chronic idiopathic myelofibrosis (CIMF) or AMM. The ECMP criteria reduced the platelet count of 600 $x10^9$/l to the upper limit of normal (>400 $x10^9$/l) as inclusion criterion for the diagnosis of thrombocythemia in various MPDs. When WHO and ECMP criteria are applied, PVSG-defined ET includes three distinct entities: true ET, early PV mimicking ET, and thrombocythemia associated with early CIMF-0 mimicking ET. Compared to characteristic bone marrow features pathognomonic for MPD, spontaneous EEC and low serum EPO levels are not sensitive enough as isolated markers for the diagnosis and differential diagnosis of prefibrotic ET, PV and CIMF-0. Bone marrow histology assessment remains the gold standard criterion for the diagnosis and staging of the MPDs. PVSG-defined ET and the presence of the $JAK2^{V617F}$ mutation, EEC, PRV-1 overexpression and low serum EPO levels is consistent with early PV ("forme fruste" PV) when ECMP criteria are applied. The combination of JAK2V617F mutation and increased hematocrit (>0.051 males and >0.48 females) is consistent with the diagnosis PV (specificity 100%, sensitivity 95%) without the need of red cell mass measurement. About half of the ET and CIMF patients are $JAK2^{V617F}$ positive (sensitivity 50%). The degree of $JAK2^{V617F}$ positivity of granulocytes is related to disease stage: heterozygous in true ET and early PV and mixed hetero/homozygous to homozygous in overt and advanced PV and CIMF-1 to 3. The proposed ECMP criteria for the differential diagnosis of ET, PV, CIMF in patients with $JAK2^{V617F}$ positive and JAK2 wild type MPD should be evaluated in prospective management studies in search for the most relevant prognostic factors of therapeutic significance.

Key words: myeloproliferative disorders, essential thrombocythemia, polycythemia vera, chronic idiopathic myelofibrosis, erythropoietin, endogenous erythroid colony assay, JAK2 V617F mutation, bone marrow pathology

Introduction

In the 19th century chronic myeloid leukemia (CML) and polycythemia vera (PV) have been described as primary distinct disease entities[1,2,3]. In 1960 Nowell and Hungerford described the presence of a minute chromosome in leukemic cells of patients with chronic myeloid leukemia (CML)[4]. This minute chromosome was called Philadelphia chromosome or Ph after the city of discovery[4]. Using banding techniques Janet Rowley (1973) discovered that the Ph originated from a translocation between the long arms of chromosomes 9 and 22, 9(9;:22)(q34;q11)[5]. Two groups working together in scientific friendship discovered that a hybrid gene is generated by the translocation consisting of the *BCR* gene on chromosome 22 and the *ABL* oncogene originating from chromosome 9[6]. This results in a *BCR/ABL* fusion gene with high tyrokinase activity and CML-transformation capacity[7,8]. Ninety-five percent of all CML patients are Ph^+; 90% are $Ph^+/BCR/ABL^+$, 5% are $Ph^-/BCR/ABL^+$, and 5% are $Ph^-/BCR/ABL^-$, the latter group usually diagnosed as atypical CML, juvenile CML, chronic neutrophilic leukemia or chronic myelomonocytic leukemia[9]. According to strict morphological, biochemical, cytogenetic and molecular criteria including the Ph^+ chromosome and bcr/abl fusion gene and protein, CML is a malignant disease with an obligate transition into acute leukemia, whereas essential thrombocythemia (ET), PV and agnogenic

Table 1. Polycythemia Vera Study Group (PVSG) criteria for the diagnosis of essential thrombocythemia (ET), polycythemia vera (PV) and modified criteria for the diagnosis of chronic idiopathic myelofibrosis (CIMF)

PVSG criteria for the diagnosis of ET17,18
Platelet count > 600 x 109/l (ECMP >400 x109/L)
• No known cause of Reactive Thrombocytosis
• Normal hemoglobin and red cell mass to exclude overt PV
• Stainable iron bone marrow to exclude PV
• No tear drop erythrocytes and no leuko-erythroblastosis
• No features of MDS in bone marrow smear and biopsy
• Absence of Ph1+ chromosome (brc/abl) to exclude CML
• Myelofibrosis grade 1 up to grade 2 is allowed in stead of collagen fibrosis of bone marrow is allowed up to <1/3 of bone marrow biopsy area.
PVSG defined ET is a clinical diagnosis of exclusion of reactive thrombocytosis, overt PV, MDS, CML and inclusion of early PV, prefibrotic CIMF-0 and early fibrotic stage of CIMF-1 without leuko-erythroblastosis in the peripheral blood (early clinical stage CIMF-0 and CIMF-1, table 3) when ECMP criteria are applied.

PVSG criteria for the diagnosis of PV14,15
A. Major criteria
A 1. Increased red cell mass: male >36 ml/kg, female >32 ml/kg
A 2. normal arterial oxygen (O2) saturation >92%
A 3. Splenomegaly on palpation
B. Minor criteria
B 1. thrombocytosis, platelet count >400 x10/l
B 2. Leukocytosis >12 x10/l (no fever or infection)
B 3. Increased leukocyte alkaline phosphatase score (LAP) (no fever or infection)
B 4. Increased serum B12 (>900 pg/ml) or unsaturated B12 binding capacity (>2200 pg/ml)
Diagnosis PV
A1 + A2 +A3 or A1 + A2 + any two from category B establish the diagnosis of PV.
Increased red cell mass is a crude criterion and overlook the early thrombocythemic and erythrocythemic stages of PV

Criteria for CIMF with leuko-erythroblastosis24,66,79,80
A. Pathological criteria
1. The presence of CIMF with grade 1, 2 or 3 myelofibrosis100.
2. WHO defined bone marrow features of CIMF (P1 and P2 in table 3) and no evidence of preceding CML, MDS, or PV, but associated with or preceded by PVSG defined ET24,72,73
B. Clinical criteria
- Splenomegaly at any grade on echogram or palpation
- Anisopoikolicytosis with tear drop erythrocytes
- Presence of circulating immature myeloid cells (CD34+).
- Presence of circulating erythroblasts.
- Anemia, hemoglobin <12 g/dl
- Increased LDH.
Diagnosis CIMF with leuko-erythroblastosis:
A1 and A2 plus any other two from B when splenomegaly or anemia is present
JAK2V617F and MPL515 confirm clonal MPD

Table 2. WHO bone marrow features and European clinical, molecular and pathological (ECMP) criteria for the diagnosis of essential thrombocythemia (true ET)[24,72,73]

Clinical and molecular criteria	Pathological criteria (WHO)
C1. Sustained platelet count above the upper limit of normal: >400 x10^9/l **C2.** Presence of large or giant platelets in a peripheral blood smear **C3.** Presence of MPL^{515} or JA2^{V617F} mutation **C4.** Normal values for hemoglobin, hematocrit, white blood cell differential count **C5.** Absence of the Philadelphia chromosome or any other cytogenetic fusion-gene abnormality	**P1)** Increase of dispersed or loosely clustered, predominantly enlarged megakaryocytes with mature cytoplasm and hyperlobulated nuclei (figures 2 and 3) **P2)** Normal cellularity, no proliferation or immaturity of erythropoiesis and granulopoiesis and no increase of reticulin fibers (myelofibrosis grade 0, MF-0)[100]

According to WHO/ECMP criteria C1 + P1 and P2 establish the diagnosis of true ET.
A typical ET bone marrow histological picture (figure 2, 3 and 4A) excludes PV, CIMF, CML, MDS, RARS-T and reactive thrombocytosis[72,73].

myeloid metaplasia (AMM) form the Ph-chromosome and brc/abl negative chronic myeloproliferative disorder featured by a benign proliferation of the 3 hematopoietic cell lines (figure 1)[10].

In 1950, William Dameshek described polycythemia vera (PV) as a chronic disorder of the bone marrow characterized by excessive production of nucleated red cells, granulocytes and megakaryocytes peripheral blood erythrocytosis, leukocytosis and thrombocytosis (figure 1)[11]. Some cases however show only a moderate elevation of erythrocytes with an extreme degree of thrombocytosis, while in others the leukocyte counts may be at or close to leukemic levels, with only slight increase in red cells or platelets[11]. According to Dameshek all "stops" to blood production in the bone marrow seem to have been pulled out in PV. As to the etiology of PV, Dameshek proposed two highly speculative possibilities: first, the presence of excessive bone marrow stimulation by an unknown factor or factors, and second, a lack or a diminution in the normal inhibitory factor or factors[10]. This hypothesis of Dameshek has recently confirmed by the discovery of the JAK2^{V617F} mutation[12] demonstrating that the V617F mutation 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, which makes the mutated hematopoietic stem cells hypersensitive to hematopoietic growth factors TPO EPO, IGF1, SCF and GCSF, resulting in trilinear myeloproliferation (Figure 1)[13].

The concept of PV as a trilinear MPD

Wasserman extended in 1954 the 1950 concept of Dameshek on PV as a trilinear MPD and distinguished at least five subsequent stages in the natural history of PV[3].

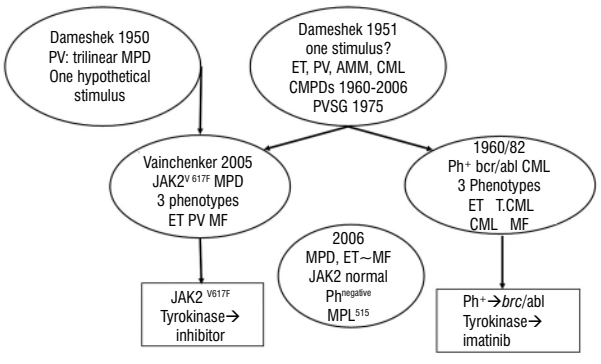


Figure 1. The concept of Dameshek in 1950 on polycythemia vera (PV) as a trilinear myeloproliferative disorder (MPD) due to one hypothetical stimulans, appeared to be caused by the acquired JAK2^{V617F} mutation discovered by Vainchenker et al in 2005.
The unifying concept of Dameshek in 1951 on the chronic myeloproliferative disorders (CMPDs) essential thrombocythemia (ET), polycythemia vera (PV), agnogenic myeloid metaplasia (AMM) has been broken up by the PVSG into Ph-positive thrombocythemia and CML complicated by myelofibrosis (MF) and the Ph-negative MPDs ET, PV and MF either positive or negative for the acquired JAK2^{V617F} mutation.

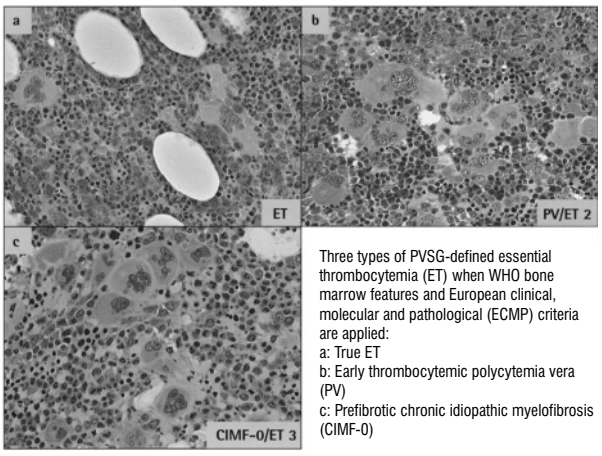
Table 3. WHO bone marrow features and European clinical, molecular and pathological (ECMP) criteria for diagnosis and staging chronic idiopathic myelofibrosis CIMF[24,72,73]

Clinical and molecular criteria	Pathological criteria (WHO)
C 1. Usually associated with or preceded by PVSG-defined ET and no preceding true ET, PV CML, CMML, HES or MDS. Absence of Philadelphia chromosome Presence of JAK2^{V617F} or MPL 515 mutation	**P 1.** Increased cellularity due to chronic megakaryocytic and granulocytic myeloproliferation (CMGM)[19] and no or relative reduction of erythroid precursors. **P 2.** Dense clustering and increase in atypical giant to medium sized megakaryocytes containing bulbous (cloud-like) hypolobulated nuclei and definitive maturation defects (figures 2, 3, 4C)
ECMP staging	
Early Clinical Stage Platelet count >400 x10^9/l, usually pronounced around 1000 x10^9/l No leuko-erythroblastosis, no anemia No or slight splenomegaly on echogram CIMF-0 or CIMF-1	**Grading of myelofibrosis (MF)[100]** **MF 0:** prefibrotic CIMF-0: scattered linear reticulin with no intersections (cross-over) corresponding to normal bone marrow
Intermediate Clinical Stage Definitive leuko-erythroblastosis Anemia grade 1: Hb <12 to >10 g/dl, or 7.5to 6.25 momol/lmmol/l Splenomegaly on palpation CIMF-1 and 2	**MF 1**: early fibrotic CIMF-1: loose network of reticulin with many intersections, especially in peripheral areas, no collagenization
Advanced Clinical Stage Pronounced leuko-erythroblastosis Anemia grade 2: Hb <10 g/dl or Hb >10 g/dl with the presence of adverse signs* Pronounced splenomegaly Leukocotosis, leukopenia Normal or decreased platelet count CIMF-2 and 3	**MF 2**: fibrotic CIMF-2: diffuse and dense increase in reticulin with extensive intersections, occasionally with only focal bundles of collagen and/or focal osteosclerosis.
	MF 3: classic CIMF-3: diffuse and dense increased in reticulin with extensive interactions with course bundles of collagen often associated with significant osteosclerosis
	MF >3: endstage hypocellular with extensive osteomyelosclerosis

According to WHO/ECMP criteria, C 1 and P1 plus P2 establish CIMF – any other peripheral blood criterion and grading of secondary myelofibrosis (MF) contribute to ECMP staging of WHO defined CIMF[72,73].

Stage 1). Pure erythrocythemia is featured by increased hemoglobin, hematocrit and red cell mass with normal leukocytes, thrombocytes and spleen size, which is labelled as idiopathic erythrocytosis.

Stage 2). The polycythemic stage of PV is featured by thrombocythemia, erythrocythemia and



Three types of PVSG-defined essential thrombocytemia (ET) when WHO bone marrow features and European clinical, molecular and pathological (ECMP) criteria are applied:
a: True ET
b: Early thrombocytemic polycytemia vera (PV)
c: Prefibrotic chronic idiopathic myelofibrosis (CIMF-0)

Figure 2. Three variants of thrombocythemia in MPD

Table 4. WHO bone marrow features and European clinical, molecular and pathological (ECMP) Criteria for the Diagnosis of Polycythemia Vera (PV) and diagnostic differentiation between PV and congenital or acquired erythrocytosis[24,72,73]

Clinical and molecular criteria	Pathological criteria (WHO)
Major	
A 0. Early PV. Hematocrit in the upper limit of normal: Ht: 0.45 to 0.51 in male and 0.43 to 0.48 in female) **A 1.** Classical PV: Hematocrit >0.51/>0.48 in male/female **A 2. P**resence of JAK2V617F mutation **A 3.** Low serum Epo level	**P 1.** Bone marrow pathology: increased cellularity due to trilinear increase of erythropoiesis, megakaryopoiesis and granulopoiesis and clustering of small to giant (pleomorph) megakaryocytes with hyperlobulated nuclei (figures 2 and 3) Absence of stainable iron. No pronounced inflammatory reaction (plasmacytosis, cellular debris)
Minor	
B 1. Persistent increase of platelet count: grade I: 400-1500, grade II: >1500. **B 2.** Granulocytes >10 x10^9/l or Leukocytes >12 x10^9/l and/or raised LAP-score or increased PRV-1 expression in the absence of fever or infection **B 3.** Splenomegaly on palpation or on ultrasound echogram (>12 cm length in diameter). **B 4**. Spontaneous endogenous erythroid colony (EEC) formation (optional)	**P 2.** Selective increase of erythropoiesis, normal granulopoiesis and megakaryocytes of normal size, morphology and no clustering in primary/secondary erythrocytosis (figure 3) P3. Grading of myelofibrosis (MF)[100] Post PV-MF-1, MF-22 and MF-3 (table 3)

WHO/ECMP criteria for early and overt PV[21,24,72,73]
A0, A2, B1 and P1 establish early PV (mimicking ET) PV ECP stage 0, or masked PV
A1, A2, P1 and none of B establish polycythemic PV ECP stage 1
A1, A2, P1 and one or more of B establish classic and advanced PV ECP stage 2 and 3
A1 and P2 with normal or increased values of serum EPO is consistent with erythrocytosis.
A3 confirms PV. B4 is an important research option.

no or slight myeloid metaplasia, leukocytosis and/or splenomegaly.

Stage 3). Myeloid metaplasia in PV patients presents with no or different grades of reticulin and collagen fibrosis in the bone marrow and progressive splenomegaly during long-term follow in about one third of the cases.

Stage 4). The polycythemic stage with various degrees myelofibrosis and splenomegaly following PV may elapse 5 to 25 years before a period of normal red cell values so-called spontaneous remission of PV occurs. This stage must be considered as the beginning of spent phase PV and may last a few to several years. At this point the spleen is frequently large and very firm to palpation, the liver is enlarged to a moderately degree in most patients, thrombocythemia is frequent and may be pronounced with bizarre and giant platelets, and white cells are usually increased with granulocytic leukocytosis (leukocythemia) accompanied by a small percentage of immature forms.

Stage 5). Post-PV myeloid metaplasia shows various degrees of leuko-erythroblastosis of the peripheral blood and may progress to extreme myelofibrosis with a dry tap on aspiration and massive splenomegaly. At this end-stage histopathology of bone marrow biopsy shows a similar picture and

Table 5. ET according to PVSG17,18, WHO21 and ECMP72,73 criteria

ECMP PVSG →	Hereditary ET	True ET ET type 1	Earlyl PV ET Type 2	Prefibrotic CIMF ET Type 3
Incidence	< 0.001	20-30	20-30	40-60
Serum EPO	Normal	Normal	Decreased ↓	Normal
Platelets	↑/↑↑	>400 ECP >600 WHO	>400 ECP >600 ECP	>400 ECP >600 WHO
Erythrocytes	N	N	N/↑	N/↓
Hematocrit	N	N	N/↑	N/↓
Bone marrow:	ET picture	ET picture	PV picture	CIMF picture
Megakaryocytes	Normal large / giant and mature			Abnormal
Splenomegaly	-	- / +	- / +	+ / -
JAK2 V617F	Neg: -	- / +	++	+ / -
EEC	Neg: -	- / +	++	+ / -
PVR-1		- / +	++	+ / -
Clonality	polyclonal	monoclonal	Monoclonal	Monoclonal

can not been differentiated from agnogenic myeloid metaplasia with no previous history of PV.

Three phenotypes of MPD: ET, PV and CIMF

The criteria the Polycythemia Vera Study Group (PVSG) originate from the early 1970s to classify the chronic myeloproliferative disorders (MPD) as 3 disease entities ET, PV and idiopathic myelofibrosis (IMF) and separate these 3 MPDs from Philadelphia chromosome-positive chronic myeloid leukaemia (CML) (figure 1, table 1)[14-18]. The criteria of Ph-chromosome-negative myeloproliferative disorders according to the 1990 Hannover bone marrow classification[19], the Rotterdam clinical and pathological criteria between 1997 and 2000[21] and the World Health Organisation (WHO) in 2001[21] are an attempt to integrate bone marrow morphological criteria alongside PVSG clinical criteria for essential thrombocythemia (ET), polycythemia vera (PV) and chronic idiopathic myelofibrosis (CIMF). However, early stages of PV and ET are not recognized by the PVSG and 2001 WHO classifications and many patients will be diagnosed as unclassifiable MPD, indicating the need to modify the diagnostic inclusion criteria for early and overt stages of MPD. In the present study we propose a new set of European clinical, molecular and pathological (ECMP) criteria in a joint effort by clinicians and pathologists to integrate PVSG criteria, WHO bone marrow features and the use of new laboratory and molecular markers for diagnostic differentiation of each of the early and overt MPD phenotypes more accurately as a sound basis on which proper treatment guidelines are to be recommended.

Table 6. Staging of PV patients according to WHO/ECMP criteria: therapeutic implications[24,72,73]

PV, ECP stage	0	1	2	3	4	5
Clinical Diagnosis	Early PV ET type 2	polycythemic PV	Classic PV	Advanced PV	Post-PV myelofibrosis	Spent phase PV
LAP-score and/or PRV-1	↑	↑	↑	↑/↑↑	variable	variable
Red cell mass	N	↑	↑	↑	variable	N/↓
Serum EPO	N/↓	↓	↓	↓	variable	N/↓
Leukocytes x109/l	<12	<12	N->12	>15	>20	>20
Platelets x109/l	>400	<400	>400	>1000	variable	variable
Peripheral blood red cells						
Hemoglobin g/dl (mmol/l)	<16 (10)	>16 (10)	>16 (10)	>16 (10)	variable	N/↓
Hematocrit	<0.51	>0.51	>0.51	>0.51	variable	N↓
Erythrocytes x1012/l	<6	>6	>6	>6	variable	N/↓
WHO bone marrow	Early PV	Early PV	Trilinear PV	Trilinear PV	Trilinear /MF	MF
Bone marrow cellularity (%) Grading myelofibrosis57	50-80 MF 0	50-80 MF 0	80-100 MF 0/1	80-100 MF 1/2	Decreased MF 3	Decreased MF 3
Splenomegaly	slight	no	Slight/moderate	moderate	large	large
Spleen size, echogram cm	12-15	<12	12-15	12-20	>20	>20
Specific MPD markers						
EEC	+	+	+	+	+	+/?
Molecular JAK2V617F Granulocytes BFU-e	 + +(++)	 + +(++)	 +/++ ++	 +/++ ++	 ++ ++	 ++ ++
Therapeutic implications						
First line treatment	Aspirin	Phlebotomy aspirin	Phlebotomy* aspirin	INF/HU* aspirin	HU	Supportive Splenectomy

*↑ = increased, ↓ = decreased, N = normal, + = present or heterozygous; ++ = homozygous

Tabel 7. **Translation of PVSG defined ET into WHO-defined True ET and Chronic Idiopathic Myelofibrosis (CIMF) grade 0, 1, 2, and 3 in a retrospective study of 865 thrombocythemia patients[70,71].**

PVSG clinical diagnosis WHO pathological diagnosis	ET True ET	ET CIMF-0	ET CIMF-1	Classic IMF CIMF-2	Classic IMF CIMF-3
WHO bone marrow EC Grading MF[100]	Normocellular MF-0	Hypercellular MF-0	Early fibrotic MF-1	Fibrotic MF-2	Advanced fibrotic MF-3
Number of evaluable patients	111	232	333	146	154
Age years (mean)	59	67	69	70	-
Platelets (mean x10^9/l)	1176	902	841	649	308
Leukocytes (mean x10^9/l)	11.1	12.4	12.2	11.0	10.9
Anemia	No	No	No	moderate	Moderate/severe
Hemoglobin: g/dl (mean)	13.8	13.9	13.1	12.0	10.6
mmol/l (mean)	8.6	8.7	8.2	7.5	6.6
Leuko-erytroblastosis and tear drop erythrocytes:	No	No	No/slight	Clearly present	Pronounced present
Splenomegaly* Palpable spleen cm* (mean)	No/slight 0.4	no/slight 1.1	No*/Slight 1.4	Moderate 3.2	Pronounced 5.5
LDH increase above the upper limit of normal	no	no	No*/slight	yes	yes
Adverse signs**	no	no	no	no	1 or 2
Mean life expectancy, years	>20	>15	>15 to >10	Around 10	<5

* Spleen and liver size on palpation in cm below the costal margin.
**Adverse signs include age > 70 years, hemoglobin < 10 g/dl, myeloblasts PB >2 %, erythro-normoblasts PB > 2%, leukocytosis > 20 x 10^9/l , thrombocytopenia < 300 x 10^9/l, severe constitutional symptoms, massive splenomegaly, cytogenetic abnormalities.

Limitations of PVSG and 2001 WHO criteria for the diagnoses of ET, PV and CIMF

The Polycythemia Vera Study Group (PVSG) reduced since 1986 the platelet count of 1000 to 600 x10^9/l as the arbitrary minimum for the diagnosis of ET (table 1)[17,18]. Lengfelder et al showed that this minimum of 600x10^9/l platelets according to the PVSG excluded early stage ET at platelets between normal and 600 x10^9/l in 29% of 143 ET cases when bone marrow biopsy was included in the investigations employed to diagnose ET[22]. These data confirmed the need to modify the PVSG criteria by lowering the platelet counts to 400 x10^9/l as the upper limit of normal for the clinical diagnosis of ET (table 2)[20,23,24].

The 2001 WHO bone marrow criteria separated true ET (table 2) from thrombocythemia associated with prefibrotic and early fibrotic stages of CIMF-0 and CIMF-1 (table 3)[21,24,25]. Early stage CIMF-0 and CIMF-1 manifest typical clinical and laboratory features of ET with no features of leuko-erythroblastosis and therefore are diagnosed as ET by the PVSG criteria (table 1)[24]. In retrospect, 97% of PVSG-defined ET patients according to Lengfelder et al showed bone marrow features with typical increase and/or clustering of enlarged megakaryocytes with abnormal morphology diagnostic for MPD[22]. This was associated with normal cellularity in 52% consistent with true ET, with increased erythropoiesis in 17% consistent with early PV, and with increased cellularity due to pronounced granulopoiesis in 45% consistent with prefibrotic CIMF[22].

The 2001 WHO criteria combined a typical ET histological bone marrow picture with platelet count in excess of 600 x10^9/l for the diagnosis of ET[21], thereby excluding early stage ET[22-24]. Only one-third of PVSG-defined ET is diagnosed as true ET when the 2001 WHO clinical and bone marrow criteria are applied[25]. Unclassifiable early stage CMPD according to the 2001 WHO criteria include 3 types of early stage MPD: 1) initial ET with a typical ET bone marrow but platelet count below 600 x10^9/l; 2) initial PV with a typical PV bone marrow, platelet count less than 600 x10^9/l, low serum erythropoietin (EPO), normal red cell mass and hematocrit less than 0.51; and 3) initial masked MPD with splenomegaly and normal or slightly increased platelet count and hematocrit[21].

According to Wasserman, PV frequently presents with initial erythrocytosis (idiopathic erythrocytosis), and distinguished at least five subsequent stages in the natural history of PV[3]. The PVSG used 3 major and 4 minor clinical criteria as inclusion criteria for the diagnosis of PV in the PVSG-01 study (Table 1)[16,17]. Increased red cell mass is a crude inclusion criterion for the diagnosis of PV (Louis Wasserman personal communication ASH 1995) and corresponds to high hematocrit values between 0.48 and 0.76, increased platelet count above 400 x10^9/L in two-third and palpable spleen in two-third of about 400 PV patients in the PVSG-

Table 8. **European Consensus (EC) on grading of bone marrow fibrosis (myelofibrosis: MF) in CML, PV and CIMF according to Thiele et al100**

Grading MF European consensus: EC	UK	USA	EC	CIMF
Focal scattered linearfine fibers with no intersections and only rare coarse fibers	1+	MF 1	MF-0	Prefibrotic
Loose network of reticulin with many intersections, especially in perivascular areas, no collagenization	2+ /3+	MF 2	MF-1	Early fibrotic
Diffuse and dense increase in reticulin with extensive intersection and only focal bundles of collagen/osteosclerosis	4+	MF 3	MF-2	Fibrotic
Diffuse and dense increased reticulin with extensive interactions with coarse bundles of collagen and significant osteosclerosis	-	MF 4	MF-3	Sclerotic

01 study[17]. One third of PV patients have normal platelet count and spleen at time of presentation. Pre-treatment bone marrow biopsy specimens in 103 PV patients of the PVSG-01 study with increased red cell mass showed increased cellularity from 50 to 80% in two-thirds consistent with early stage PV and from 80 to 100% in one-third consistent with overt stage PV at the bone marrow level[26].

Evidence accumulates that the very early prefibrotic stage of chronic myeloproliferative disease is featured by a platelet count in the upper limit of normal (around 400 x10^9/L), the presence of enlarged platelets in a peripheral blood smear in the complete absence of any underlying disorder for reactive thrombocytosis. ET according to the PVSG criteria comprises 3 types of prefibrotic MPDs including true ET, thrombocythemia associated with early polycythemia vera (PV), and thrombocythemia associated with prefibrotic chronic idiopathic myelofibrosis (CIMF-0) when the recently defined WHO bone marrow features and the European clinical molecular and pathological (ECMP) criteria are applied (figure 2, table 5). We produced good evidence that characteristic PV bone marrow histology features (irrespective of red cell mass measurements) are seen in four different stages of newly diagnosed MPD patients (figures 3, tables 4 and 6)[23,24,27-29]: First, early PV mimicking ET with a hematocrit in the upper limit of normal but increased platelet count without or with slight splenomegaly (stage 0 PV, table 6); Second, erythrocytosis with increased red cell mass, high hematocrit, low serum erythropoietin (EPO), but normal platelet count and spleen size (so-called idiopathic erythrocytosis = stage 1 PV, table 6); Third, classic PV with increased red cell mass, high hematocrit and one or more PVSG B criteria PV (overt stage 2 and 3 PV, table 6); Fourth, unclassifiable MPD or

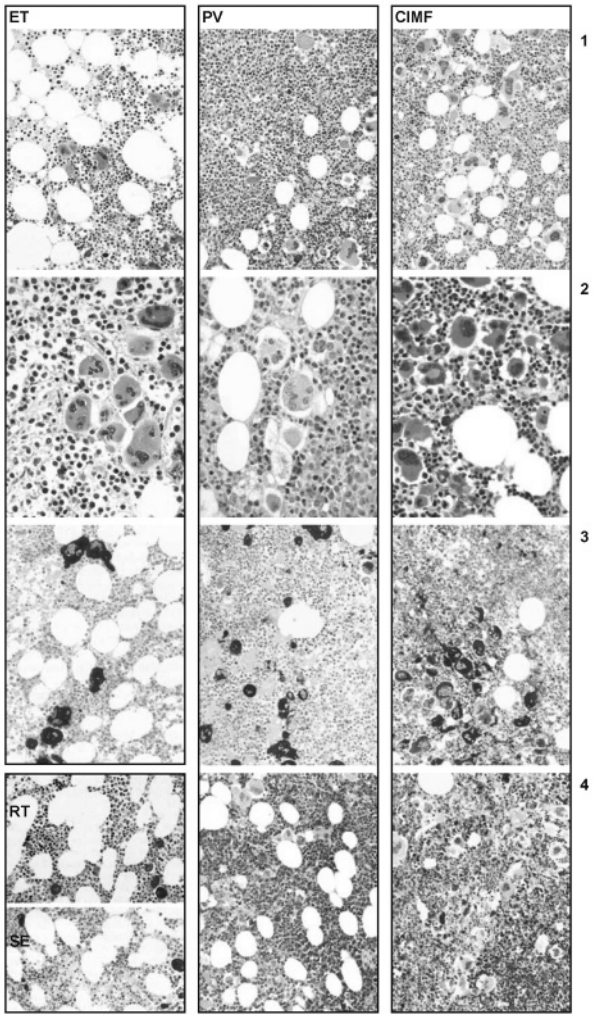


Figure 3. The World Health Organization (WHO) bone marrow (pathological) features for the differential diagnosis of the 3 prefibrotic myeloproliferative disorders (MPDs): essential thrombocythemia (ET), polycythemia vera (PV) and chronic idiopathic myelofibrosis (CIMF) according to Michiels & Thiele[24,28,66,72,73].
In contrast to normal sized and mature megakaryocytes in reactive thrombocytosis (RT) and secondary erythrocytosis (SE) essential thrombocythemia (ET) is characterized by loose clusters of enlarged to megakaryocytes with hyperploid nuclei, normal cellularity, normal erythropoiesis, normal granulopoiesis, and no increase of reticulin.
In polycythemia vera (PV), there is prominent proliferation of the 3 hematopoietic cell lineages(increased erythropoietin, granulopiesis and megakaryopoiesis) with loose clustering of small enlarged and giant (pleomorphic) mature megakaryocytes with hyperploid nuclei, especially in patients with thrombocythemia.
In prefibrotic chronic idiopathic myelofibrosis (CIMF-0) and early fibrotic CIMF-1, there is dense clustering of small, enlarged to giant immature megakaryocytes, with increased cellularity due to increased granulopoiesis and relative reduction of erythropoiesis. The megakaryocytes in CIMF are giant to medium size, and show bulbous (cloud-like) hypolobulated immature nuclei and definitive maturation defects, which are not seen in ET and PV.
Hematoxillin-eosine (HE) or perjodic acid Schiff (PAS) reagens staining for morphology. CD61 immunostaining of megakaryocytes. Chloro acetate esterase staining (Leder) for demonstration of increased granulopoiesis in PV and CIMF, silver staining following Gommorri's techniques for grading reticulin and collagen fibers[28].

masked PV with pronounced splenomegaly, normal hemoglobin and hematocrit, normal or slightly elevated platelet count. Thiele et al has nicely worked out this concept and confirmed that characteristic PV bone marrow histopathological features are seen in classic PV and in early PV mimicking ET (tables 4 and 6)[30-35]. The 2001 WHO criteria com-

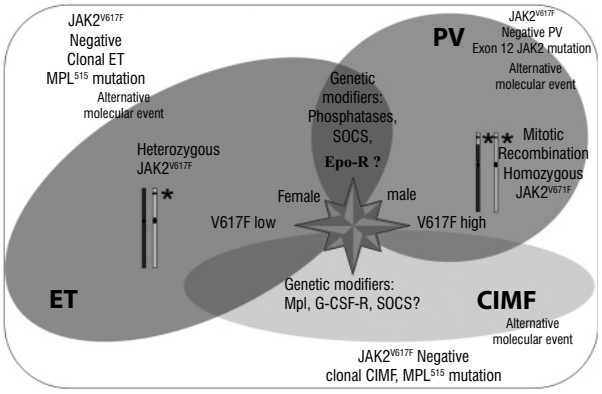


Figure 4. The JAK2^{V617F} "dosage" and "additional events" hypothesis of Vainchenker and co-workers[53-55]. Heterozygosity is the first acquired genetic step in which one allele of hematopoietic cells (HSCs) acquires the Jak2^{V617F} mutation and homozygosity is the second genetic step in which both alleles of HSCs carry the JAK2^{V617F} mutation due the mitotic recombination occurring at times that HSCs are dividing. Progression of heterozygous ET from 1 to 100% is associated with progression of ET to early stage PV, very likely with mild splenomegaly and no tendency to develop myelofibrosis.
Homozygosity for the JAK2^{V617F} mutation is associated with a more rapid onset (still rather slow), and with pronounced trilinear myeloproliferation of megakaryopoiesis, erythropoiesis and granulopoiesis leading to panmyelosis of the bone marrow.
The degree of homozygosity for the JAK2^{V617F} mutation in PV may range from 1% to 100% during long-term follow-up. If less than 25% of the HSCs cells are homozygous for the JAK2V617F mutation the result of JAK2 measurement in granulocytes using PCR technique will be less than 50%. If more than 25%of the HSCs carry the JAK2^{V617F} mutation the result of the JAK2^{V617F} measurement in granulocytes will be more than 50%.
If during follow-up all HSCs carry the JAK2V617F mutation the maximum positivity of the JAK2^{V617F} mutation using PCR technique is 100%. Consequently, PV patients may progress from low percentage to high percentage positivity for the homozygous JAK2^{V617F} mutation, which is associated with progressive splenomegaly and post-PV myelofibrosis during long-term follow.
About 95% of PV patients are positive for the JAK2^{V617F} mutation eitherheterozygous or homozygous or combined. Among the 5% of PV negativeJAK2^{V617F}, the majority do carry an exon 12 mutation in the JAK2 gene.
About 60% of ET and CIMF patients are positive for the JAK2^{V617F} mutation: true ET heterozygous and CIMF heterozygous or homozygous or both.
About 40% of ET and CIMF are negative for the JAK2V617F mutation do not show PV features and do have another etiology including MPL^{515} mutations.
ET is more frequent in females and PV in males.

bined a characteristic PV histological bone marrow picture as a minor criterion with increased red cell mass as a major crude inclusion criterion for the diagnosis of PV[7], thereby excluding early stage PV mimicking ET[23,24,27-29]. To overcome the shortcomings of the 2001 WHO classification of the MPDs, we here propose the updated European clinical, molecular and pathological (ECMP) for the diagnosis, classification and staging of true ET, PV and CIMF (figures 2, and 3, tables 2, 3 and 4)[24,28].

Limitations of laboratory markers for the diagnosis of ET and PV

Red cell mass measurement (RCM) is cumbersome, time consuming, costly, and not specific for MPD. Increased RCM separates patients with increased erythrocytosis from apparent erythrocytosis (pseudo-polycythemia), but does not distinguish PV from congenital polycythemia (CP) or secondary erythrocytosis (SE)[27-29]. The ECMP separates patients with increased erythrocytosis into patients with polycythemia vera (true polycythemia, figure 2 and 3) as a trilinear MPD and

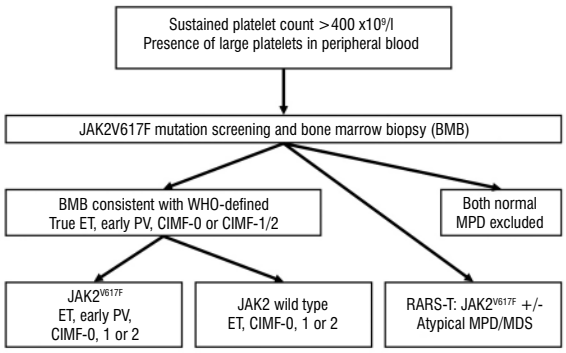


Figure 5. Algorithm for diagnostic work-up for patients with suspected thrombocythemia as the presenting feature of ET, early PV, prefibrotic CIMF-0, early fibrotic CIMF-1 or refractory anemia with increased ringed sideroblasts (RARS-T)[72,73].

non-clonal polycythemia (erythrocytosis, table 4), either congenital, acquired or idiopathic. In a consecutive cohort of 105 patients with both PV and non-clonal polycythemia as well as ET, in whom diagnostic categories were established based on clinical data, laboratory parameters and bone marrow histology, RCM had a sensitivity of 76% in the diagnosis of PV and a specificity of 79% in distinguishing PV and non-clonal polycythemia[34]. PV patients with RCM may have normal hemoglobin and hematocrit because of associated iron deficiency and/or significant splenomergaly[35]. Bone marrow histopathology has a sensitivity and specificity of near to 100% (gold standard) to differentiate a typical trilinear hypercellular bone marrow with small and enlarged (pleomorphic) megakaryocytes in early and overt PV (table 4) from the presence of isolated increased erythrocytosis and normal megakaryocytes in congenital (primary) polycythemia (CP), congenital erythrocytosis (CE) idiopathic erythrocytosis (IE) or acquired (secondary) erythrocytosis (SE) without the need for red cell mass measurement[27-33]. In none of the PV patients is the information from RCM measurement found to be of additional diagnostic value, because all PV patients with increased red cell mass not only show a typical PV bone marrow histology picture but also the presence of one or more specific markers endogenous erythroid colony (EEC), low serum EPO, increased platelet count, and/or splenomegaly for the diagnosis of PV[23,27].

Spontaneous EEC and low serum EPO levels are specific confirmative criteria for the diagnosis of PV, but have insufficient diagnostic sensitivity as isolated parameters to differentiate between PV, CP, CE, SE, ET and normal controls[36-41]. About 50% of PVSG defined ET patients show not

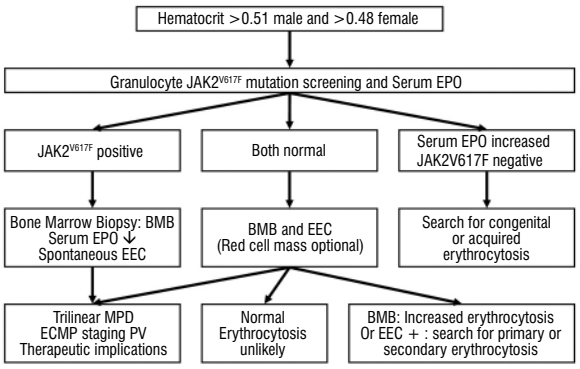


Figure 6. Algorithm for diagnostic work-up of patients with suspected polycythemia vera or erythrocytosis[72,73].

only spontaneous EEC but also increased PRV-1 expression[42-46] together with low serum EPO levels[42,46,47] indicating that EEC/PRV-1-positive ET comprises a biologically distinct subgroup of ET patients reflecting early PV ("forme fruste" PV) that is at risk for progression to overt PV (tables 5 and 6). Considering the finding of clustered enlarged or giant megakaryocytes according to ECMP criteria as diagnostic for MPD (ET, PV or CIMF) in 46 MPD patients with splanchnic thrombosis, the sensitivity of increased red cell mass for the diagnosis of MPD was 63%, of low serum EPO level 52%, of EEC 72%, and of splenomegaly 74%, indicating the superiority of bone marrow histopathology to detect masked, early and overt stages of MPD[48]. In patients with splanchnic vein thrombosis (Budd-Chiari syndrome or portal vein thrombosis) without signs of overt MPD, the laboratory markers EEC, PRV-1 expression and low serum EPO are insensitive but the combination of JAK2^{V617F} mutation and bone marrow histology assessment is highly sensitive and specific to diagnose early ET and PV [48-52].

The role of JAK2^{V617F} mutation in the pathogenesis and classification of trilinear MPD

The discovery of the JAK2 ^{V617F} mutation in 2004 by William Vainchenker and his team in France (JAK2 ^{Vainchenker} 617 ^{France}) was immediately appreciated as a real evolutionary event, and rapidly confirmed in 2005 by several investigators[24,53]. JAK2 plays an essential role in hematopoiesis by mediating signals from several hematopoietic cytokines including EPO, TPO IL-3 G-CSF, and GMSCF[24,53-57]. The JAK2 V617F mutation makes the mutated hematopoietic progenitor cells hypersensitive to these cytokines, thereby leading to growth advantage of the mutated above the non-mutated normal trilinear hematopopoietic cells in the bone marrow (figure 4)[24,53]. The JAK2^{V617F} mutation is detectable in CD34^{+} hematopoietic bone marrow cells, erythroblasts, in cells of spontaneous EEC, blood platelets and granulocytes[53-55]. Applying allele-specific polymerase chain reaction (PCR) analysis in PVSG-defined MPD patients, a high frequency of the JAK2^{V617F} mutation of 95% (92-97%) in PV, and a lower frequency of 53% (49-57%) in ET and 52% (44-55%) in idiopathic myelofibrosis (IMF) are described[24,56]. Only 3 to 4% of ET, 24 to 27% of PV and 6 to 18% of IMF patients are homozygous for the JAK2^{V617F} mutation[24,56]. Two hypotheses have been proposed by Vainchenker's group of French investigators Delhommeau, James, Pisani, Villeval and Casadevall to explain why three different phenotypes of MPD are caused by the same JAK2^{V617F} mutation: the "dosage" hypothesis and the "additional events" hypothesis (figure 4)[53-55]. According to the dosage hypothesis (based on animal studies and different mutation states of JAK2^{V617F} in MPD patients), the level and duration of JAK2^{V617F} directly contribute to the phenotypic diversity of trilinear MPDs. According to this model (based on animal studies and different mutation states of JAK2^{V617F} in MPD patients), the level and duration of JAK2^{V617F} directly contribute to the phenotypic diversity of trilinear MPDs (figure 4)[53,55]. The hypothesis to explain that the level of kinase activity regulates the disease phenotype of MPD is based on different densities of thrombopoietin (TPO) and EPO receptors (TPOR and EPOR) on hematopoietic progenitor cells and on difference of response of TPOR and EPOR to various levels of JAK2^{V617F} activity[55]. TPOR or MPL is expressed at high levels in megakaryocytic cells where it controls TPO physiologic levels. It is possible that activation of a few TPO receptors by low levels of JAK2^{V617F} (heterozygous) is sufficient to send a signal to megakaryocytic cells. A slight increase in numbers of mutated megakaryocytes and platelets (about 200 x10^9/l mutated platelets) might be enough to produce platelet-mediated microvascular circulation disturbances[24,53]. Conversely, EPOR is expressed at low levels on hematopoietic progenitor cells and therefore high levels of JAK2^{V617F} may be required to activate EPOR and generate PV-like phenotype[24,53]. Sustained high levels of JAK2^{V617F} during long-term follow-up subsequently may lead to a high level activation of EPOR and granulocyte colony stimulating factor receptor (G-CSFR) leading to extramedullary hematopoiesis (splenomegaly) and cytokine mediated secondary myelofibrosis[53,55]. The degree of JAK2^{V617F} positivity (progression from heterozygous to homozygous in figure 4) is strongly cor-

related with (polycythemia rubra vera-1 gene) PRV-1 over expression in granulocytes, with the ability to form spontaneous EEC and with progressive post-PV myelofibrosis[45,55,57]. Scott et al recently showed that BFU-e colonies are already homozygous for the $JAK2^{V617F}$ mutation in PV patients with a heterozygous pattern of $JAK2^{V617F}$ in their peripheral blood granulocytes[58]. In contrast, the BFU-E colonies from heterozygous patients with ET did not contain a subpopulation of $JAK2^{V617F}$ homozygous cells[58]. In a recent study, $JAK2^{V617F}$ was detected in 75% of ET (n=60) and in 97% of PV patients (n=62)[59]. Allelic ratios exceeding 50% $JAK2^{V617F}$ indicating the presence of granulocytes homozygous for $JAK2^{V617F}$ were found in 70% of PV at diagnosis but never in ET[59]. Passamonti et al produced good evidence that transition from heterozygosity to homozygosity for the $JAK2^{V617F}$ mutation represents a very important step in the progression from classic PV to post-PV myelofibrosis[57].

$JAK2^{V617F}$ may be may be dependent not only on the amount of heterozygous and homozygous mutant protein, but also on the various pathway regulating JAK2 activity including MPL, JAK2, STAT-3 signalling pathway[53]. This has led to the recent discovery of the MPL^{W515L} and MPL^{W515K} mutations as the underlying etiology in some ET and CIMF patients[60,61].Pikman et al identified a somatic activating mutation in the transmembrane domain of MPL, the thrombopoietin receptor (TPOR), in 4 of 45 (9%) of JAK2 wild type myelofibrosis patients[60]. In a mouse model transplant assay, expression of the MPL^{W515L}, but not wild type MPL, resulted in a fully penetrant MPD characterized by marked thrombocytosis and leukocytosis with no evidence of polycythemia. Thrombocythemia with leukocytosis was caused by dual proliferation of megakaryopoiesis and granulopoiesis (myelopoiesis) with increased numbers and clustering of atypical dysmorphic megakaryocytes in the bone marrow, myelofibrosis and marked splenomegaly due to extramedullary hemopoiesis consistent with true CIMF[60]. Pardanani et al screened 1182 PVSG-defined MPD patients (318 ET, 242 PV, and 290 IMF) and 64 controls for MPL^{515} mutations regardless of $JAK2^{V617F}$ mutation[46]. MPL mutations either MPL^{W515L} (n=17) or MPL^{W515K} (n=5) was detected in 20 patients (de novo IMF in 12 = 4%, ET in 4 = 1%) and post-ET myelofibrosis in 1, but not in PV and controls[61]. Six cases carried the MPL^{W515L} and $JAK2^{V617F}$ alleles indicating that these alleles have functional complementation in IMF. These experimental and clinical observations indicate that MPL^{515} mutation related myelofibrosis may represent a distinct entity of JAK2 wild type IMF distinct from $JAK2^{V617F}$ trilinear MPD. According to the "additional events" hypothesis, alternative and/or additional molecular abnormalities modify, or precede a homozygous state deferred to by the $JAK2^{V617}$ mutation alone, combinations carrying the $JAK^{V617F\ and}$ MPL^{515} mutations[60,61] or other combinations of still unknown mutations (figure 4)[53]. Mechanisms other than mitotic recombination such as duplication of the mutated allele is observed in a proportion of PV and CIMF patients displaying a gain of 9p, mostly due to trisomy 9[53]. Therefore, there may be an overlap between "dosage" and "additional molecular events" hypotheses. Long-term studies are warranted to delineate the chronology and impact of various putative additional molecular abnormalities especially in cases of progressive disease from $JAK2^{V671F}$ positive true ET to PV and subsequent CIMF and in cases with combined $JAK2^{V617F}$/MPL^{515} mutations or JAK2-wild-type/MPL^{515} positive ET and CIMF. Sex appears to be a powerful genetic background modifier in $JAK2^{V617F}$-positive MPDs as ET is more common in females and PV in males.

WHO bone marrow features and ECMP criteria for the diagnosis of ET, PV and CIMF

In 1980s Georgii and Thiele independently defined the pathological features of ET, PV and idiopathic myelofibrosis (IMF) or agnogenic myeloid metaplasia (AMM) as derived from histopathological morphology of bone marrow biopsies[19,62]. ET is defined by persistent increase of platelets in excess of 400 $x10^9$/l without the Ph^{1+} chromosome together with monolinear proliferation of mature enlarged megakaryocytes in the bone marrow with normal cellularity, normal erythropoiesis and normal granulopoiesis (figure 2)[19,62]. PV is defined as a trilinear proliferation of megakaryopoiesis, erythropoiesis and granulopoiesis in which the erythropoiesis was most prominent together with variable degrees of increased platelets, erythrocytes and granulocytes in the peripheral blood in the absence of the Ph^{1+} chromosome (figure 2)[19,62]. Georgii regarded myelofibrosis (MF) as a reactive feature secondary to progressive disease[9,10] seen in CMGM, PV and CML[19,63,64]. Therefore, according to Georgii the terms agnogenic myloid metaplasia (AMM) or idiopathic myelofibrosis (IMF) lack accuracy since they are applied to both the prefibrotic hypercellular and advanced fibrotic stages of AMM or IMF. Consequently, Georgii replaced the terms

prefibrotic AMM and IMF by chronic megakaryocytic granulocytic myeloproliferation (CMGM) and proposed in 1990 the Hannover Classification to distinguish 3 distinct primary CMPDs ET, PV and CMGM[19]. The diagnosis of CMGM according to the Hannover classification[19] or prefibrotic CIMF-0 according to the Cologne criteria proposed by Thiele[66] (figure 3) is based on 3 main features. First, the presence of large megakaryocytes with immature cytoplasm and immature cloud-like nuclei not seen in ET and PV. Second, increased granulopoiesis but never disturbed in maturation. Third, erythropoiesis is usually relatively decreased[19,62,63]. The Hannover classification uses the term CMGM[19,62,63], and the Cologne classification uses the term prefibrotic chronic IMF (CIMF-0) for the third entity of prefibtotic MPD[65-71]. The Hannover and Cologne classifications[19,66] are based on specific bone marrow histology (pathological) features for the three prefibrotic CMPDs true ET, PV and CIMF-0 at the bone marrow level, and have been taken over in 2001 by the WHO[21]. Michiels and Thiele subsequently proposed the European clinical and pathological (ECP) criteria for the diagnosis of the Ph-negative MPDs to describe the full spectrum of clinical, laboratory, and WHO bone marrow features for true ET, early and overt PV and CIMF-0 (figure 3)[24,28,72,73,] In a recent joint effort by clinicians and pathologists, we added the molecular marker and updated the European clinical, molecular and pathological (ECMP) criteria to extend PVSG criteria by including WHO bone marrow features and the use of laboratory and molecular markers for the diagnosis and staging of the three prefibrotic MPDs true ET, PV and CIMF (tables 2, 3 and 4)[24,72,73]. These ECMP criteria allow a cross talk between clinicians and pathologists to translate PVSG clinical criteria (table 1) by including WHO bone marrow features and new biological and molecular markers (figures 2, and 3, tables 2, 3 and 4) to reach agreement on diagnosis, classification and staging of the MPDs[72,73].

For the diagnosis of MPD bone marrow trephine biopsy specimens should be embedded in paraffin or plastic, both with its technical limitations. Paraffin requires decalcification with EDTA (preferable, allows reasonable DNA quality) or acid electrolysis[28,72,73]. The specimens should have at least 4 evaluable bone marrow spaces with hematopoiesis. Recommended stains include: hematoxylin and eosin (H&E), Giemsa (3 μm sections), periodic acid-Schiff (PAS); Perls for estimation of hemosiderin content; chloro-acetate esterase (Leder) for identification of granulocytic differentiation; silver stain for reticulin; and trichrome-Masson for collagen staining. Immunostains of paraffin embedded specimens should include glycophorine C for erythropoiesis, myeloperoxydase for granulopoiesis, CD42b, CD61 or FVIII- related antigen for megakaryocytes; CD34 for CD34-positive blasts and CD117 for myeloid differentiation. Regarding MPD, clinicians want to receive from their pathologist[28,72,73] a detailed report according to the Cologne bone marrow evaluation form[72,73]. According to WHO and ECMP criteria, increase and loose clustering of enlarged mature megakaryocytes with hyperlobulated nuclei in a normocellular bone marrow and platelet count >400 x10^9/l represent the hallmark of true ET (table 2, figures 2 and 3). In true ET there is no proliferation or immaturity of granulopoiesis or erythropoiesis. In congenital and acquired erythrocytosis and in reactive thrombocytosis the megakaryocytes are of normal size and morphology and there is no tendency to cluster. A typical histopathological ET picture of the bone marrow excludes RT and distinguishes true ET from early PV, CIMF-0, CIMF-1 (figure 2), and thrombocythemia associated with atypical MPD, MDS, refractory anemia with increased ringed sideroblasts (RARS-T) or Ph^1–positive thrombocythemia in CML. The characteristic increase and clustering of small and enlarged pleomorphic megakaryocytes and increased erythropoiesis with increased granulopoiesis and increased cellularity (80-100%) are the diagnostic characteristics of classic PV with increased hematocrit >0.51, low serum EPO and/or $JAK2^{V617F}$ (tables 4 and 6) distinguishing it from congenital and secondary erythrocytosis. A typical histological PV picture with moderately increased bone marrow cellularity (according to age) is seen in patients with early PV mimicking ET or "forme fruste" PV featured by platelet count >400x10^9/l and hematocrit <0.51, low serum EPO and/or the presence of the $JAK2^{V617F}$ mutation (tables 4 and 6). A typical histological PV bone marrow picture is also seen in the early erythrocythemic stage 1 PV featured by increased hematocrit >0.51, normal platelet count <400 x10^9/l, normal spleen, low serum EPO and/or the presence of the $JAK2^{V617F}$ mutation (tables 4 and 6).

According to Hannover[19], Cologne[66], WHO[21] and ECMP criteria[24,72,73], CIMF-0 is characterized by hypercellularity of the bone marrow (60-100%) due to increased granulopoiesis, relative decrease of erythropoiesis and the presence of dense clusters of immature megakaryocytes with maturation defects

of cytoplasm and nuclei with bulky nuclei showing clumpy lobuli and irregular roundish shaped forms (so-called cloud-like or clumsy nuclei), which are almost never seen in ET and PV (figures 2 and 3, table 3)[28,64-73]. The degree of dysmegakaryopoiesis in CIMF-0 may range from slight maturation defect of megakaryocytes with no cloud-like nuclei to manifest maturation defects of megakaryocytes with typical cloud-like nuclei or a mixture of both. The risk of CIMF-0 to transform into early CIMF-1 and subsequent CIMF-2/3 with extramedullary hematopoiesis is clearly dependent on the degree of hypercellularity and on the degree of maturation defects of megakaryopoiesis[70,71]. High quality histological bone marrow preparations in the hands of experienced hematopathologists are required to distinguish CIMF-0 from true ET and PV in about 85 to 90% of the cases, which is only feasible in centers of excellence (figures 2 and 3)[57-73]. There are no studies that have examined the concordance between a number of pathologists who have used characteristic histological bone marrow features to assign cases to the prefibrotic stages of true ET, PV and CIMF-0 without knowledge of the clinical findings and biological MPD markers except age. Data on the very long-term natural history of patients with ECMP defined true ET, early PV and CIMF-0 as derived from large scale prospective studies are lacking. We don't really know whether the early stages of PV patients are at no, low or high risk of progression to post-PV myelofibrosis or whether true ET never progress to CIMF as it is claimed. The bone marrow histology of CIMF-0 with slight dysmegakaryopoiesis can appear to overlap with early PV (hematocrit <0.51) presenting with a trilinear hypercellular bone marrow with relative increased granulopoiesis as compared to erythropoiesis and a similar degree of thrombocythemia, leukocytosis, increased LAP-score and slight splenomegaly. The histology of CIMF-0 slight dysmorphic megakaryopoiiesis may overlap with that of true ET in cases of very mild hyperplasia of granulopoiesis and/or a mixture of mild dysmorphic megakaryocytes and mature enlarged megakaryocytes with hyperploid nuclei. Diagnostic differentiation between true ET, early PV, CIMF-0 at the bone marrow level will be feasible in well equipped pathology laboratories by experienced trained pathologists in the majority of cases, but significant overlap (grey zones) between CIMF-0 with slight dysmegakaryopoiesis versus true ET or early PV due to a rather high inter-observer disagreement between hematopathologists in routine daily practice is very likely.

Clinical relevance of ECMP criteria for diagnosis and staging of ET, PV and CIMF

The PVSG-defined ET patients (table 1) comprise three ECMP-defined phenotypes of thrombocythemia at the bone marrow level: true ET, early PV mimicking ET, and CIMF-0 or CIMF-1 without features of leuko-erytroblastosis in the peripheral blood[25,70,71]. These three ECMP defined ET phenotypes do not differ significantly with regard to peripheral blood features, thrombocythemia related clinical presentation and laboratory findings during long-term follow-up (table 7)[70,71]. Therefore, patients with true ET and early PV and CIMF-0 mimicking ET are to be treated equally based on clinical risk stratification for thrombotic and bleeding complications irrespective of bone marrow features[56]. The relevance of recognition of CIMF-0 and CIMF-1 lies in the increased risk of myelofibrotic transformation and decreased prognosis in terms of survival compared to true ET[70,71]. The prognostic importance of the WHO/ECMP criteria is demonstrated in a large retrospective study of 476 PVSG-defined ET patients (platelet count >600 $x10^9$/l), who were reclassified according to the WHO bone marrow criteria: true ET in 167, CIMF-0 in 174 and CIMF-1 in 135[71]. Mean age of true ET patients was 59 years, which is 8 to 10 years younger compared to CIMF-0 (67 years) and CIMF-1 (69 years) patients. The differences in relative 10 years survival rates: 99 ± 7.8% for true ET, 81 ± 11.7% for CIMF-0, and 67 ±17.8% for CIMF-1 patients, are significant due to an increased risk of myelofibrosis and splenomegaly during follow-up. In this retrospective "one-center-study" the majority of CIMF-0 patients have early stage disease without features of leuko-erythroblastosis (table 7), whereas CIMF-1 patients are a mixture of early and intermediated stage disease without and with leuko-erythroblastosis[71] (as defined in tables 1 and 3 when applying ECMP criteria). The ECMP criteria for diagnosis and staging of CIMF patients do clearly separate early stage CIMF-0 and CIMF-1 without leuko-erythroblastosis from intermediate stage CIMF-1 and CIMF-2 with leuko-erythroblastosis of the peripheral blood (table 3)[72,73]. Clinicians should realise that the overall survival curves of patients with early stage CIMF-0 or CIMF-1 and no leuko-erythroblastosis will be still as good as for newly diagnosed PV patients, and that the life expectancy of patients with CIMF-2 and CIMF-3 with leuko-erythroblastosis, splenomegaly and anemia will be significantly shortened (table 7).

A recent report of 116 PVSG-defined ET patients and reclassified based on WHO bone marrow cri-

teria confirmed that such cohort of patients with the clinical picture of ET in fact comprises true ET in 19%, CIMF-0 in 21%, CIMF-1 in 37%, CIMF-2 in 12%, early PV in 8%, and unclassified MPD in 3%[74]. Median age of true ET and CIMF-0 patients was 54 and 52 years respectively, which is 7 to 14 years younger compared to CIMF-1 (59 years) and CIMF-2 (66 years), which points to the unexplored question whether true ET will progress to PV of CIMF after very long-term follow-up[74]. Thromboembolic events were equally frequent in ET, CIMF-0 and 1, but relevant life threatening events including acute myeloid leukemia, advanced CIMF and second malignancies were more frequent in CIMF-1 and 2 during long-term follow-up[74]

In ECMP-defined true ET, progression into myelofibrosis grade 1 or 2 was not seen five years after diagnosis in two recent studies, but data on very long-term follow-up of more than 10 to 15 years are lacking[75,76]. Kvasnicka and Thiele calculated that the estimated risk of transformation within 3 years into clinically defined IMF (with anemia, leuko-erythroblastosis and extramedullary hematopoiesis) was 2.2% in PVSG-defined ET and 2.8% in WHO-defined CIMF-0 and -1 (nearly 1% per year)[71]. In a large series of 195 PVSG-defined ET patients and a median follow-up of 7.2 years, evolution into CIMF-2/3 (classic IMF with anemia, leuko-erythroblastosis and splenomegaly) occurred in 2.7% at 5 years, 8.3% at 10 years and 15% at 15 years (6.7% after a median of 8.3 years)[77]. In another retrospective study of 322 PVSG-defined ET, the cumulative risks of CIMF-2/3 and leukemia were 3.8% and 1.4% at 10 years, and 19.9% and 8.1% at 20 years respectively[78]. In these two studies of PVSG-defined ET patients, the overall survival was similar to that of the age-matched control population in the first decade, but significantly worse beyond the first decade of the disease. Applying ECMP criteria to PVSG-defined ET patients at time presentation will separate patients with true ET from early PV and CIMF-0 mimicking ET and therefore surely will become of prognostic importance to distinguish true ET from prefibrotic CIMF-0 and early fibrotic CIMF-1 in the context of new prospective clinical management studies.

Diagnostic work-up of patients with thrombocythemia in various MPD

Clinical features suspicious for thrombocythemia in various MPDs (true ET, early PV and CIMF-0) include a sustained increased platelet counts (>400 $x10^9$/l) in the absence of any cause for reactive thrombocytosis[20,23,24,72,73]. The presence of giant platelets in a peripheral blood smear is indicative for MPD and precludes reactive thrombocytosis. Sustained increase of platelet counts (>400 $x10^9$/l) associated with slight splenomegaly on echogram (>12cm), increased leukocytes (>12 $x10^9$/l) or LAP score with normal ESR is highly suspicious of myeloproliferative thrombocythemia. Clinical manifestations of thrombocythemia in various MPDs consist of microvascular circulation disturbances including atypical and typical TIAs, ocular ischemic attacks, erythromelalgia, splanchnic or cerebral vein thrombosis[41]. Clinicians and pathologists should realise that PVSG-defined ET includes true ET, early PV mimicking ET, and thrombocythemia associated with CIMF-0 or CIMF-1 when ECMP criteria are applied (tables 1, 2, 3 and 4). The presence of numerous abnormal enlarged or giant mature megakaryocytes with hyperlobulated nuclei and preserved nuclear/cytoplasmic ratio or the presence of pleiomorphic small and enlarged or giant megakaryocytes with hypolobulated cloud-like nuclei, and/or the evidence of several clusters of enlarged megakaryocytes are the pathognomonic clues to the diagnosis of prefibrotic MPD (ET, PV or CIMF)[3,19-33]. The diagnostic work-up of patients with ET and thrombocythemia associated with CIMF according to ECMP criteria[28,72,73] is based on positive criteria in peripheral blood and bone marrow (figure 5). These include:

1. Thrombocythemia patients should fulfil the peripheral blood (clinical) criteria for the diagnosis of thrombocythemia irrespective of bone marrow features (tables 2 and 3).

2. The screening for $JAK2^{V617F}$ as a first intention diagnostic test is very helpful in the diagnostic work-up of patients with suspected thrombocythemia in various MPDs, but only half of ET and CIMF patients carry this mutation.

3. Pretreatment bone marrow biopsy will allow clinicians and pathologists to diagnose the early stages of MPDs including $JAK2^{V617F}$ positive and JAK2 wild type thrombocythemia. The ECMP criteria classify the PVSG-defined ET (table 1) as: true ET (table 2); early PV mimicking ET (table 4); early stage CIMF-0 or CIMF-1 without features of leuko-erythrocytosis and extramedullary hematopoiesis (table 3); and intermediate CIMF-1, 2 and 3 with features of leucoerythroblastosis[79,80] (table 7).

4. The ECMP criteria distinguish thrombocythemia in various MPDs from thrombocythemia

associated with Ph[1]-chromosome and *bcr/abl* positive chronic myeloid leukemia (CML)[81] or myelodysplastic syndromes (MDS) including the so-called 5q-syndrome, which clearly differs from refractory anemia with ringed sideroblasts and significant thrombocytosis (RARS-T) (figure 5)[82-84]. Among 9 RARS-T patients in a recent study, 6 showed the presence of JAK2^{V617F} mutation[84].

Comparing the laboratory features of JAK2^{V617F} positive (in granulocytes) and JAK2 wild type PVSG-defined ET patients in the PT-1 study showed that JAK2^{V617F} positive ET is characterized by higher values for hemoglobin, hematocrit, neutrophil counts, LAP score, by lower values for serum EPO levels, serum ferritin and MCV, and by increased cellularity of the bone marrow in biopsy material[85,86]. This observation confirms the ECMP concept that JAK2^{V617F} and EEC positive ET patients represent an early PV mimicking ET ("forme fruste" PV, stage 1 PV, table 5)[24,56,72,73]. As compared to JAK2^{V617F} positive ET (early PV), JAK2 wild type ET patients had significantly higher platelet counts, normal serum EPO levels, a typical bone marrow picture of true ET, no features of early PV, and are at lower risk for the development of thrombotic complications[85,86]. These data are in line with the hypothesis that JAK2^{V617} positive and JAK2 wild type ET patients at diagnosis represent two distinct entities with a related pathophysiology in the JAK-2/STAT signalling pathway but different molecular etiology similar to the gain of function mutation in the TPO or MPL genes causing hereditary ET[87,88].

Diagnostic work-up of patients with polycythemia vera

Suspected polycythemia (PV) with characteristic PV features include increased hematocrit (>0.51), increased erythrocytes (>6 x10^{12}/l), slight splenomegaly, increased leukocytes (>12 x10^{9}/l) or LAP score with normal ESR, increased platelets (>400 x10^{9}/l). PV patients usually show the presence of large platelet in peripheral blood smear. PV patients frequently present with headache, TIAs, erythromelalgia, splanchnic or cerebral vein thrombosis and microcytosis of erythrocytes due to iron deficiency[56]. Patients with congenital or acquired erythrocytosis lack the clinical and laboratory features of MPD are usually asymptomatic. The presence of JAK2^{V617F} has a sensitivity of about 95% and positive predictive value of 100% for the diagnosis of PV in the context of absolute erythrocytosis (hematocrit >0.51 in males and >0.48 in females) and excludes congenital and secondary erythrocytosis (figure 6)[89]. Subsequent red cell mass measurement will distinguish apparent from absolute erythrocytosis but does not differentiate between PV and congenital or secondary erythrocytosis. In contrast, bone marrow histology not only differentiates trilinear hypercellularity in PV (figures 2 and 3) from isolated increase of erythropoiesis (figure 3) in congenital polycythemia and secondary erythrocytosis, but also significantly contributes to phenotyping and staging of PV patients[59,89].

The detection of JAK2^{V617F} in granulocytes with sensitive PCR techniques as to play a key-role as a first intention diagnostic test for erythrocytosis (hematocrit >0.51), because it simplifies the diagnostic work-up of PV (table 4, figure 6)[89,90]. The presence of the JAK2^{V617F} mutation[59,89] combined with increased hematocrit (>0.51), and EEC or low serum EPO[91-93] is diagnostic for PV without the need of red cell mass measurement (table 4), but is not enough to define the broad spectrum of PV phenotypes according to EMCP criteria (table 6). Since EEC is time consuming and difficult to establish in many (non-specialized) laboratories, clinicians and pathologists tend to replace it by the wide spread available bone marrow histology assessment as a gold standard criterion for the diagnosis of PV[94,95] and its differentiation from the EMCP-defined true ET and prefibrotic or early fibrotic CIMF (tables 3 and 4, figures 2 and 3)[19,24,28,72,73].

Comparing 45 JAK2^{V617F} heterozygous and 13 homozygous PV patients showed that homozygote JAK2^{V617F} PV patients displayed significantly higher hemoglobin at time of diagnosis, increased incidence of pruritus, higher PRV-1 transcripts in their blood granulocytes, and higher rate of fibrotic transformation[96]. These observations indicate that pretreatment and follow-up bone marrow histology examinations will be helpful for proper staging of PV patients for reasons of prognosis assessment and therapeutic implications (table 6)[73]. In the context of a prospective clinical study to discriminate between early and advanced PV and to monitor disease progression to post-PV myelofibrosis, bone marrow histology, cytogenetic analysis and JAK2^{V617F} mutation detection in granulocytes, and EEC should always be performed.

Grading of myelofibrosis in myeloproliferative disorders (MPD)

Myelofibrosis (MF) itself is not a disease because reticulin and collagen fibrosis are produced by pol-

yclonal fibroblasts as the consequence of cytokines released from the clonal granulocytic and megakaryocytic proliferative cells in both PV and CIMF[19,97]. The Baumeister scoring system of MF was developed on aspirated bone marrow samples, but proved to be not reliable for the proper grading of myelofibrosis in bone marrow biopsies by pathologist (table 8)[98]. The Manoharan system used silver stain according to Gordon and Sweet and scored the degree of reticulin in bone marrow biopsy in a completely different (table 8)[99]. A scoring system based on morphometric analysis (point intersection with an ocular grid) and quality of fibers (reticulin and collagen fibers) and the bone marrow fiber density (fine or course reticulin and some or course bundles of collagen) have been proposed by Georgii et al[19,63,64] and Thiele et al[65-68]. All these different scoring systems for MF use different criteria for grading of reticulin and collagen, are subjective and not comparable by lack of strict criteria. A panel of experienced European pathologists and a USA expert reached a consensus on how to grade bone fibrosis in bone marrow biopsies of patients with CIMF or PV (EC, tables 3 and 8)[100] Grading of MF was simplified by using four easily reproducible categories that included differentiation between reticulin and collagen[100]. According to defined standardized semiquantitative grading of reticulin and collagen fibrosis in the bone marrow, MF can reliably be graded at the pathological bone marrow level as 0 in prefibrotic, as 1 in early fibrotic, as 2 in classical fibrotic and as 3 in classical sclerotic CIMF (table 3 and 8)[100]. Myelofibrosis is rare in ET and does occur in about one third of PV and in the majority of CIMF-0 patients during long-term follow-up[63,64,69,70,76].

Conclusion: The diagnosis CIMF according to WHO bone marrow features does not distinguish between CIMF-0/CIMF-1 without leuko-erythroblastosis versus CIMF-1/CIMF-2 with leuko-erythroblastosis when ECMP criteria are applied (tables 3 and 7) indicating the need to use clinical score assessment on top of bone marrow features for prognosis prediction.

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