Journal of Hematology & Thromboembolic Diseases

Michiels, et al., J Hematol Thromb Dis 2015, S1:001
http://dx.doi.org/10.4172/2329-8790.1000S1-001

Research Article Open Access

Increased Erythrocyte Count on Top of Bone Marrow Histology but not Serum EPO Level or JAK2 Mutation Load Discriminates between JAK2V617F Mutated Essential Thrombocythemia and Polycythemia Vera

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Abstract

Bone marrow histology is a powerful tool to differentiate between the myeloproliferative neoplasms (MPN) of essential thrombocythemia (ET) and polycythemia vera (PV) from all variants of primary or secondary erythrocytosis and reactive thrombocytosis with a sensitivity and specificity of 100%. Bone marrow histopathology on its own is not reliable to differentiate between WHO defined ET and PV. The majority of JAK2 mutated ET and all PV patients have increased scores for the leukocyte alkaline phosphatase (LAP) stain. The morphology of large pleomorphic megakarocytes were not different in JAK2 mutated ET, prodromal PV, and overt PV. Serum EPO level or JAK2 allele mutation load do not discriminate between ET and prodromal PV versus classical and masked PV in $JAK2^{V617F}$ positive trilinear MPN. A typical MPN bone marrow histology, erythrocytes above 5.8 × 10^{12}/L in males and 5.6 × 10^{12}/l in females (normal cut-off value is 5.5 × 10^{12}/L in females) separates overt and masked PV from ET and prodromal PV obviating the need of RCM measurement.

Keywords: Essential thrombocythemia; Polycythemia vera; Myelofibrosis; Red cell mass; Bone marrow pathology; JAK2 mutation; Myelproliferative neoplasm

Introduction

Aspirin responsive erythromelalgia and migraine-like microvascular cerebral transient ischemic attacks (MIA) are the presenting features of early stage essential thrombocythemia (ET) and polycythemia vera (PV) [1-4]. Prefibrotic stages of ET and PV are diagnosed by the combined use of clinical, laboratory and bone marrow histopathology features. The 1980 RCP criteria of ET were determined by careful prospective documentation of peripheral blood and bone marrow smears and bone marrow biopsy material. In 1980 we modified the PVSG criteria for PV by including bone marrow histology as a pathognomonic clue to myeloproliferative disease (Table 1) [1,2]. We replaced O_2-saturation of >92% by bone marrow biopsy, changed splenomegaly by bone marrow histology as a major criterion (A3), and used splenomegaly as a minor criterion (Table 1B) [1-10]. We skipped raised B12 (>900 ng/L) or raised B12 binding capacity (>2200 ng/L) as completely irrelevant for early stage PV.

Platelets in excess of 400 × 10^9/L, and an increase of clustered enlarged megakaryocytes in a bone marrow biopsy material was found to be pathognomonic diagnostic for ET and PV and excluded reactive thrombocytosis (Figure 1). The 1980 RCP criteria for PV, bone marrow histopathology and erythrocyte count above 6 × 10^{12}/L are diagnostic for PV and do exclude all variants of primary and secondary erythrocytosis (Table 1) [4-6]. The combined use of clinical and bone marrow features on top of JAK2 and MPL mutation screening significantly improved the 2006 European Clinical Molecular and Pathological (2006 ECMP) [5,6] and the 2008 WHO classification of the $JAK2^{V617}$ and JAK2 wild type MPNs ET, PV and myelofibrosis (MF) [11,12]. The WHO-ECMP criteria [4-6] for prefibrotic normocellular ET, hypercellular prodromal PV and PV combined the use blood cell counts, bone marrow histology and $JAK2^{V617F}$ mutation screening to clearly distinguish $JAK2^{V617F}$ mutated trilinear MPN entity (Tables 2 and 3) from JAK2 wild type MPN [6]. In the present prospective clinical research study of newly diagnosed ET and PV at presentation we evaluated the 1980 RCP and the WHO. ECMP features of $JAK2^{V617F}$ ET and PV patients.

Methods

Hemoglobin, hematocrit, erythrocytes, leukocytes, platelets, iron status and chemical parameters were routinely performed. Erythrocyte volume (red cell mass RCM) was measured using Cr51 (natriumchromate) labeled autologous erythrocyte and plasma volume was measures with J131-human serum albumin. The leukocyte-alkaline-

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Received April 29, 2015; **Accepted** June 17, 2015; **Published** June 30, 2015

Citation: Michiels JJ, Medinger M, Raeve HD, Schroyens W, Schelfout K, et al. (2015) Increased Erythrocyte Count on Top of Bone Marrow Histology but not Serum EPO Level or JAK2 Mutation Load Discriminates between JAK2V617F Mutated Essential Thrombocythemia and Polycythemia Vera. J Hematol Thromb Dis 3: S1-001. doi:10.4172/2329-8790.1000S1-001

1 A. The RCP Thrombocythemia (Emajor (A) and confirmative (B) criteria for prefibrotic ET [1,2]

A1 Persistent platelet count in excess of 400 × 10^9/L.
A2 Increase and clustering of enlarged megakaryocytes in bone marrow biopsy.
A3 No or slight increase of reticulin fibers (RF 0 or RF 1)
B1 Presence of large platelets in a peripheral blood smear
B2 Absence of any underlying disease for reactive thrombocytosis and normal ESR.
B3 No or slight splenomegaly on palpation or scan (<15 cm)
B4 Increase of LAP-score and no signs of fever or inflammation

Exclusion criterion

Ph+ chromosome and any other cytogenetic abnormality in blood or bone marrow cells

1 B. The RCP major (A) and minor (B) criteria for prefibrotic PV [1,2]

A1 Raised red cell mass (RCM). Male >36 ml/kg, female >32 ml/kg [7], or erythrocyte count above 6 × 10^{12}/L (Dameshek 1940-1950) [9,10]
A2 Absence of primary or secondary erythrocytosis by clinical and laboratory tests.
A3 Slight, moderate or marked increase in bone marrow biopsy of clustered, enlarged pleomorphic megakaryocytes with hyperlobulated nuclei and moderate to marked increase cellularity of megakaryopoiesis/erythropoiesis or typically trilinear mega-erythro-granulopoiesis.
B1 Thrombocythemia, persistant increase of platelet >400 × 10^9/L
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 Splenomegaly on palpation or on isotope/ultrasound scanning

A1+ A3 plus one of B establishes PV and excludes any variant of erythrocytosis.

1 C. Grading of bone marrow reticulin fibrosis (RF) according to Ellis et al. [13] and grading of myelofibrosis (MF) according to Thiele et al. [14]

Table 1: The 1980 Rotterdam Clinical and Pathological (RCP) criteria for Essential T) and Polycythemia Vera (PV) [1,2].

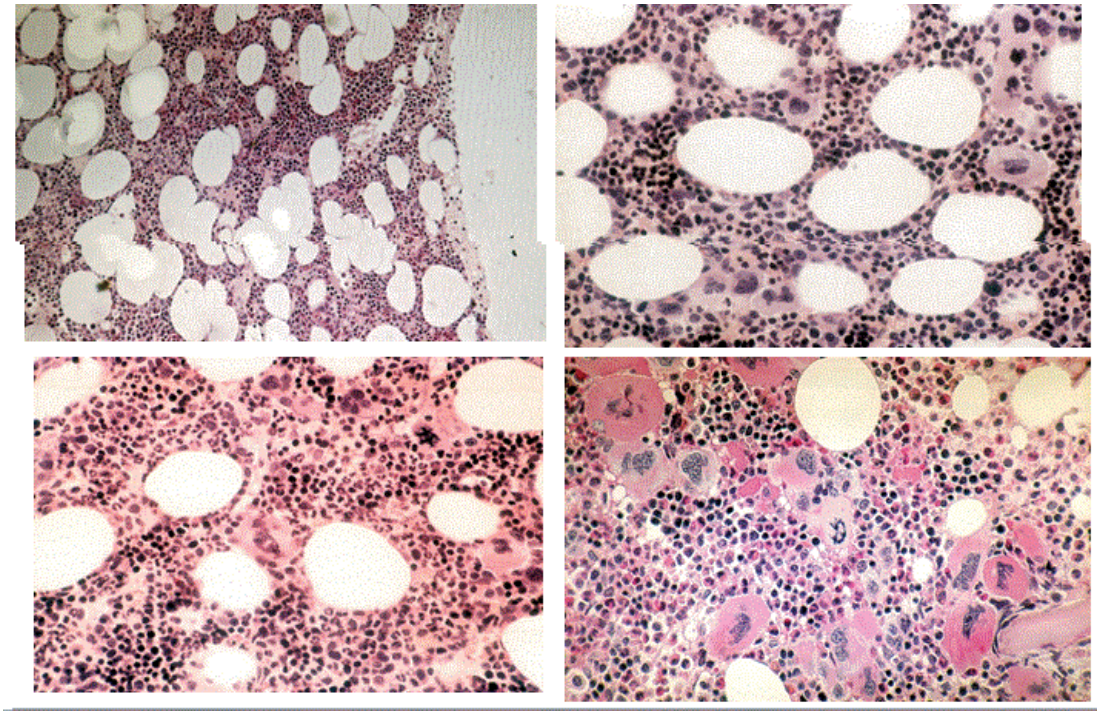

Figure 1: 1980 RCP ET (upper) , ET/PV (lower left) , and PV (lower right) bone marrow features in ET and PV patients. Pleiomorphic megakaryocytes in ET (upper panels) have less hyperlobulated nuclei as compared to PV (left bottum). The LAP score is increased in ET and PV complicated by erythromelalgia and the clustered pleiomorphic megakaryocytes in prefibrotic ET ET/PV and PV patients complicated by erythromelalgia are identical. Observations between 1975-1985 [1,2].

Grading reticulin fibrosis (RF) [1,13]	Grading MF [14]	Description of reticulin fibers (RF) [17] and reticulin/collagen fibers (RCF) in myelofibrosis (MF) as a secondary event in myeloproliferative neoplasms (MPN)
Normal RF-0	N MF 0	No reticulin fibers, occasional individual fibers or focal areas with tiny amount of reticulin fiber network
Slight increase RF 1	+ MF 0	Fine reticulin fiber network throughout much of section and no course reticukin fibers
Moderate increase RF 2	+ + MF 1	Diffuse fine reticuline network with focal collections of thick course reticulin fibers and no collagenisation
Marked increase BM dry tap RF 3	+++ RCF=MF 2	Diffuse and dense increase in reticulin with extensive intersections, and presence of collagen fibers and no or minor osteosclerosis
OS Dry tap RF 4	Sclerotic RCF&O=MF 3	Diffuse and dense reticulin with with coarse bundles of collagen associated with significant osteosclerosis (O)

Table 2: The 2015 WHO-European Clinical Molecular and Pathobiological (2015 WHO-ECMP) criteria for the classification of prefibrotic JAK2^{V617F} mutated essential thrombocythemia (ET) [5,6,28-30].

Clinical and molecular (CM) criteria	Bone marrow pathology (P) criteria (WHO)
ET	**Normocellular ET**
1. Platelet count of >350 × 10^9/l and the presence of large platelets in a blood smear 2. Presence of JAK2-^{V617F} mutation 3. Normal erythrocytes <5.8 × 10^{12}/L males, <5.6 × 10^{12}/L females 4. Normal haemoglobin (Hb) and hematocrit (ht)	Predominant proliferation of enlarged mature megakaryocytes with hyperlobulated nuclei and mature cytoplasm, lacking conspicuous morphological abnormalities. No increase, proliferation or immaturity of granulopoiesis or erythropoiesis. Reticuline fibrosis (RF) 0 or 1
Prodromal PV	ET with bone marrow features of PV
1. Platelet count of ≥350 × 10^9/l and normal ht male <0.51, female <0.48, normal erythrocyte <5.8 × 10^{12}/L males, <5.6 × 10^{12}/L females is mandatory. 2. Presence of JAK2-^{V617F} mutation 3. Low serum EPO level and/or increased LAP score 4. Spontaneous EEC.	Increased cellularity with due to icreased erytropoiesis or trilineage myeloproliferation (i.e. panmyelosis). Proliferation and clustering of small to giant (pleomorphic) megakaryocytes. Absence bone marrow features consistent with congenital polycythemia and secondary erythrocytosis. RF 0 or 1
Prefibrotic hypercellular ET	EMGM
1. Platelet count of ≥350 × 10^9/l, 2. No signs of leuko-erythroblastosis 3. Slight or moderate splenomegaly on ultrasound 4. No anemia with hb and ht in the normal low normal range: hb >13 g/dl 5. Presence of JAK2-^{V617F} mutation 6. No preceding or allied CML, PV, RARS-T or MDS.	Hypercellular ET due to chronic megakaryocytic and granulocytic myeloproliferation (EMGM) and normal or reduced erythroid precursors. Loose to dense clustering of more pleiomorphic megakaryocytes with hyperploid or clumpsy nuclei (not or some cloud-like). RF grading (Table 1C): Prefibrotic: RF- 0/1, MF-0, no/minor splenomegaly
ET stage 4, borderline anemia and LDH↑ ET stage 5 Hb <12 g/dL, LDH↑↑, CD34+	Early fibrotic ET:RF 2, MF 1, splenomegaly no/minor Fibrotic ET: RF3, RCF or MF2, overt splenomegaly
ET stage 6, Post-ET MF	**Post-ET MF: RF3/4, or MF-2/3**

Table 3a: The 2015 WHO-ECMP criteria for the diagnosis of prodromal and overt polycythemia vera (PV) and primary or secondary erythrocytoses [5,6,28-30].

Clinical and molecular (CM) criteria	Bone marrow pathology (P) criteria (WHO)
Major Prodromal PV (ET stage 2). Hematocrit upper limit of normal: Ht: 0.45 to 0.51 in male and 0.43 to 0.48 in female), Erythrocytes <5.8 × 10^{12}/L males, <5.6 × 10^{12}/L females Classical PV A 1. Hematocrit >0.51/>0.48 in male/female Erythrocytes >5.8 × 10^{12}/L males >5.6 × 10^{12}/L females A 2. Presence of heterozygous or homozygous JAK2^{V617F} or JAK2 exon 12 mutation A 3. Low serum Epo level Minor B 1. Persistent increase of platelet count: grade I: 400-1500, grade II: >1500. B 2. Granulocytes >10 × 10^9/l or Leukocytes >12 × 10^9/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)	PV. Bone marrow pathology: increased cellularity (60-100%) due to trilinear increase of erythropoiesis, megakaryopoiesis and granulopoiesis and clustering of small to giant (pleomorph) megakaryocytes with hyperlobulated nuclei. Absence of stainable iron. No pronounced inflammatory reaction (plasmacytosis, cellular debris) Erythrocytosis. Selective increase of erythropoiesis, normal granulopoiesis and megakaryocytes of normal size, morphology and no clustering in primary/secondary erythrocytosis. Grading of reticulin fibrosis (RF Table 1C) and myelofibrosis (MF, Table 1C) Erythrocythemic stage: A1 and P1 Prefibrotic: RF-0/1=MF-0 Early fibrotic: RF-2=MF-1 Fibrotic: RCF 3=MF-2 Post-PV MF: RF 4=MF-3

Table 3b: WHO bone marrow histology and CMP criteria for staging of early, overt, and advanced PV. A0, A2, B1 establish early PV (mimicking ET) prodromal PV CMP stage 0 [6,35].
A1, A2, and none of B establish erythrocythemic PV CMP stage 1 [6,35].
A1, A2, and one or more of B establish classic and advanced PV CMP stage 2 and 3 [6,35].
A2, B3 and a PV bone marrow histology detect masked cases of ET or PV in particular in cases who present with Budd-Chiari syndrome or splanchnic vein thrombosis.

phosphatase (LAP) stain was performed with Na-naphtyl and Fast Garnet G.B.C Salt. Iron stain of bone marrow smears were performed with Prussian blue reagents. Bone marrow biopsies from the iliac crest were stained with hematoxylin and eosin for histopathology evaluation. All bone marrow biopsies were evaluated by expert hematopathologists for morphology, grading of cellularity and scoring of silver stained reticulin fibers according to PVSG (Ellis et al. [13], Table 1C) and WHO recommendations [14]. On top of JAK2^{V617F} mutation screening using the PCR test according to Baxter et al. [15], and quantitative JAK2 mutation allele burden, the diagnostic work-up of ET and PV patients included serum erythropoietin (EPO) levels, bone marrow aspirate for morphology and endogenous erythroid colony formation (EEC), red cell mass (RCM) measurement according to Pearson et al. [16] and spleen size on echogram. The minimum criterion for the diagnosis of ET or thrombocythemia associated with PV is 400 × 10^9/L[1-10]. Collagen staining of Masson was used for objective detection of collagenisation of reticulin fibers to clearly distinguish between early stage reticulin fibrosis (RF) from advanced reticulin/collagen fibrosis (RCF) for grading of myelofibrosis (MF, Table 1C) according to WHO recommendations [12,14].

Results

In the period of January 1975 to December 31, 1980, we prospectively studied 30 consecutive early prefibrotic stage MPD patients (mean age 56.7 years, range 33-86), who presented with erythromelalgic thrombotic thrombocythemia, 14 ET and 16 PV patients. The clinical features of erythromelalgia and transient neurologic and ocular ischemic manifestations caused by platelet-mediated arteriolar inflammation and thrombosis in thrombocythemia in 26 MPN (ET and PV cases A in Table 4) have been reported in great detail [1-3]. Eleven of 14 ET patients had platelet counts between 400 and 1000 × 10^9/L. Spleen size on scan was slightly increased in 5 of 14 ET and in

13 of 16 PV patients. Leukocyte count counts was increased (>10 × 10^9/L) in 5 out of 14 ET and in 14 of 16 PV patients meeting the WHO criteria. LAP score was increased (>100) in 12 out of 14 ET and in all PV patients. Increase of clustered large pleomorphic megakaryocytes in bone marrow smears and biopsies was diagnostic for MPD in all 14 ET and 16 PV patients (Table 4). A normocellular bone marrow picture (cellularity <60%) with increase of clustered pleomorphic megakaryocytes and no increase of cellularity was seen in 7 of 14 ET and in one of 16 PV patients. A moderate increase of cellularity (1 ± 60-80%) in the bone marrow due to increased erythropoiesis leading to a WHO-CMP defined prodromal PV picture was seen in 3 ET and 4 PV patients. A typical trilinear PV bone marrow picture with pronounced increase of cellularity (2 ± 80-100%) due to predominantly increased megakaryo-erythropoiesis or megakaryocytic erythrocytic and granulocytic proliferation (panmyelosis according to Dameshek [9,10]) was seen in 2 of 14 ET and in 11 of 16 PV. These results indicate that bone marrow histopathology on its own is not reliable to differentiate between ET and PV but appeared to be a powerful tool to differentiate ET and PV from all variants of primary or secondary erythrocytosis and reactive thrombocytosis with a sensitivity and specificity near to 100% [5,6]. The morphology of large pleomorphic megakarocytes was not different in ET and PV. Pleomorphism of megakaryocytes became more pronounced in the hypercellular (80-100%) bone marrows with increased reticulin fibrosis (RCF) in advanced PV. The clinical manifestations and laboratory features in 50 consecutive cases of thrombocythemia, (30 ET and 20 PV) are described in great detail [16]. The peripheral blood findings in 30 ET (primary thrombocythemia; PT) and 20 PV seen between 1975 and 1985 are shown in Figure 1 [17]. Hemoglobin levels are normal in ET and elevated in PV. Leukocytes were normal or elevated in ET and PV. LAP score was much more elevated in PV than in ET (Figure 2). Out of 30 ET patients LAP score was increased in 24 and normal in 6. Platelet counts were between 400 and 1000 × 10^9/L in the majority of the 30 ET patients. Serum uric acid levels were normal in ET and usually elevated in PV (Figure 2) [17].

Between 1997 and 2014 we prospectively studied 10 early stages of $JAK2^{V617F}$ mutated MPN patients (6 ET and 4 PV), who presented with erythromelalgia or migraine-like microvascular cerebral ischemic attacks (MIA). The clinical diagnoses without the use of bone marrow histopathology (The French Approach according to Villeval, James, Pisani Casadevall and Vainchenker 2006 [18]) were ET in 6 and PV in 4 (Table 5A). The 6 ET were heterozygous for the $JAK2^{V617F}$ mutation. A typical example of hetrozygous JAK2V617F mutated ET with asymptomatic stable disease for more than 20 years is shown in Figure 3. Three PV patients were homozygous for the $JAK2^{V617F}$ mutation (case 8-10, Table 5). All ET patients had an erythrocyte count far below 6 × 10^{12}/L and all PV had erythrocyte counts above 6 × 10^{12}/L (Table 5A). Increase of erythrocytes counts above 6 × 10^{12}/L for the diagnosis of PV appears to be independent from the iron deficient status and persists in PV in a clinical remission obtained by repeated venesection (Figures 3

Number	Leukoc	**LAF**	Ery/Plasm	BM	BM	BM	Platelets	Hb	Ht	Erythroc
	10^9/L	score	volume	RF	megakar	cellularity	10^9/L	mmol/l		10^{12} /L
A1 ET PV	10	183	31 / 41	N	1+	N	792	10.4	0.49	6.7
A2 ET PV	9	155		N	1+	N	887	10	0.51	6
A3 ET	8	109		1+	2+	2+	911	8.9	0.47	5.4
A4 ET	9	101		1+			614	8	0.39	4.5
A5 ET	16	128	26 / 37	N	1+	N	939	8.3	0.4	4.4
A6 ET	7	139		N	1+	N	742	9.8	0.49	5.8
A7 ET	8	127		N	1+	N	567	9.5	0.46	5.2
A8 ET	10	38		1+	2+	1+	875	8.8	0.43	4.9
A9 ET	10	103		1+	1+	1+	690	8.8	0.45	5.5
A10 ET	11	60		N	1+	N	1440	8.6	0.43	4.7
A11 ET PV	13	113	32 / 43	2+	2+	1+	1435	9.4	0.46	6.1
A12 PV	10	207	59 / 52	1+	1+	2+	1932	11.1	0.56	6.5
A13 PV	28	193		N	2+	2+	1800	12.1	0.62	7.6
A14 PVT	*13*	*236*		*2+*	*2+*	*2+*	*952*	*8.3*	*0.45*	*5.6*
A15 PVT	*11*	*103*		*1+*	*2+*	*N*	*636*	*7.7*	*0.39*	*5.4*
A16 PV	17	243	45 / 38	1+	2+	2+	1065	13.4	0.68	7.9
A17 PV	8	186	60 / 51	1+	1+	1+	728	10.9	0.57	7.5
A18 PV	14	184	63 / 50	1+	2+	1+	1035	12.2	0.64	7.1
A19 PV	16	219	50 / 40	1+	2+	2+	1320	13.3	0.7	6.4
A20 PV	18	128	38 / 52	1+	2+	1+	1300	11.9	0.65	7.6
A21 PV	13	170	43 / 37	2+	2+	2+	1085	12.1	0.61	7.1
A22 PV	17	168	42 / 35	1+	2+	2+	708	11	0.59	7.5
A23 PV	9	219	54 / 42	1+	2+	2+	959	13.1	0.72	9.1
A24 PV	18	215	82 / 46	2+			609	12.5	0.66	9.9
B 1 PV		235	38 / 36	2+	2+	2+	2975	5.3	0.32	4.4
B 2 ET	5	20	28 / 58	1+		2+	699	8.3	0.42	4
B 3 PV	14	140	58 / 62	1+	2+	2+	918	11.3	0.38	7.3
B 4 PV	23	140	44 / 51	2+	2+	2+	2500	12.7	0.63	7.7
B 5 ET	18	118	30 / 38	1+	1+	N	810	10	0.5	5.1

Table 4: Peripheral blood and bone marrow data in 14 ET and 16 PV patients observed between 1975 and 1981, University Hospital, Dijkzigt, Rotterdam [1]. A=complicated by erythromelalgia and/or migraine-like atypical transient ischemic attacks. B=asymptomatic ET or PV. ET:L Essential Thrombocythemia. PV: Polycythemia Vera. *PVT=PV after phlebotomy.*

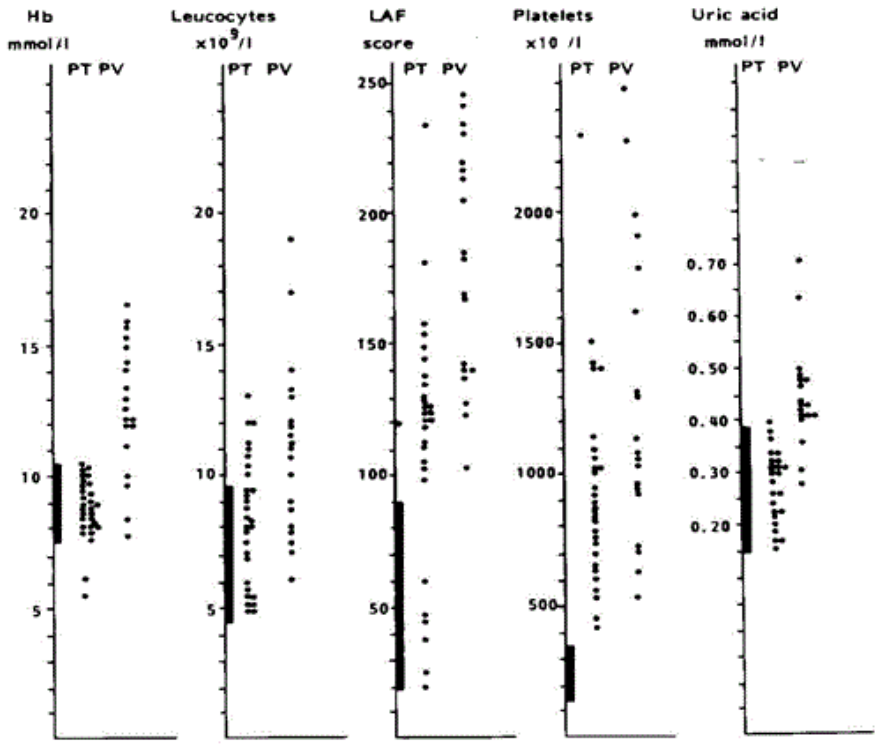

Figure 2: Laboratory findings at time of presentation in symptomatic with thrombocythemia subdivided in 30 PT (=ET) and 20 PV patients seen between 1975 and 1985 [17]. Hb levels are normal in PT and elevated in PV [16]. Leukocytes were normal or elevated in PT and PV. LAP score was much more elevated in PV than in PT with normal values in 6 PT patients. Platelet counts were were between 400 and 1000 × 10/L in the majority of the 30 PT patients. Serum uric acid levels werenormal in PT and usually elevated in PV.

Case	1	2	3	4	5	6	7	8	9	10
A. Clinical data										
Age (years) F/M	56/M	60/M	66/F	37/F	31/F	40/F	43/F	50/M	47/F	38/M
Platelets 10^9/L	575	814	544	553	576	425	405	397	924	384
Duration of MIAs	2 yrs	11 yrs	8 yrs	<1 yr	8 yrs	14 yrs	<1 yr	1 yr	<1 yr	1 yr
$JAK2^{V617F}$ *	+	+	+	+	+	+	+	+→+/+	+/+	++
Serum EPO	Normal	zero	decreased	decreasd.	decreased.	NT	decreased	zero	zero	zero
Leukocytes 10^9/l	6.7	5.3	12.9	8.2	6.2	6.1	14.3	7.3	13.1	8.0
LAP score	.	160	197	N	186	N	263	163	232	284
Hemoglobin g/dl	13.6	15.5	14.2	14.4	140	13.4	18.31	18.6	16.3	17.8
Hematocrit	0.40	0.45	0.44	0.44	0.41	0.40	0.52	0.63	0.53	0.60
Erythrocytes 10^{12}/L	4.5	5.3	4.7	4.8	4.9	4.6	6.1.	6.3	7.4	6.7
EEC	+	+	+	+	+	NT	+	+	+	+
Red cell mass ml/kg	26.7	27.1	NT	28.1	NT	24.9.	35.7	32.0	37.5	39.7
Spleen, cm Clinical Diagnosis	. ET	13 ET	16 ET	13 ET	11.8 ET	16.5 ET	13 PV	13.7 PV	14.3 PV	16 PV

Table 5A: Clinical and laboratory data, and clinical diagnosis of ET or PV $JAK2^{V617F}$ *: + is heterozygous, ++ is homozygous . NT=not tested, PV patients with documented increased RCM had erythrocytes above 6.0 × 10^{12}/L at diagnosis and at time of transition of prodromal PV into classic PV.

and 4).

The diagnosis of the 10 $JAK2^{V617F}$ positive MPN patients based on bone marrow histology alone, as blindly judged by pathologists, was consistent with ET in 3 (Table 5B) and PV in 7 cases (Table 4B). The 3 ET patients with PV bone marrow histology (Figure 2) had very low serum EPO levels and EEC, but erythrocyte counts far below 6 × 10^{12}/L consistent with the diagnosis of prodromal PV (Table 5B). These three ET patients with prodromal PV bone marrow histology developed a slow onset PV after long-term follow-up of 8, 9 and more than 10 years (Figure 3 and Table 5).

The diagnoses according to WHO-CMP criteria for diagnosis and staging of ET and PV patients (Table 5) were normocellular ET in 2, prodromal PV in 3 cases, hypercellular ET with a megakaryocytic granulocytic myeloproliferation on bone marrow histopathology

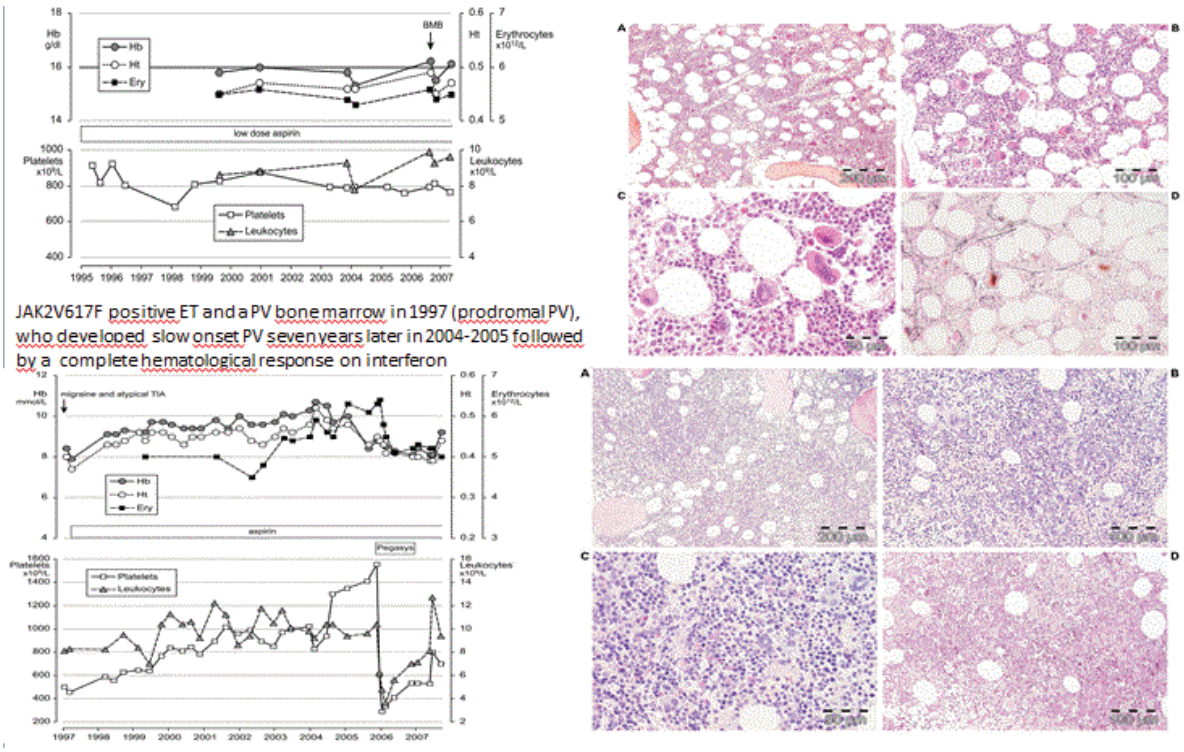

Figure 3: Heterozygous JAK2V617F positive normocellular ET with stable platelet counts during 20 years follow-up 1995-2015.

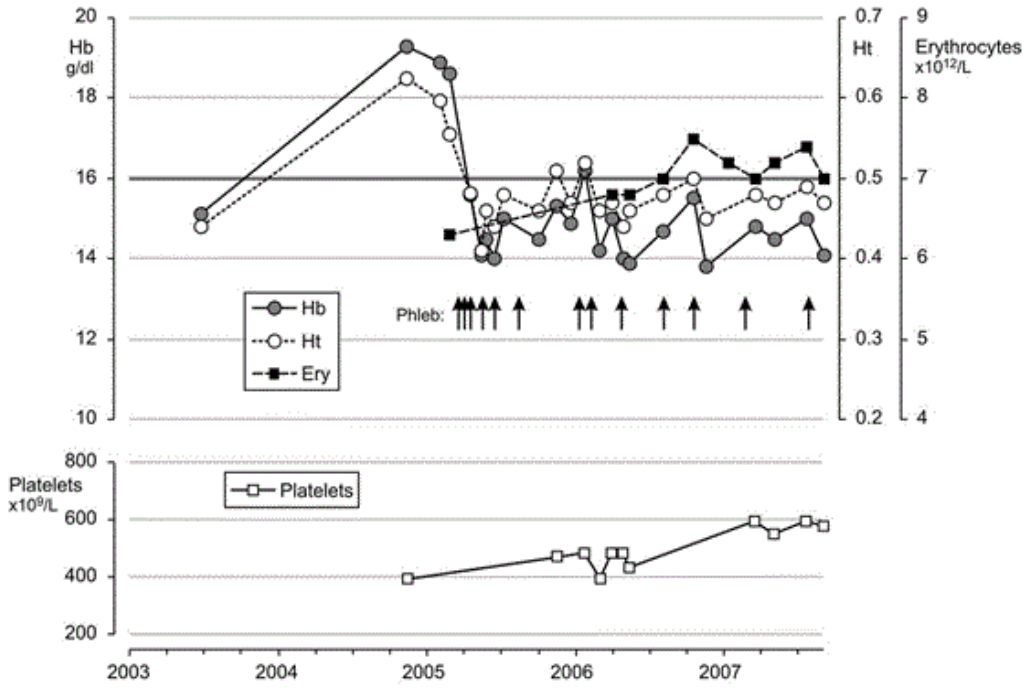

Figure 4: Clinical course of acute onset Idiopathic Erythrocythemic (IE)=PV stage zero ➔ in complete remission by phlebotomy according to Dameshek 1950 [10] on top of low dose aspirin according to Michiels et al 1985 [2].

Case	1	2	3	4	5	6	7	8	9	10
B. BM Histology		Figure 3			Figure 3				Figure 2	
Cellularity	60%	65%	90%	75%	75%	80%	75%	80%	80%	80%
M:E ratio	1	1	1	0,5	0.7	4	1	0.7	1.5	-
Megakaryocytes	MPN	MPN	MPN	MPN	MPN	MPN	MPN	MPN	MPN	MPN
Myeloid lineage	N	N	Increased	N	N	increased	N	N	increased	Increased
Erythroid lineage	Increased	Increased	Increased	Increased	increased	N	Increased	Increased	Increased	Increased
Iron	Stainable	Stainable	-	US	US	US	US	US	US	US
Fibrosis	MF-0	MF-1	MF-0	MF-0	MF-0	MF-0	MF-0	MF-0	MF-1	MF-0
BM Diagnosis	ET	ET	PV	PV	PV	EMGM [16]	PV	PV	PV	PV
WHO 2008	ET	ET	ET	ET	ET	pPMF	PV	PV	PV	PV
WHO-CMP 2014 Follow-up yrs → PV Treatment 2014	ET ET aspirin	ET Figure 3 aspirin	Pro-PV → 10 yrs PV IFN	Pro-PV → 7 yrs PV HU	Pro-PV → 11 yrs PV IFN/Phleb	EMGM [16] 'pPMF' aspirin asp	PV Figure 6 Phleb/asp	PV Phleb/asp	PV HU	PV HU

Table 5B: Bone marrow histology features diagnosis according to 2008 WHO and WHO-CMP criteria in 10 patients with JAK2V617F positive MPN at diagnosis and during long-term follow-up. M/E=myeloid erythroid ratio, US: unstainable. uc: unclassifiable. Pro-PV=prodromal PV [16].

(EMGM or masked PV) in 1, and acute onset PV in 4 patients (Table 4B). Bone marrow histopathology alone cannot distinguish JAK2^{V617F} mutated ET, prodromal PV and PV.

Bone marrow cellularity of the 10 JAK2V617F mutated MPN patients (6 ET and 4 PV) ranged from range 60% to 90% (Table 5B). There was an increased erythropoiesis in 8/10 and increased granulopoiesis in 4/10 patients with JAK2^{V617F} mutated thrombocythemia (Table 5B).

RCM, red cell counts and bone marrow histology in ET and PV

The 2008 WHO cut-of levels for the diagnosis of PV are: Hb>18.5 g/dl and Ht>0.60 in men and Hb>16.5 and Ht>0.56 in women for the diagnosis of PV with the compelling need to measure red cell mass (RCM) to distinguish JAK2^{V617F} mutated ET from PV in border line cases with hemoglobin (Hb) and hematocrit (Ht) in the upper level of normal [4-6,11,12]. In the two prospective Rotterdam studies we assessed the RCP and used the WHO-ECMP criteria related to RCM, Hb, Ht and erythrocyte counts in 10 ET and 16 PV patients in whom RCM, peripheral blood and bone marrow data were available (Table 6). The correlation curves between erythrocyte count, Hb or Ht versus RCM showed the best correlation between erythrocyte counts and RCM (Figure 5). At RCM above 30 ml/kg the erythrocytes are above 5.8 $\times 10^{12}$/L in all 19 WHO-ECMP defined PV patients (Table 6 and Figure 5). At erythrocyte counts above 5.8 $\times 10^{12}$/L the hematocrit values range from 0.46 to 0.72 in WHO-ECMP defined PV (Figure 5). At erythrocyte counts below 5.8 $\times 10^{12}$/L the hematocrit values range from 0.40 to 0.45 in WHO-ECMP defined ET who had normal RCM (Figure 5 and Table 6). At erythrocytes above 5.8 x10^{12}/L in PV patients the Hb values ranged from 15.0 to 20.9 and are below 2008 WHO criteria in 3 females and 2 males (Table 6 in blue), who had incraesed RCM. At erythrocytes above 5.8 $\times 10^{12}$/L in PV patients the Ht values ranged from 0.46 to 0.72 and are below 2008 WHO criteria but had increased RCM in 7 females and 1 male (Table 6 in blue). Seven ET patients had normal RCM at erythrocyte counts between 4.4 to 5.3 $\times 10^{12}$/L of whom 4 had WHO normocellular (<60%) ET and 3 had hypercellular (60-80%) prodromal PV bone marrow histology. The morphology of clustered medium to large megakaryocytes in bone marrow smears and biopsies were not different in ET and PV patients. Increase of erythrocytes counts above 5.8 $\times 10^{12}$/L for the diagnosis of PV appears to be independent from the iron deficient status and persists in PV in a clinical remission obtained by repeated venesection (Figure 4) thereby confirming the observations of Dameshek [9,10].

In the Basel cohort of 100 MPN patients the JAK2 status and allele burden was determined from peripheral blood samples of all included patients. Isolation of granulocytes, T lymphocytes, platelets, buccal mucosa, RNA, and DNA, as well as cDNA synthesis were performed as described (Kralovics et al.) [19,20]. The clinical, laboratory and patholgical features of 60 MPN patients subdivided in WHO defined 24 ET and 46 PV are reported in 2009 and shown in Table 7 [20]. We could correlate erythrocyte count and serum EPO levels to hemoglobin (Hb), hematocrit (Ht), platelet count and JAK2^{V617F} mutation load in these 60 evaluable MPN patients including 24 ET and in 46 PV when the 2015 WHO-ECMP criteria (Tables 2 and 3) are applied to each individual case. The JAK2^{V617F} mutation load in 24 ET patients was zero in 10 (JAK2 wild type ET) and positive in 14 ranging from 3 to 20%, from 20 to 42% and above 50% in 6, 5 and 2 cases respectively. The JAK2^{V617F} mutation load in 36 evaluable PV patients ranged from 3 to 20%, from 20 to 50% and above 50% in 5, 12 and 19 PV cases respectively. Increased erythrocyte counts above the normal level of normal (5.5 $\times 10^{12}$/L) correlated with increased red cell mass measurement in 8 PV patients and the 6 ET patients had normal values for erythrocyte count and RCM (real life observations, Table 8). In 8 JAK2^{V617F} mutated PV increased red cell mass (RCM) was associated with erythrocyte counts above 5.5 $\times 10^{12}$/L in 7 cases except one with prodromal PV (Table 7 and Figure 6). Increased RCM and erythrocytes above 5.5 $\times 10^{12}$/L was related to hemoglobin from 14.6 to 18.9 g/L, to hematocrit from 0.46 to 0.57, and platelet count between 122 to 1158 $\times 10^{9}$/L. In 6 ET patients, normal red cell mass (RCM) was related to erythrocyte counts of 4.6 to 5.4 $\times 10^{12}$/L, to Hb from 14.0 to 16.1 g/L, to Ht from 0.39 to 0.47, and platelets from 575 to758 $\times 10^{9}$/L in 5 and 1579 $\times 10^{9}$/L in 1. These findings in the Basel cohort of ET and PV patients confirm that increase of erythrocytes counts above 5.5 $\times 10^{9}$/L in males and females is related to increase RCM. The increased erythrocyte counts above the normal value of 5.5 $\times 10^{12}$/L is independent from the iron deficient status and persists in PV in a hematological remission by phlebotomy alone (Figure 4).

Discussion

Four studies showed that WHO defined elevated hemoglobin concentration cannot be used as a surrogate marker for absolute erythrocytosis in PV patients indicating the need that RCM is mandatory for patients who do not meet the WHO defined, rather crude hemoglobin and hematocrit value [21-24]. In a series of 77 consecutive patients (31 males and 46 females) with PV in the study of Johansson et al. only 35% of male and 63% of female PV patients had Hb values above 18.5 and 16.5 g/dL respectively[21]. Laboratory features at diagnosis of 266 PV and 381 ET patients diagnosed according to

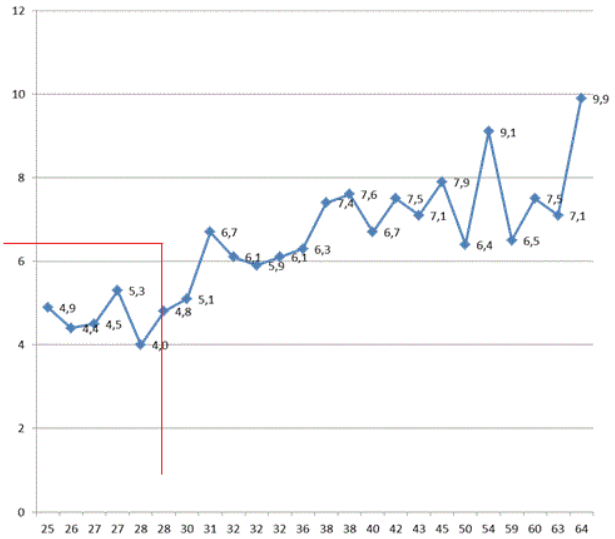

Figure 5: Erythrocyte counts x10 ^{12}/L vertical axis versus red cell mass (RCM) horizontal axis. At erythrocyte values below 5.8 × 10^{12}/L the red cell mass (RCM) values are between 25 and 30 ml/kg in ET. At erythrocyte values above 5.8 × 10^{12}/L all values of RCM area bove 30/kg in all PV cases indicating that the erythrocyte cut-off level of 5.8 × 10^{12}/L dicriminates between ET and PV (see details in Table 6) The numbers in the blue line are erythrocyte counts x10^{12}/l.

ET PV	Age M/F	Hb mmol/L	Ht	Ery x10^9/L	RCM ml/kg	Hb g/dL	Plt x10^9/L	WBC x10^9/L	BM Iron	BM histology
1 ET	56 M	8.5	0.4	4.5	27	13.6	575	7	Pos	**ET**
2 ET	46 M	8.3	0.4	4.4	26	13.2	939	16	Pos	**ET**
3 ET	60 F	9.7	0.45	5.3	27	15.5	814	7	Pos	**ET**
4 ET	37 M	8.4	0.42	4	28	13.4	699	18	pos	**ET**
5 ET	58 M	10	0.45	5.1	30	16	810	10	neg	**PV**
6 ET	47 F	8.9	0.44	4.8	28	16.3	553	8	neg	**PV**
7 ET	31 F	8.6	0.41	4.9	25	17.8	576	6	neg	**PV**
8 ET □PV	60 F	10.4	0.49	6.7	31	16.6	792	10	neg	**PV**
9 ET□PV	72 F	9.4	0.46	6.1	32	**15**	1436	13	neg	**PV**
10 ET□PV	44 F	10.5	0.49	5.9	32	16.8	1304	14	neg	**PV**
1 PV	43 F	10.8	0.52	6.1	32	17.2	405	14	neg	**PV**
2 PV	50 M	11.6	**0.63**	6.3	36	18.5	397	7	neg	**PV**
3 PV	47 F	10.2	0.53	7.4	38	**16.3**	924	13	neg	**PV**
4 PV	38 M	11.1	**0.6**	6.7	40	**17.8**	384	8	neg	**PV**
5 PV	63 M	11.1	0.56	6.5	59	**17.8**	1932	10	neg	**PV**
6 PV	60 F	13.4	**0.68**	7.9	45	21.4	1065	17	neg	**PV**
7 PV	49 F	10.9	**0.57**	7.5	60	17.4	728	8	neg	**PV**
8 PV	66 M	12.2	**0.64**	7.1	63	19.5	1035	14	neg	**PV**
9 PV	71 M	13.3	**0.7**	6.4	50	21.2	1320	16	neg	**PV**
10 PV	65 M	11.9	**0.65**	7.6	38	19	1300	18	neg	**PV**
11 PV	55 F	12.1	**0.61**	7.1	43	19.3	1085	13	neg	**PV**
12 PV	59 F	11	**0.59**	7.5	42	17.6	708	17	neg	**PV**
13 PV	74 F	13.1	**0.72**	9.1	54	20.9	959	9	neg	**PV**
14 PV	71 M	12.5	**0.66**	9.9	64	20	609	18	neg	**PV**
15 PV	66 F	9.5	**0.51**	6.7	33	**15.2**	646	18	neg	**PV**
16 PV	44 F	10.5	**0.49**	5.9	32	16.8	1302	14.5	neg	**PV**

At RCM above 30 ml/kg (Red) the erythrocytes are above 5.8 × 10^{12}/L=PV (Red).Of 10 ET cases 7 had ET and 3 had PV with erythrocytes above 5.8 × 1012/L (**Bold**)
At erythrocytes above 5.8 × 10^{12}/L Hb ranges from 15.0 to 20.9 and are below WHO criteria in 3 females and 2 males (Blue)
At erythrocytes above 5.8 × 10^{12}/L the Ht ranges from 0.46 to 0.72 and are below WHO criteria in 7 females and 1 male (Blue)
For further interpretation of these data see Figure 6. Laboratory features in 5 cases of Jak2 wild type polycythemia or idiopathic erythrocytosis (IE)

Table 6: The relation between RCM, erythrocyte count and bone marrow histology findings at time of diagnosis in 10 ET and 16 PV. Three ET cases could be diagnosed as PV according to the 2008 ECMP criteria. PV cases not meeting the crude 2008 WHO cut-of levels (hemoglobin (Hb) and hematocrit (Ht) for PV: Hb >18.5 g/dl and Ht >0.60 in men and Hb>16.5 and Ht >0.56 in women) for the diagnosis of PV [11,12] are indicated in blue.All PV cases had increased red cell mass (RCM) and increased erythrocytes above 6 × 10^{12}/L (red) meeting the 1980 RCP and the ECMP criteria for the diagnosis of classical polycythemia vera (PV).

Case	Erythrocytes g/l	Hb*10^9	Ht	Platelets percentage (%)	BM cellularity BM Iron*10^{12} Pos/Neg	
1	6.4	21.1	0.61	425 (75%)	Pos	IE
2	6.2	20.9	0.59	266 (65%)	Neg	IE
3	5.6	16.6	0.48	245 (50%)	Pos	IE
4	6.0	20.3	0.52	477 (40%)	Pos	IE
5	5.9	15.1	0.45	**1319 (85%)**	Neg	**MPN**

Table 7: The Basel data in 13 cases with documented MPN in bone marrow biopsy and 5 case of erythrocytosis: direct comparison and erythrocytes, serum EPO and RCM.

A. Laboratory features at diagnosis of PVSG defined PV and ET patients from a nationwide survey of 647 patients wit chronic myeloproliferative disease in Japan [24] PVSG diagnosis according to Pearson et al.		
	PV	**ET**
Number of patients	266	381
Erythrocytes x10^{12}/L	6.61+1.03 (5.6-7.6)	4.74+0.79 (3.9-5.3)
Hemoglobin g/dL	18.0+2.3	13.6+1.9
Hematocrit, %	55.6+6.4	41.8+5.6
Platelets x10^9/L	531+332	1063+434
White blood cells x10^9/L	13.5+7.3	11.3+4.2

B. Laboratory features of initial PV who did not meet WHO criteria with the presence of clustered pleiomorphic larged megakaryocytes and increased bone marrow cellualarity (60-80%) diagnosed by Thiele et al. [25] as ET mimicking PV in 23 patients. In this study, the erythrocyte counts were above 6 × 10^{12}/L in males and above 5.5 in the majority of females consistent with the diagnosis of PV when WHO-ECMP criteria are applied (Table 3)			
	Gender	**Initial PV**	**Normal values**
Erythrocytes, x10^{12}/L	Males	7.2 (6.1-8.6)	4.5-5.9
	Females	6.5 (5.2-7.6)	4.0-5.5
Hemoglobin, g/dL	Males	17.8 (17.0-18.3)	13.2-16.4
	Females	15.6 (15.0-16.4)	11.6-15.0
Hematocrit, %	Males	53.4 (50.3-60.4)	0.40-0.50
	Females	50.0 (43.0-58.0)	0.35-0.45
Leukocytes, x10^9/L		14.2 (6.0-17.3)	4.0-10.0
Thrombocytes, x10^9/L		780 (608-1,260)	140-360
LAP		193 (85-391)	10-80
Spleen palpation cm below costal margin		2.0 (0-3.9)	Not palpable

A. Laboratory features at diagnosis of PVSG defined PV and ET patients from a nationwide survey of 647 patients wit chronic myeloproliferative disease in Japan [24] PVSG diagnosis according to Pearson et al.

	PV	**ET**
Number of patients	266	381
Erythrocytes x10^{12}/L	6.61+1.03 (5.6-7.6)	4.74+0.79 (3.9-5.3)
Hemoglobin g/dL	18.0+2.3	13.6+1.9
Hematocrit, %	55.6+6.4	41.8+5.6
Platelets x10^9/L	531+332	1063+434
White blood cells x10^9/L	13.5+7.3	11.3+4.2

B. Laboratory features of initial PV who did not meet WHO criteria with the presence of clustered pleiomorphic larged megakaryocytes and increased bone marrow cellualarity (60-80%) diagnosed by Thiele et al. [25] as ET mimicking PV in 23 patients. In this study, the erythrocyte counts were above 6 × 10^{12}/L in males and above 5.5 in the majority of females consistent with the diagnosis of PV when WHO-ECMP criteria are applied (Table 3)

	Gender	**Initial PV**	**Normal values**
Erythrocytes, x10^{12}/L	Males	7.2 (6.1-8.6)	4.5-5.9
	Females	6.5 (5.2-7.6)	4.0-5.5
Hemoglobin, g/dL	Males	17.8 (17.0-18.3)	13.2-16.4
	Females	15.6 (15.0-16.4)	11.6-15.0
Hematocrit, %	Males	53.4 (50.3-60.4)	0.40-0.50
	Females	50.0 (43.0-58.0)	0.35-0.45
Leukocytes, x10^9/L		14.2 (6.0-17.3)	4.0-10.0
Thrombocytes, x10^9/L		780 (608-1,260)	140-360
LAP		193 (85-391)	10-80
Spleen palpation cm below costal margin		2.0 (0-3.9)	Not palpable

Table 8: WHO-CMP criteria for PV including increased erythrocyte counts.

Pearson et al. [16] from a nation-wide survey of 647 patients with chronic myeloproliferative disease in Japan are summarized in Table 8 [24]. In this study of 266 PV patients with increased red cell mass, erythrocytes counts were in the range of 5.6 to 7.6 × 10^{12}/L (mean 6.61 × 10^{12}/L). All PV patients had increased erythrocytes above the normal value of 5.5 × 10^{12}/L in 100%, whereas hemoglobin was above 18 gm/dL in 50%, hematocrit above 0.55 in 46%, and decreased serum EPO (<3.3 u/mL) in 94% [24]. The corresponding values in 381 PVSG defined ET patients had completely normal values for 4.74 + 0.79 × 10^{12}/L (range 3.9-5.3 × 10^{12}/L), hemoglobin and hematocrit. The platelet counts in PVSG defined ET and PV of 531 + 332 × 10^{9}/L and 1063 ± 434 × 10^{9}/L are significantly different. A PV bone marrow histology with clustered pleiomorphic large megakaryocytes and increased of erythropietic cellularity (60-80%) has been observed by Thiele in cases of so-called initial (latent) PV with thrombocythemia at platelet counts between 600 × 10^{9}/L and 1260 × 10^{9}/L mimicking WHO ET [25]. The laboratory data of 23 cases diagnosed as initial (latent) PV did not meet the PVSG and WHO defined levels of hemoglobin and hematocrit required for diagnosis of PV [25], but did meet the WHO-CMP criteria for PV including increased erythrocyte counts above 5.8 × 10^{12}/L in men and above 5.6 × 10^{12}/L in female (Table 8) [25].

The present study demonstrates that erythrocyte count at a cutoff level of 5.8 × 10/^{12}L in males and 5.6 × 10/^{12}L in females differentiates ET and prodromal PV from classical PV (Tables 6, 7, Figures 5 and 6) obviating the need to measure RCM in JAK2^{V617F} and exon 12 mutated patients. It is the degree of erythrocythosis on top of characteristic bone marrow histology, increased LAP score and decreased serum EPO levels that distinguishes WHO JAK2^{V617F} mutated classical PV from ET and prodromal PV and in particular also from the JAK2 wild type MPN phenotypes carrying the MPL or calcireticulin (CALR) mutation (Figure 7) [26-30]. The reduction in iron reserve in PV leads to an insufficient amount of iron for the synthesis of haemoglobin in the developing red cells, and as a result that bone marrow iron stain is negative in PV [10,26]. JAK2 wild type ET lacks features of PV at the clinical laboratory and bone marrow level [27-30]. As iron deficiency develops in PV on treatment with phlebotomy, the mature red cells produced are smaller than normal and occupy less room in the circulation, which is associated with the relief of hypervolemic symptoms [10,26]. The haemoglobin and hematocrit levels remain low for periods of months to years in PV patients in complete haematological remission by phlebotomy alone, but the erythrocyte count persist to remain above 5.8 × 10^{12}/L (Figures 5 and 6) [10,26]. As the mean corpuscular volume of red cells becomes reduced to levels below 70 cubic micron due to the chronic iron deficiency state, the discrepancy between the high red cell count far above 6 × 10^{12}/L and low hemoglobin level becomes increasingly more striking [10,26].

Piche et al. described the bone marrow histopathology findings in 59 JAK2^{V617F} positive ET and 44 JAK2 wild ET cases [27]. These original observations confirm our findings in the present study that ET patients with JAK2^{V617F} mutation indeed have PV-like morphological bone marrow changes of pleomorphic large megakaryocytes similar to our findings in WHO-CMP defined ET, prodromal PV patients and PV patients [6,28-30]. At time of first presentation symptomatic JAK2V617F positive ET and prodromal PV usually have platelet count between 400 and 1000 × 10^{9}/L, low serum EPO, increased LAP score, and slight to moderate increased bone marrow cellularity due to increased erythropoiesis. Increase of bone marrow erythropoiesis, granulopoiesis and serum LDH levels and spleen size are more pronounced in JAK2^{V617F} positive ET in particular at higher JAK2^{V617F} mutation load but clusters of large and giant megakaryocyte with 'staghorn' nuclei are rare [27].

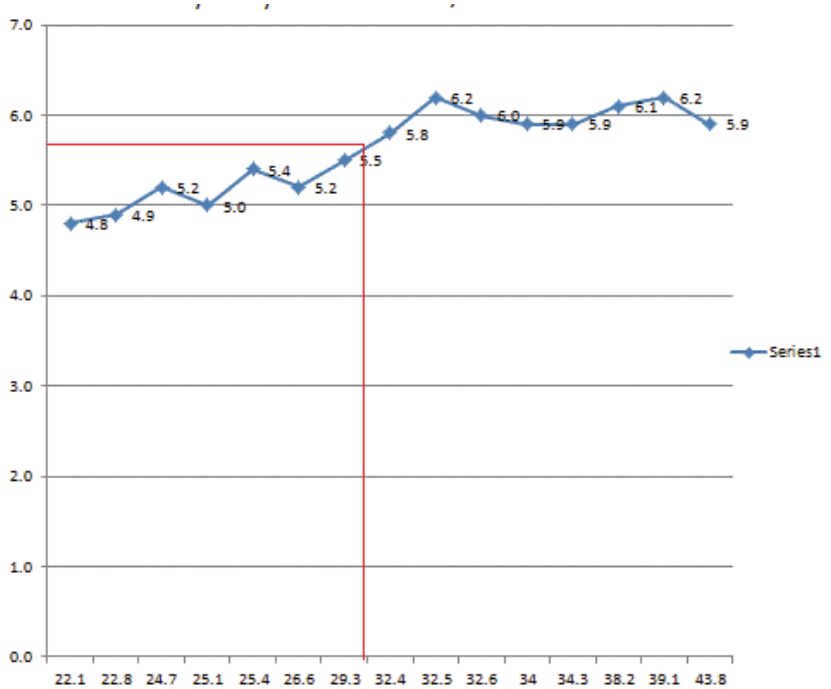

Figure 6: Erythrocyte counts x10 ^{12}/L vertical axis versus red cell mass (RCM) horizontal axis. At erythrocyte values below 5.5 × 10^{12}/L the red cell mass (RCM) values are between 25 and 30 ml/kg in ET. At erythrocyte values above 5.5 × 10^{12}/L all values of RCM area bove 30/kg in all PV cases indicating that the erythrocyte cut-off level of 5.5 × 10^{12}/L discriminates between ET and PV (see details in Table 7). The numbers in the blue line are erythrocyte counts x10^{12}/l.

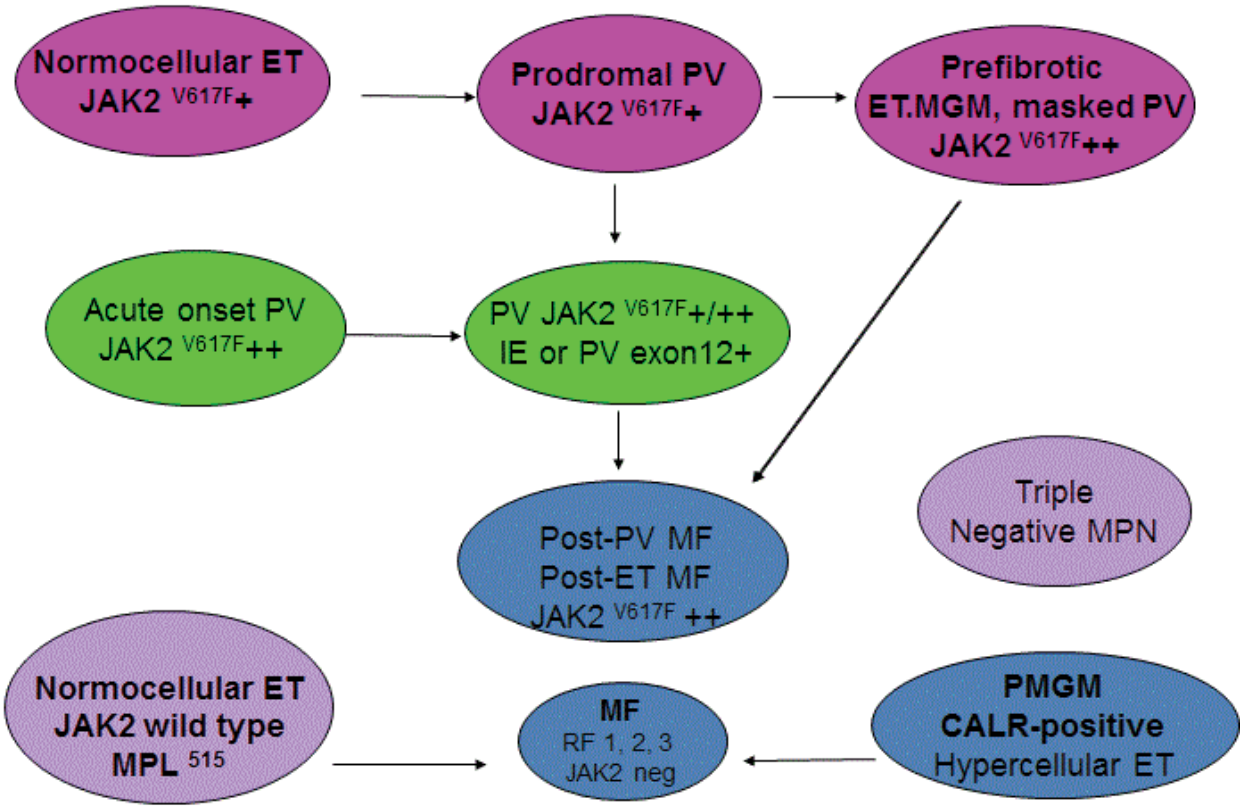

Figure 7: WHO-CMP Classification of five distinct clonal Myeloproliferative Neoplasms (MPNs) and Transitional states according to Michiels et al. [6,28-30].

In conclusion, a typical MPN bone marrow histology, erythrocytes above 5.8×10^{12}/L in males and 5.6×10^{12}/l in females (normal cut-off value is 5.5×10^{12}/L in females) separates overt and masked PV from ET and prodromal PV obviating the need of RCM measurement. The superiority of 2015 WHO-CMP [28-30] above the 2008 WHO [11,12] criteria is that the 2015 WHO-CMP clearly distinguish within the JAK2^{V617F} mutated MPN normocellular ET (WHO-ET), hypercelluar ET due to increased erythropoiesis (prodromal PV) and ET with hypercellular megakaryocytic-granulocytic myeloproliferation (EMGM or masked PV) from overt and advanced PV with splenomegaly and bone marrow reticulin fibrosis (Table 3 and Figure 7). With the advent of molecular screening for JAK2, MPL and CALR since 2013 at least four main types of clonal myeloproliferative neoplasm (MPN) can be distinguished (Figure 7) [28-30]. First, JAK2^{V617F}-positive ET, prodromal PV and masked PV, slow onset PV and rapid onset PV. Second, JAK2 wild type ET and myelofibrosis (MF) carrying the MPL515. Third, JAK2 wild calreticulin (*CALR*) mutated ET and MF. Fourth, a small proportion of ET and MF patients are JAK2, MPL/CALR wild type.

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This article was originally published in a special issue, **Epigenetics in Hematology** handled by Editor(s). Dr. Adel Gouri, Badji Mokhtar University; Algeria

Citation: Michiels JJ, Medinger M, Raeve HD, Schroyens W, Schelfout K, et al. (2015) Increased Erythrocyte Count on Top of Bone Marrow Histology but not Serum EPO Level or JAK2 Mutation Load Discriminates between JAK2V617F Mutated Essential Thrombocythemia and Polycythemia Vera. J Hematol Thromb Dis 3: S1-001. doi:10.4172/2329-8790.1000S1-001