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Review Article

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Novel Clinical, Laboratory, Molecular and Pathological (2018 CLMP) Criteria for the Differential Diagnosis of three Distinct JAK2, CALR and MPL Mutated Myeloproliferative Neoplasms: The Role of Driver Mutation Analysis and Bone Marrow Histology

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Abstract

The broad spectrum of JAK2^{V617F} mutated trilinear phenotypes varies from essential thrombocythemia (ET), prodromal polycythemia vera (PV), masked PV, erythrocythemic PV, classical PV, and PV complicated by splenomegaly and myelofibrosis (MF). ET heterozygous for the JAK2^{V617F} mutation is associated with normal life expecancy. JAK2^{V617F} mutation load increases from low to 40% in ET, from below to above 50% in early stage PV and above 50% up to 100% in overt and advanced PV and MF. Pretreatment bone marrow morphology and cellularity distinguish JAK2^{V617F} mutated trilinear MPN from calreticulin (CALR) and MPL mutated MPN. The morphology of clustered mature enlarged pleomorphic megakaryocytes with hyperlobulated nuclei are similar in JAK2^{V67F} ET and PV patients. MPL515 mutated thrombocythemia is featured by monolinear proliferation of large to giant mature megakaryocytes with hyperlobulated nuclei in a normocellular or hypocellular bone marrow. CALR mutated thrombocythemia shows characteristic bone marrow features of primary dual megakaryocytic granulocytic myeloproliferation (PMGM) in a normocellular or hypercellular bone marrow without features of PV. JAK2^{V617F}, CALR and MPL^{515} allele burden slowly increases to values around 50% together with the degree of splenomegaly, myelofibrosis and constitutional symptoms during life long follow-up. Natural history and life expectancy relate to the degree of splenomegaly, bone marrow fibrosis, anemia and the acquisition of epigenetic mutations at increasing age predict unfavorable outcome in JAK2^{V617F}, CALR and MPL^{515} mutated MPN. Low dose aspirin in JAK2^{V617F} mutated ET and PV and phlebotomy on top of aspirin in PV is mandatory to prevent platelet-mediated microvascular circulation disturbances. Pegylated interferon is the first line myeloreductive treatment option in prodromal and early stage JAK2^{V617F} mutated PV and in CALR and MPL mutated thrombocythemia to postpone the use of hydroxyurea and ruxolitinib as long as possible.

Keywords: Myeloproliferative Neoplasms; Essential Thrombocythemia; Polycythemia vera; Primary Megakaryocytic Granulocytic Myeloproliferation; Myelofibrosis; JAK2^{V617F} mutation; MPL^{515} mutation; Calreticulin Mutation; JAK2 Wild Type; Bone Marrow Pathology

Introduction

The combination of plethoric appearance, splenomegaly, erythocyte count above 6×10^{12}/L, elevated platelet count and the presence of large megakaryocytes and panmyelosis in the bone marrow has been used by Dameshek & Henthel as diagnostic for polycythemia vera since 1940 [1,2]. Venesection aiming at a haematocrit of 0.40

is the treatment of choice in PV to relieve symptoms and control hypervolemia for several years [1-5]. The one cause origin of an unknown stimulatory or inhibitory factor according to Dameshek (1950) for PV as trilinear myeloproliferative disorder (Figure 1) has been confirmed by Vainchenker's ground breaking discovery in 2005 that the acquired $JAK2^{V617F}$ mutation is the cause of three phenotypes of myeloproliferative disorders (MPD) essential thrombocythemia (ET), PV and myelofibrosis (MF) (Figure 1) [2,3,6,7]. The $JAK2^{V617F}$ mutation induces a loss of inhibitory activity of the JH2 pseudokinase part on the JH1 kinase part of Janus kinase 2 (JAK2) [4,6,7]. This leads to enhanced activity of the normal JH1 kinase activity of JAK2, which makes the mutated hematopoietic stem cells hypersensitive to the hematopoietic growth factors thrombopoietin (TPO), erythropoietin (EPO), insulin-like growth factor-1, stem cell factor (SCF) and granulocyte colony-stimulating factor, resulting in $JAK2^{V617F}$ induced clonal trilinear hematopoietic neoproliferation in the bone marrow [3,6]. The $JAK2^{V617F}$ mutation is detectable in hematopoietic progenitor cells, endogenous erythroid colonies (EEC), platelets and granulocytes [5,6].

Dameshek (1951) speculated on an unifying theory that the various conditions of myeloproliferative syndromes are all somewhat variable manifestations of proliferative activity of bone marrow cells due to one hypothetical stimulus, which may affect the marrow cells diffusely or irregeularly resulting in overlapping myeloproliferative syndromes (Figure 1). Putting together such apparently dissimilar diseases as chronic granulocytic leukemia, polycythemia vera, agnogenic myeloid metaplasia of the spleen and megakaryocytic leukemia without features of PV was conceivable at that time but without scientific foundation (Figure 1). Dameshek (1951) recognized the existence of agnogenic myeloid metaplasia of the spleen (AMM) and megakaryocytic leukemia (ML) in patients without features of PV and left the question open whether ML belonged to the spectrum of the myeloproliferative syndromes (MPS) (Figure 1) [3,5].

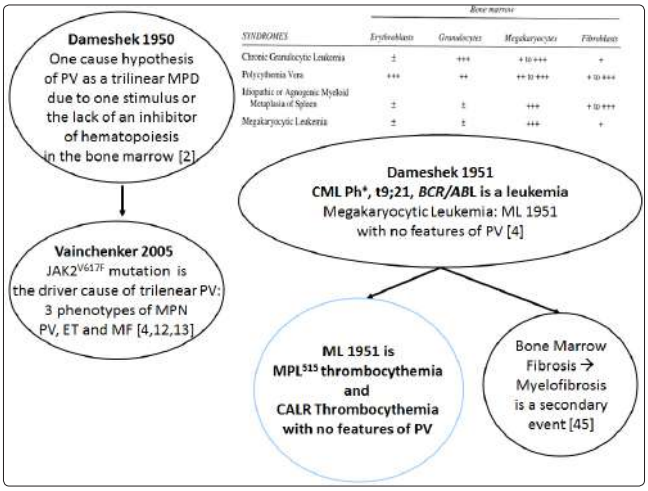

Figure 1: The one cause origin defined by Dameshek (1950) for PV as trilinear erythrocythemia (E), megakaryocytic (M) and granulocytic (G) bone marrow proliferation by an unknown factor or factors, or the lack or diminution of an inhibitory factor of bone marrow hematopoiesis has been confirmed by Vainchenker's ground breaking discovery in 2005 that the acquired $JAK2^{V617F}$ mutation is the cause of three phenotypes of myeloproliferative disorders (MPD) essential thrombocythemia (ET), PV and myelofibrosis (MF) [2,3,6,7].

Dameshek's (1951) speculated that the various conditions of myeloprolferative syndromes are due to one hypothetical stimulus resulting in overlapping myeloproliferative syndromes. Putting together chronic granulocytic leukemia, polycythemia vera, agnogenic myeloid metaplasia of the spleen and megakaryocytic leukemia without features of PV was conceivable at that time but without scientific foundation. Dameshek (1951) recognized the existence of megakaryocytic leukemia (ML) in patients without features of PV and left the question open whether they belonged to the spectrum of the myeloproliferative syndromes (MPS) [3,5]. With the advent of MPL^{515} and CALR as driver causes of thrombocythemia or ML without features of PV both MPL^{515} ET and CALR ET belong to the category of ML as defined by Dameshek in 1951.

Here we update and define the novel Clinical Laboratory, Molecular and Pathological (2018 CLMP) criteria for classification and staging of newly diagnosed MPN patients caused by JAK2, MPL and CALR driver mutations as the replacement of the 2008 and 2016 WHO MPN classifications [8-12]. We have produced good evidence that ML of Dameshek fits with the diagnosis of MPL or CALR mutated thrombocythemia without features of PV [5,8-10].

Red cell mass versus red cell count and bone marrow histology in $JAK2^{V617F}$ mutated trilinear ET and PV

The WHO criteria for PV did not include bone marrow morphology and used crude cut-off levels for hemoglobin and hematocrit (Hb >18.5 g/dl and Ht >0.60 in men and Hb>16.5 and Ht >0.56 in women). The WHO classification measured red cell mass (RCM) to separate ET from PV. MPL^{515} mutated thrombocythemia is featured by monolinear proliferation of large to giant megakaryocytes with hyperlobulated nuclei (Figure 2) [11,12]. Since 1980 we have used bone marrow histology as a pathognomic clue to distinguish all variants of MPN from reactive thrombocytosis, *BCR/ABL* positive thrombocythemia in chronic myeloid leukemia (CML), and thrombocythemia in myelodysplastic syndromes (MDS, 5q minus syndrome). The megakaryocytes in MPN are large megakaryocytes but small monolobulated megakaryocytes in CML and dysmorphic in MDS [6-9]. Megakaryocytes are identical pleomorphic in prefibrotic $JAK2^{V617F}$ positive ET and PV patients (Tables 1 and 2) and clearly different from the large mature and giant megakaryocytes with hyperlobated nuclei in MPL mutated ET (Table 3) and also differ from the large immature megakaryocytes with 'cloud-like' nuclei in CALR positive thrombocythemia (Table 4). We assessed the diagnostic value of red cell mass (RCM) related to erythrocyte count, hemoglobin (Hb) and hematocrit (Ht) to separate $JAK2^{V617F}$ mutated ET from PV in 10 ET and 16 PV patients (Tables 1 and 2). Comparing erythrocyte count and Hb or Ht versus RCM, we found the best correlation between erythrocyte counts and RCM (Figure 2). At RCM above 30 ml/kg the erythrocytes are above $5.8x10^{12}$/L which is diagnostic for PV in all 19 WHO-CMP defined PV patients. At erythrocyte counts above $5.8x10^{12}$/L, hematocrit values ranged from 0.46 to 0.72 (Figure 2). WHO defined ET had normal RCM and erythrocyte counts below 5.8 $x10^{12}$/L with hematocrit values ranging from 0.40 to 0.45 (figure 2, table 1). At erythrocytes above 5.8 $x10^{12}$/L, (diagnostic for PV), Hb values ranged from 15.0 to 20.9 g/dL and were below the 2008 WHO criteria for PV in 5 cases, who had increased RCM. Ht values ranged from 0.46 to 0.72 which are below the 2008 WHO PV criteria but 8 cases had increased RCM. Seven ET patients had normal RCM at erythrocyte counts between 4.4 to 5.3 $x10^{12}$/L of whom 4 had normocellular (<60%) ET and 3 had hypercellular (60-80%) prodromal PV bone marrow

histology [10-12]. Erythrocyte counts remain above $6x10^{12}$/L in PV in hematological remission due to iron deficiency by repeated phlebotomy alone [2,3]. Erythrocyte count above the upper limit of normal (>$5.8x10^{12}$/L in males and >$5.6 x10^{12}$/L in females on top of characteristic bone marrow histology, increased LAP score and decreased serum EPO levels appeared to be diagnostic for WHO $JAK2^{V617F}$ mutated classical PV. Erythrocyte count in the normal range separates ET and prodromal PV from classical PV (Figure 2, Tables 1 and 2) [6-9]. Bone marrow iron stain is negative in PV, but usually positive in ET [2,3,6-9]. In PV in remission by phlebotomy alone, the erythrocyte counts remain above $5.8x10^{12}$/L because the erythrocytes are becoming microcytic (MCV below 70 fl) due to iron deficiency.

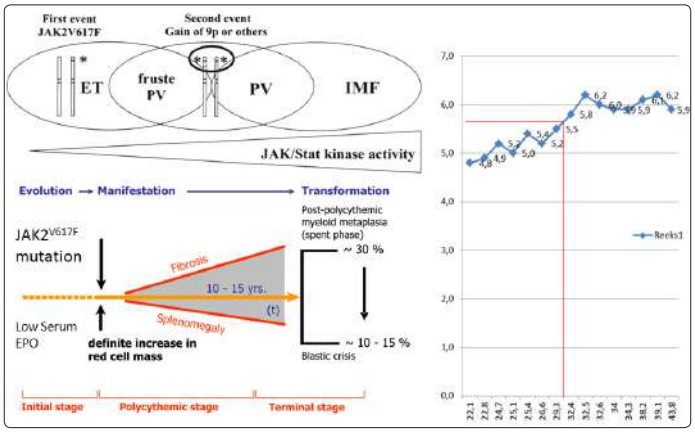

Figure 2: Upper. The discovery of the somatic $JAK2^{V617F}$ gain mutation can explain the three sequential phenotypes of ET, PV and MF. Slight increase (changes) in the V617F JAK2 kinase activity in heterozygous mutated MPN is enough to produce the clinical phenotype of essential thrombocythemia (ET), and that increasing $JAK2^{V617F}$ kinase activity is associated with early, overt and advanced polycythemia vara (PV) due to mitotic recombination resulting in heterozygous/homozygous and predominantly homozygous mutated MPN respectively (Figure 5). This concept has been confirmed to be true at the EEC bone marrow level by studies from the UK and Europe [30,31]**.**

Lower. Dynamics of the $JAK2^{V617F}$ disease processes in PV as a broad spectrum (Tables 1 and 2) ranging from normocellular ET, prodromal PV mimicking ET and the defintive increase in red cells (>$5.8x10^{12}$/L) followed by masked PV, PV complicated by fibrosis and splenomegaly, spent phase PV and blastic transformation.

Right. Initial stage of $JAK2^{V617F}$ mutated ET and prodromal PV with normal RCM and erythrocytes <$5.7x10^{12}$/L, versus manifest PV with definitive increase of RCM and erythrocytes above $5.7x10^{12}$/L.

Table 1: 2018 Clinical, Laboratory, Molecular and Pathobiological (2018 CLMP) criteria for diagnosis of $JAK2^{V617F}$ mutated essential thrombocythemia (ET) [6-9]

Clinical and molecular (CLM) criteria	Bone marrow pathology (P) criteria
Prefibrotic ET	**Normocellular ET**
1. Platelet count of >350 $x10^9$/l 2. Heterozygous JAK2-^{V617F} mutation, and low JAK2 allele mutation load 3. Normal erythrocytes <$5.8x10^{12}$/L males, <$5.6 x10^{12}$/L females 4. Hemoglobin (Hb) and hematocrit (Ht) normal or upper range of normal	Normocellular bone marrow (<60%), Megakaryocytic (M) proliferation of clustered of medium sized to large (pleomorphic) mature megakaryocytes in a normocellular bone marrow (<60%), no proliferation of erythropoiesis and granulopoiesis. Reticuline fibrosis (RF) 0 or 1
Prefibrotic prodromal PV	**ET with PV bone marrow features**
1. Platelet count of >350 $x10^9$/l. Hb and Ht in upper range of normal, but erythrocyte count <$5.8x10^{12}$/L males, < $5.6x10^{12}$/L females. 2. Presence of JAK2-^{V617F} mutation and variable JAK mutation load 3. Low serum EPO, increased LAP score	Increased cellularity (60-80%) due to increased erythrocytic, megakaryocytic (**EM**) proliferation or trilinear erythrocytic, megakaryocytic, granulocytic (**EMG**) proliferation. Increase of clustered medium sized to large (pleomorphic) mature megakaryocytes. Spontaneous EEC. RF 0 or 1
Prefibrotic hypercellular ET	**EMG, masked PV**
1. Platelet count of >350 $x10^9$/l, 2. Presence of JAK2-^{V617F} mutation and high JAK2 mutation load 3. Moderate myeloid neoplasia of the spleen → splenomegaly 4. No preceding or allied CML, PMGM, RARS-T or MDS. ..... **Clinical stage 1**: Hb and Ht in lower range of normal: hb >12 g/dl, normal LDH and CD34+ **Clinical stage 2**: anemia Hb <12 to >10 g/dL, LDH↑, and splenomegaly **Clinical stage 3**: severe anemia, Hb <10 g/dL, LDH↑↑, CD34+ , leukoerythrobastosis, tear drop erythrocytes, and large spleen	Hypercellular ET due to increased erythrocytic, megakaryocytic and granulocytic myeloproliferation (**EMG, masked PV, prefibrotic**) or increased megakaryocytic, granulocytic (**MG, fibrotic**) proliferation with relative reduced erythroid precursors. Loose to dense clustering of more pleomorphic megakaryocytes with hyperploid or clumpsy nuclei **Grading of reticulin fibrosis and MF** Prefibrotic: RF- 0/1 = MF-0, no/minor splenomegaly Early fibrotic EMGM: RF 2 = MF 1 and minor or moderate splenomegaly Fibrotic EMGM: RF3, RCF = MF2 and overt splenomegaly **Post-ET MF: RF3/4 = MF-2/3 (WHO criteria)**

Table 2: 2018 Clinical, Laboratory, Molecular and Pathological (2018 CLMP) criteria for the diagnosis of prodromal, masked and classical JAK2 mutated polycythemia vera (PV) versus primary or secondary erythrocytoses [6-9]

Clinical, laboratory, molecular (CLM) criteria	Bone marrow pathology (P) criteria
Major criteria for PV **A 1**. Erythrocytes >5.8x10^{12}/L males and >5.6x10^{12}/L females. Hemoglobin and Hematocrit upper range of normal or increased **A 2**. Heterozygous and/or homozygous JAK2^{V617F} or JAK2 exon 12 mutation **A 3**. Low serum Epo level **Confirmative criteria** **B 1**. Persistent increase of platelet count x10^9/L: grade I: 400-1500, grade II: >1500. **B 2**. Granulocytes >10 x10^9/l or Leukocytes >12x10^9/l and raised LAP-score or increased CD11b expression in the absence of fever or infection **B 3**. Myeloid metaplasia of the spleen → splenomegaly on ultrasound echogram (>12 cm length in diameter) or on palpation. **B 4**. Spontaneous endogenous erythroid colony (EEC) formation (optional)	**PV**. Increased cellularity (60-100%) due to increased erythocytic, megakaryocytic (**EM**) proliferation or trilinear erythrocytic, megakaryocytic and granulocytic (**EMG**) proliferation. Increase of clustered medium to large (pleomorphic) megakaryocytes with hyperlobulated nuclei. Absence of stainable iron. **Erythrocytosis**. Normal erythropoiesis, normal granulopoiesis and megakaryocytes of normal size, morphology and no clustering **Grading of secondary reticulin fibrosis (RF) and myelofibrosis (MF)** **Prefibrotic: RF-0/1 = MF-0** **Early fibrotic: RF-2 = MF-1** **Fibrotic: RCF 3 = MF-2** **Post-PV MF: RF 4 = MF-3**

JAK2^{V617F} mutated trilinear MPN

The JAK2^{V617F} mutated trilinear MPN phenotypic expression includes normocellular ET, prodromal PV, erythrocythemic PV with normal platelet and leukocyte count, classical PV, masked PV, and various degrees of splenomegaly and myelofibrosis (MF) [13,14]. The morphology of clustered medium to large megakaryocytes is similar in heterozygous-mutated JAK2^{V67F} ET (Figure 3, Table 1) and homozygous mutated PV patients (Figure 4, Table 2). JAK2^{V617F} ET patients are heterozygous for the JAK2 mutation with a mutation load between a few to about 40% of granulocytes [15-26]. Early stage JAK2^{V617F} PV patients are hetero-homozygous for the mutation with a load of less than 50%. PV and MF patients with advanced, long duration MPN are homozygous for the JAK2 mutation with increased JAK2 burden in 50% to 100% of granulocytes (Figure 6) [17-21]. According to the "dosage" hypothesis, heterozygosity for the JAK2^{V617F} mutation is enough to activate megakaryocytes and induce the ET clinical phenotype [23-25]. JAK2^{V617F} mutated platelets are constitutively activated, hypersensitive and cause aspirin-responsive platelet-mediated microvascular circulation disturbances, such as erythromelalgia and migraine-like atypical transient ischemic attacks (Table 3) [27-29]. According to the "dosage" hypothesis, higher intracellular levels of JAK2^{V617F} in homozygous mutated progenitor stem cells are needed to preferentially activate the erythropoietin receptor (EPOR) and generate a PV-like phenotype with erythrocytes above 5.8x10^{12}/L and slightly increased platelet count [13,16,23-25]. Homozygous JAK2^{V617F} mutated MPN is associated with extramedullary myeloid neoplasia in the spleen (MNS), splenomegaly and cytokine mediated MF (Table 4). Transition of heterozygous into homozygous JAK2^{V617F} mutation due to mitotic recombination is strongly correlated with progression of ET into PV and post-PV myelofibrosis (Figure 6) [21,25,26].

Table 3: 2018 Clinical Laboratory, Molecular and Pathological (CLMP) criteria for the diagnosis of normocelular ET carrying one of the MPL515 mutations . This entity is identical to 'true' ET as defined in 2002 by Michiels & Thiele [26]

Clinical, laboratory, molecular (CLM)	Bone marrow pathology (P)
1. Platelet count >350x10^9/L and presence of large platelets in blood smear 2. Normal hemoglobin, haematocrit and erythrocyte count 3. Presence of MPL^{515} mutation 4. Normal serum EPO 5. Normal LAP score (CD11b) 6. No or slight splenomegaly 7. No preceding or allied CML, PV, PMGM, RARS-T or MDS Clinical staging similar as in CALR thrombocythemia based on the degree of anemia, splenomegaly and myelofibrosis	**Megakaryocytic (M)** proliferation in a normocellular (<60%) bone marrow featured by large to giant mature megakaryocyte with hyperlobulated, staghorn-like nuclei. No increase of erythropoiesis, and granulopoiesis No or slight increase in reticulin RF0/1 Grading of reticulin fibrosis (RF) and myelofibrosis (MF) similar as described for CALR thrombocythemia

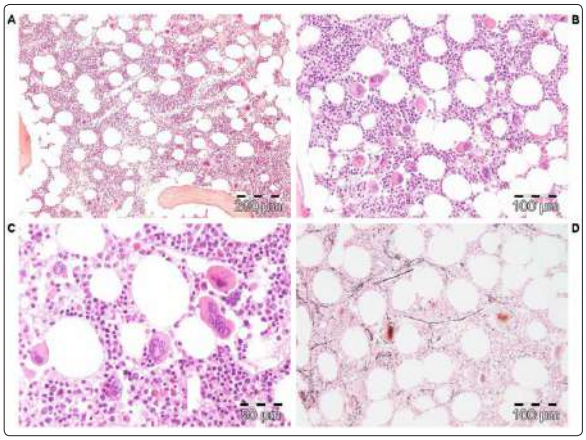

Figure 3: Typical JAK2^{V617F} heterozygous ET bone marrow histology with increase and clustering of mature pleomorphic megakaryocytes with hyperlobulated nuclei in a normocellular bone marrow with slight increased of erythropoiesis and no increase of reticulin fibers.

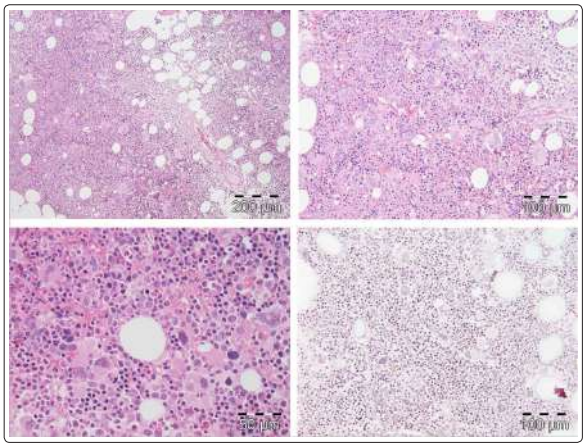

Figure 4: Typical JAK2^{V617F} homozygous PV bone marrow histology with increased cellularity (90-100%) due to increased erythocytic, megakaryocytic **(EM)** proliferation versus trilinear erythrocytic, megakaryocytic and granulocytic **(EMG)** myeloproliferation. Increase of clustered medium to large (pleomorphic) megakaryocytes with hyperlobulated nuclei.

Table 4: 2018 Clinical Laboratory, Molecular and Pathological (CLMP) criteria for hypercellular ET associated with primary megakaryocytic, granulocytic myeloproliferation (PMGM) caused by calreticulin (CALR) mutations

CM criteria CALR thrombocythemia (ET)	Pathological (P) criteria of CALR MPN
A1 No preceding or allied other subtype of myeloproliferative neoplasm PV, CML, MDS. The main presenting features is pronounced isolated thrombocythemia with platelet count around or above $1000x10^9$/L A2 CALR mutation and JAK2 wild type **C Clinical stages of CALR Thrombocythemia** C 1. Early clinical stage: Hb >12g/dL, slight to moderate splenomegaly, thrombocytosis around or above $1000x10^9$/L, normal LAP score C2. Intermediate clinical stage: slight anemia Hb <12 to >10 g/dL, decreasing platelet count, splenomegaly, increased LDH and definitive tear drop erythrocytes C3. Advanced stage: anemia Hb <10 g/dL, tear drop erythrocytes, increased LDH, increased CD34+ cells, pronounced splenomegaly, normal or decreased platelet counts, leucocytosis or leukopenia.	Dual megakaryocytic granulocytic (**MG**) proliferation and relative or absolute reduction of erythropoiesis and erythroid precursors. Abnormal dense clustering and increase in atypical medium sized, large to giant immature megakaryocytes containing bulbous (cloud-like) hypolobulated nuclei and definitive maturation defects No features of PV in blood and bone marrow **MF grading reticulin fibrosis (RF), myelofibrosis (MF)** MF 0 Prefibrotic CALR MG, no reticulin fibrosis RF 0/1 MF 1 Early fibrotic CALR MG slight reticulin fibrosis RF 2 MF 2 Fibrotic CALR MG increase RF grade 3 and slight to moderate collagen fibrosis MF 3 Advanced fibrotic CALR MG with collagen fibrosis-osteosclerosis

Godfrey *et al* studied the genotype of individual BFU-E in 29 $JAK2^{V617F}$ mutated ET and 30 $JAK2^{V617F}$ mutated PV patients expressed as a percentage (%) of EEC colonies genotyped as homozygous (red), heterozygous (purple) or wild type (white in figure 5) [30]. All 29 $JAK2^{V617F}$ positive ET patients have heterozygous JAK2 mutated EEC colonies: 9 of them have a low percentage (<10%) and 1 has 20% of homozygous colonies. Out of 30 $JAK2^{V617F}$ positive PV patients, 8 have heterozygous JAK2 mutated EEC, 13 have homozygous EEC colonies of more than 50% and 7 of less than 50% (Figure 5). Homozygous EEC colonies were absent or rare in heterozygous ET, but prevalent in $JAK2^{V617F}$-positive PV [30], which is completely in line with the "dosage" hypothesis [23-25]. A small number of PV patients harbored a major homozygous-mutant clone that was 8-85 times the size of minor homozygous subclones in the same patient. In real field medicine, the JAK2 mutation load (in percentages of JAK2 mutated granulocytes) in the study of Rumi *et al* was low in granulocytes of 250 ET patients (median 18%), significantly higher in granulocytes of 212 PV patients (median 42%) and 18 post-ET myelofibrosis (median 42%) and predominantly high (above 50%) in granulocytes of post-PV myelofibrosis (median 93%) patients) [31]. A $JAK2^{V617F}$ allele burden in granulocytes above 50% (homozygous) was recorded in only 2% of 250 ET, in 41% of 212 PV, in 72% of 18 post-ET and in 93% of 55 post-PV patients [31].

Diagnosis by detection of $JAK2^{V617F}$ and increased erythrocytes is diagnostic for PV and distinguishes PV from all variants of JAK2 wild type erythrocytoses [32,33]. The sensitivity of the $JAK2^{V617F}$ for PV is 95%. 5% of PV patients have a PV bone marrow histology but are $JAK2^{V617F}$ negative and carry one of the JAK2 exon 12 mutations [34-36]. JAK2 exon 12 mutated MPN presents with a typical PV bone marrow morphology and the clinical features of early stage PV or idiopathic erythrocythemia (IE) with normal leukocytes and platelets counts, no splenomegaly and normal life expectancy [34-36]. Godfrey *et al* found a low percentage of homozygosity for the JAK2 *K539L*-type and *E543del*-type exon 12 mutations. The majority of heterozygous exon 12 mutated IE and early PV were stable during long-term follow-up [30].

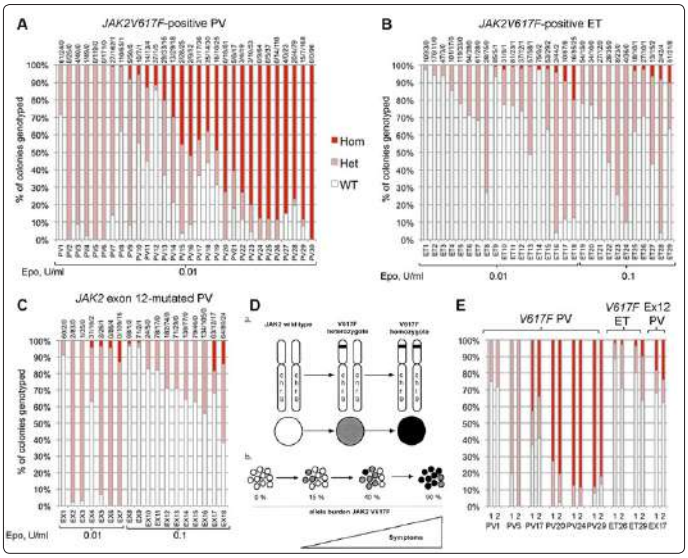

Figure 5: Proportions of $JAK2^{V617F}$ genotypes in BFU-Es from 59 patients with $JAK2^{V617F}$-mutated essential thrombocythemia (ET) and polycythemia vera (PV) (Godfrey *et al* 2012) [30]. Each vertical bar represents 1 patient, divided according to the proportion of wild-type, heterozygous, and homozygous-mutant colonies obtained, with the absolute colony numbers shown above: (wild type white), heterozygous (pink) homozygous (red). Results of EEC colony genotypes are presented for 29 $JAK2^{V617F}$-positive ET (B) patients (total 2277 colonies; mean 79 per patient) and for 30 $JAK2^{V617F}$-positive PV (A) patients (total 2287 colonies; mean 76 colonies per patient). All 29 $JAK2^{V617F}$ positive ET patients have heterozygous JAK2 mutated EEC colonies and less than 10% homozyous colonies in 9 and 20% in 1 of them. Out of 30 $JAK2^{V617F}$ positive PV patients 8 have heterozygous JAK2 mutated EEC, 13 have homozygous EEC colonies of more than 50% and 7 of less than 50%.
A. In total 29 PV patients: 5 were heterozygous, 13 heterozygous/homozygous and 11 predominant homozygous (high allele burden) for the JAK2V617F mutation.
B. In total 29 ET patients all are predomominant heterozygous (low allele burden) for the $JAK2^{V617F}$ mutation but half of them do have a minor clone of homozygous mutated BFU-Es.
C. In total 18 JAK2 exon 12 mutated PV: all are predominany heterozygous (low allele burden) for the JAK2 exon 12 mutation, but 7 of hem had a minor clone of homozygous mutated BFU-Es. Colony genotypes for 18 patients with *JAK2* exon 12-mutated PV (total 1931 colonies; mean 107 per patient (C). E show example sequence traces for patients with patients with homozygous *JAK2* exon 12 mutations in colonies. In total, 16 patients (5 "heterozygous-only" $JAK2^{V617F}$-positive PV patients, 4 $JAK2^{V617F}$-positive PV patients with homozygous and heterozygous clones, 3 $JAK2^{V617F}$-positive ET patients with small homozygous clones, and 4 JAK2 exon 12-mutated PV patients with homozygous clones) were assessed in this way (mean time between experiments, 13 months; range, 2-32 months) and showed reproducibility of proportions of heterozygous and homozygous-mutant colonies.

Acquired MPL515 mutated normocellular ET

The prevalence of the MPL^{515} mutated ET range from 3% of MPN to 8.5% of JAK2 wild type MPN [37-39]. The clinical presentation in 30 MPL^{515} mutated ET patients (9 males and 21 females, age 22-84, mean 56 years) featured major arterial thrombosis in 23%, venous thrombosis in 10%, aspirin responsive microvessel disturbances in 60%, and major hemorrhage in 7% [37]. The laboratory findings in MPL^{515} mutated ET were increased platelet count, 956±331 × 10^9/L in all, slight splenomegaly in 5 (17%), and no PV features in blood and bone marrow. Pretreatment bone marrow histology at the time of diagnosis in MPL^{515} mutated ET features large and giant megakarocytes with hyperlobulated nuclei in a normal or hypocellular bone marrow (Figure 6), clearly different from $JAK2^{V617F}$ ET (Figure 3) and PV (Figure 4). In 2015 Michiels *et al* described three main differences in bone marrow histopathology between patients with MPL^{515} mutated (N=12) versus $JAK2^{V617F}$ mutated MPN. First, the presence of clustered small and giant megakaryocytes with deeply lobulated staghorn like nuclei (figure 1) in MPL^{515} mutated ET (Figure 6) are not seen in $JAK2^{V617F}$ positive ET (Figure 3), prodromal PV, and classical PV (Figure 4) [8]. The pleomorphic medium to large megakaryocytes in $JAK2^{V617F}$ mutated ET and PV in bone marrow smears and bone marrow biopsy were comparable regarding size and degree of pleomorphy (figure 2). Second, there was local increase of erythropoiesis in areas of loose clustered pleiomorphic megakaryocytes in normocelluar $JAK2^{V617F}$ mutated ET and prodromal PV (figure 2), which is not seen in MPL^{515} mutated ET. Third, MPL^{515} mutated ET have no clinical, laboratory and bone marrow features of prodromal PV at diagnosis, do not evolve into PV during follow-up, and have normal LAP score, serum EPO and ferritin levels [3,9-11].

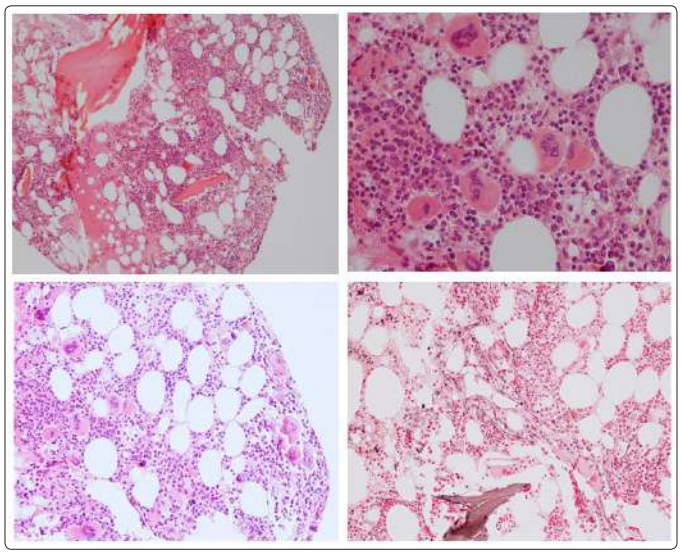

Figure 6: Bone marrow histology in a case of MPL^{515} mutated ET shows slight increased cellularity, loosely clustered mature large to giant megakaryoctyes with hypersegmented nuclei. There is no increase in erythropoiesis or granulopoiesis and slight increase in reticulin fibers without crossing-overs (RF grade 1)

CALR mutated Thrombocythemia without PV features

In 2013 Klampf *et al* first discovered the calreticulin (CALR) driver mutation as the cause of thrombocythemia in 78 of 311 (25%) ET patients and in 72 of 203 (35%) MF patients. The CALR mutation was detected in none of 382 PV, 45 CML, 73 MDS, and 64 chronic myelomonocytic leukemia (CMML) patients [40]. Three (12%) of 24 RARS-T cases were positive for both the SF3B1 and CALR mutation [40]. The Italian-Austrian study of 1235 ET and MF patients detected the $JAK2^{V617F}$, MPL^{515} and CALR mutation in 63.3%, 23.5% and 4.4% respectively with 8.8% being negative for all three mutations [31]. Evolution into MF during follow up was as high in CALR mutated ET as in JAK2 mutated PV (about 20% after 20 years). CALR mutated MPN patients lacked features of PV (normal erthrocytes and hematocrit), had higher platelet counts and a lower incidence of major thrombosis compared to JAK2 positive ET [31-40]. The UK study found somatic CALR driver mutations in 80 of 112 (70%) JAK2/MPL wild type ET patients, and in 18 of 32 (56%) JAK2/MPL wild type MF patients and in none of 120 JAK2 or MPL mutated MPN patients [41]. CALR mutations were detected in 10 of 120 (8%) MDS patients (RA in 5 of 53, RARS in 3 of 27 and RAEB-T in 2 of 27), and in one patient each with CMML and atypical CML. CALR mutations were not found in control samples, lymphoid cancers, solid tumors, or cell lines [41]. The distribution of the $JAK2^{V617F}$, CALR and MPL mutations or triple negative cases in 254 WHO-defined MF patients retrospectively analysed by Tefferi *et al* was 58%, 25%, 8.3% and 8.7% with median overall survival of 8.2, 4.1, 4.3 and 2. 5 years respectively reflecting advanced or endstage MPN disease [42].

The biological and clinical features of WHO defined $JAK2^{V617F}$ and CALR mutated ET clearly differ [31,40-44]. The mutant allele burden was lower in $JAK2^{V617F}$ mutated ET than in CALR mutated ET. $JAK2^{V617F}$ ET patients were older, had higher hemoglobin and white blood cell counts but lower platelet counts. Serum erythropoietin levels are lower and frequently decreased in $JAK2^{V617F}$ ET but normal in CALR thrombocythemia. The cumulative risk of $JAK2^{V617F}$ mutated ET to transform into PV was 29% after 15 years but no transformation into PV was observed in CALR ET. $JAK2^{V617F}$ mutated ET and PV patients had a similar two times higher risk of minor and major thrombosis than that of CALR mutated ET (thrombocythemia without features of PV) patients. A second Italian study found CALR mutations in 15.5% of 576 WHO defined ET. The CALR mutation was present in 48.9% of JAK2/MPL wild type ET patients [43]. CALR-mutated ET patients were about 10 years younger, were more frequently male, had higher platelet counts, lower hemoglobin and leukocyte count and showed a much lower risk of minor and major thrombosis than JAK2 mutated ET patients [43].

Andrikovics *et al* analysed the clinical charateristics of JAK2, CALR and MPL mutated MPN in 503 patients diagnosed as 2008 WHO defined PV (N=215), ET (N=289) and MF (N=99) [44]. All PV patients in this study were $JAK2^{V617F}$ positive. $JAK2^{V617F}$, CALR and MPL515 mutations among the 289 ET patients were found in 154 (53%), 96 (33%) and 9 (3%) respectively. Of the 99 MF patients, 56 (57%) carried $JAK2^{V617F}$, 25 (25%) carried CALR and and 7 (7%) carried MPL515. Triple negative cases were identified in 30 (11%) ET and in 11 (11%) MF patients. Comparing WHO defined $JAK2^{V617F}$ positive PV patients (N=215) versus $JAK2^{V617F}$ positive ET patients (N=154), all PV cases had increased hemoglobin by definition, lower mean platelet counts (456 vs 778x10^9/L), similar leukocyte

counts (11 vs 10x10^9/L), and higher incidences of splenomegaly (47% vs 27%), MF (13% vs 6%) and acute leukemia (8% vs 3%). Venous thrombosis was recorded in 13%, 18% and 7% of JAK2^{V617F} PV, JAK2^{V617F} ET and CALR ET patients respectively. The mean JAK2 mutation load was around 25% in ET and around to far above 50% in PV and MF patients (Figure 7). The CALR mutation load was around 35% in ET and around 50% in MF patients but did not reach values above 50% (Figure 7) [44]. The probability of overall survival in years from diagnosis was rather favorable and quite similar in JAK2 (n=150) and CALR (n=85) mutated ET patients. The probability of overall survival in years from diagnosis was significantly longer (about 10 years) in CALR mutated MF (n=21) compared with JAK2 mutated MF (n=55). It should be emphasized that CALR mutated MF patients were 12 years younger (56 years) than JAK2^{V617F} mutated MF patients (68 years) in this study [44].

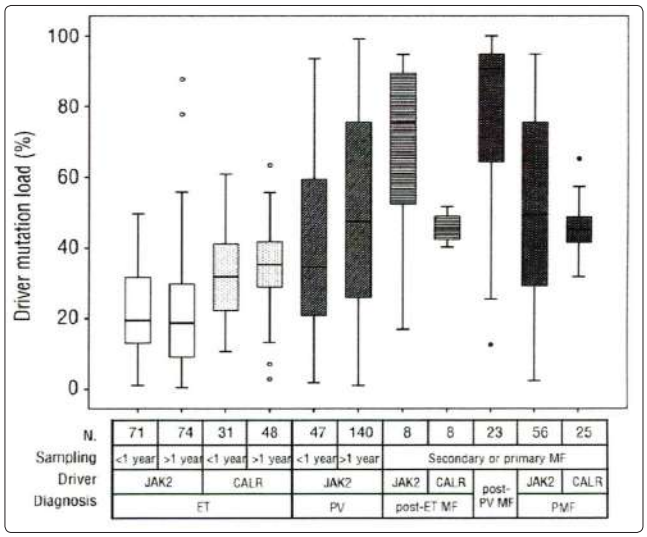

Figure 7: The driver mutation load in JAK2^{V617F} ET, in CALR ET, and in CALR myelofibrosis (MF). The driver mutation is high in JAK2^{V617F} mutated PV, post-PV myelofibrosis (MF) and primary myelofibrosis (PMF). **Courtesy of Dr Hajnalka Andrikovics**.

Bone marrow pathology of CALR mutated ET and MF

Between 1994 and 2006, Michiels *et al* documented a case of JAK2 wild type ET with a PMGM bone marrow (Table 4) in a 9-year-old boy (unpublished) with high platelet count of 1596 to 1946x10^9/l, no splenomegaly on palpation, white blood differential count (metamyelocytes 0.5%, banded forms 1%, segmented granulocytes 52%, basophiles 2.5%, lymphocytes 35% monocytes 6%), low LAP score, and thrombocythemia with a hypercellular (80-100%) PMGM bone marrow [8]. The 10 year follow-up from 1994 to 2004 featured normal blood cell counts, absence of the JAK2^{V617F} mutation, no myelofibrosis, and no splenomegaly [8]. In 2014/2015, we found typical PMGM pictures in 15 consecutive newly diagnosed CALR mutated ET and MF patients (Figure 8A and 8B. Our original observations between 2014 and 2016 in 15 consecutive CALR mutated MPN patients produced very good evidence that CALR ET patients are phenotypically identical to PMGM defined by the Michiels *et al* in 2006 and belong to the original description by Dameshek of megakaryocyte leukemia (ML) without features of PV (Figure 1) [5,10]. CALR mutated ET and MF (Table 4) are clearly dinstinct from JAK2^{V617F} ET and prodromal PV (Table 1) and classical PV (Table 2) cases with regard to clinical, hematological and bone marrow features at presentation and during follow-up.

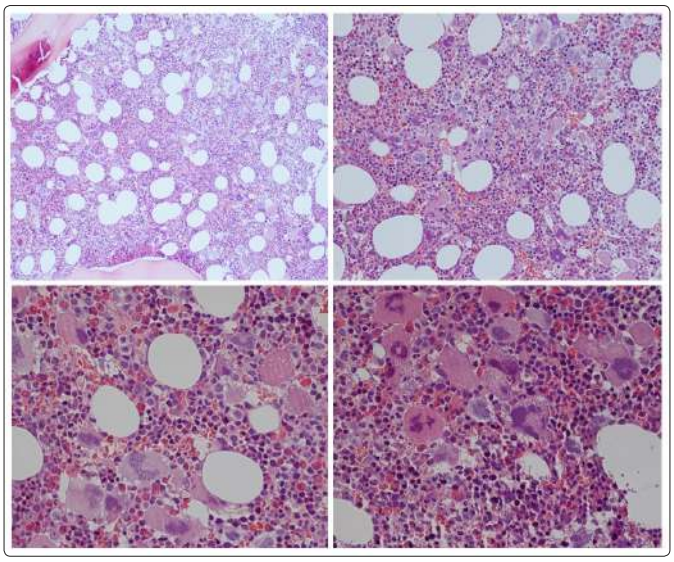

Figure 8A. Clinical case of calreticulin (CALR) positive ET who presented with aspirin responsive platelet thrombophilia (Sticky Platelet Syndrome), normal values for hemogobin, hematocrit and erythrocytes, platelet count of 1352x10^9/L and slight splenomegaly (16 cm lenght diameter on echogram). Bone marrow histology is hypercellular with relative decrease of erythropoiesis, dense cluster of large immature megakaryocytes with hypolobulated 'cloud-like' nuclei and no increase of reticulin fibrosis consistent with a typical PMGM bone marrow (Table 5) clearly distinct from JAK2^{V617F} mutated ET (Figure 3), PV (Figure 4) and MPL^{515} mutated ET (Figure 6).

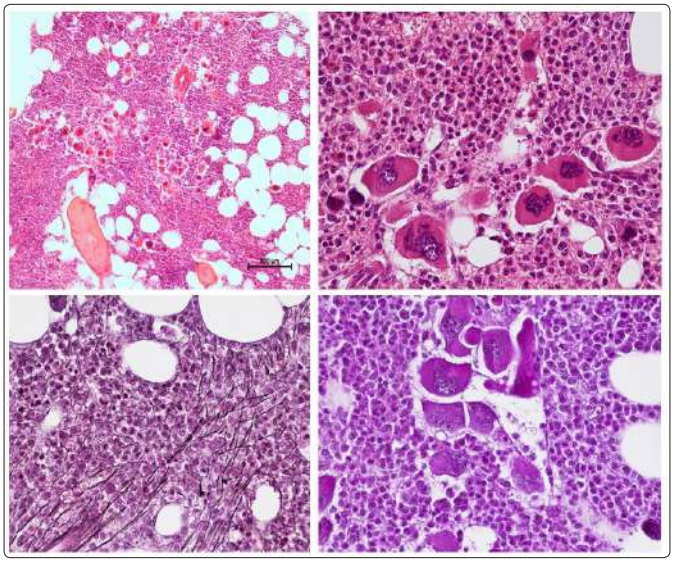

Figure 8B: Clinical case of CALR positive myelofibrosis (MF): hemoglobin 11.2 g/dL,hematocrit 0.33, leukocytes 9.2x10^9/L, platelets 347x10^9/L, LDH 1393 U/l, and the presence of tear drop erythrocytes, poikolocytosis and polychromasia in a peripheral blood smear, and hypercellular bone marrow with relative decrease of erythropoesis, dense cluster of immature megakaryocytes with hypolobulated 'cloud-like' nuclei consistent with PMGM, and reticulin fibrosis grade 2, clearly distinct from JAK2^{V617F} mutated prodromal PV and MPL^{515} mutated ET.

Secondary myelofibrosis in JAK2 mutated and JAK2 wild type MPNs

The 2008-2016 WHO classifications defined ET, PV and primary myelofibrosis (PMF) as three variants of myeloproliferative neoplasia MPD without the use of bone marrow histology [11,12]. Cytogenetic studies, isozyme markers and gene mutation studies (polymerase chain reaction: PCR) between 1969 and 1981 revealed that fibroblast proliferation in ET, PV, and PMF is polyclonal [26]. This indicates that increase of reticulin fibrosis (RF) and reticulin/collagen fibrosis (RCF, table 5) is a reactive process, whereas the hematopoietic stem cells are of clonal origin in JAK2 mutated ET, PV and MF, in CALR and MPL thrombocythemia, as well as in BCR/ABL positive ET and thrombocythemia associated with CML. Grading of bone marrow content of RF and MF according to standardized recommendations (Table 5) remains of significant prognostic importance at the time of diagnosis and during follow-up) [10,26,45-47].

Table 5: Grading of reticulin and collagen fibrosis as a secondary event in JAK2V617 trilinear MPN and in MPL and CALR mutated thrombocythemia and myelofibrosis

Grading reticulin fibrosis (RF)	WHO Grading of myelofibrosis (MF)	Description of reticulin fibers (RF) and reticulin/collagen fibers (RCF) in myelofibrosis (MF) 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 reticulin network with focal collections of thick course reticulin fibers and no collagenisation
Marked RCF 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 coarse bundles of collagen associated with significant osteosclerosis (OS)

Epigenetic factors on top of JAK2^{V671F}, MPL^{515} and CALR driver mutations in MPN

Selective expansion of one dominant homozygous subclone probably reflects additional cytogenetic, genetic or epigenetic alterations in PV and MF patients [31,46-48]. The presence of epigenetic factors on top of the JAK2, MPL and CALR driver mutations of MPN is associated with an impaired prognosis [46-48]. The targeted search for epigenetic factors has been confirmed to be of main importance in many studies to the understanding of differences in biology, prognosis and outcome of MPN patients. Using next generation sequencing (NGS) on the JAK2 or CALR mutation, Lundberg *et al* found one, two or more epigenetic somatic mutations in 65 (33%) of 197 WHO defined MPN patients (94 PV, 69 ET, 34 MF) (Figure 9) [48]. Seventeen of 69 (25%) ET patients, 11 of 34 (32%) MF and none (0%) of 94 PV patients carried mutations in CALR. In addition to JAK2^{V617F} and CALR, the most frequently observed epigenetic somatic mutations affecting the biology and natural history of MPN disease included TET2, ASXL1, DNMT3A, EZH2, and IDH1 (Figure 9) [48]. Rare epigenetic mutations were NF1, NFE2, and CUX1. The presence of one, two or more somatic mutations appeared to impair prognosis in JAK2 and CALR mutated MPN [48].

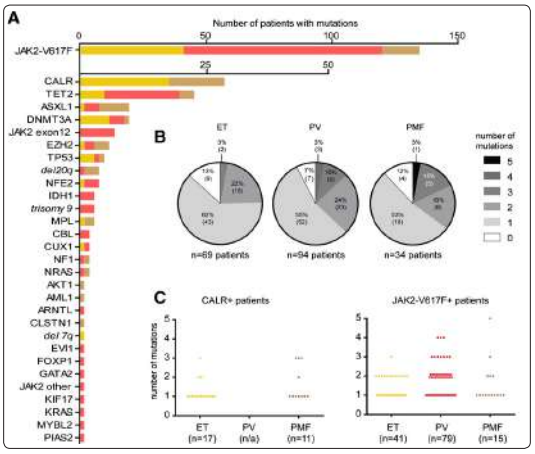

Figure 9: Distribution of somatic mutations in 197 MPN patients from the study of Lundberg *et al* (2014)[48]. None of 94 (%) PV patients, 17 of 69 (25%) ET patients and 11 of 34 (32%) MF patients carried mutations in the calreticulin (CALR) gene. After JAK2^{V617F} and CALR, the most frequently observed mutation-affected genes implicated in epigenetic regulation were TET2, ASXL1, DNMT3A, EZH2, and IDH1. Rare epigenetic mutations include NF1, NFE2, and CUX1. Recurrent somatic mutations were observed in the genes TP53, CBL, MPL, and NRAS. On top of the JAK2 or CALR mutation one additional (=2) or two (=3) or more (=4, 5) somatic mutations were found in 65 of 197 (33%) patients, which appeared to be of impaired prognostic significance (Lundberg *et al*., 2014)[48]. Number of mutations: 0=triple negative; 1= one driver mutation JAK2 or CALR

Translation of 2016 WHO into 2018 CLMP classification of MPN

The molecular genetic and pathological bone marrow characteristics in a large cross sectional study of 407 WHO defined MPN patients including PV in 111, ET in 179 and MF in 117 was used by Kim *et al* to translate the 2008 WHO into the 2018 WHO-CLMP classification [49]. The three driver mutations were detected in 82.6% of 407 MPN patients with a mutation distribution of JAK2 in 275 (67.5%), CALR in 55 (13.7%), MPL in 6 (1.5%) The distribution of clinical phenotypes in 275 JAK2 mutated MPN were PV in 101, ET in 95 and MF in 79. The distribution of clinical phenotypes in 56 CALR mutated MPN were PV in none, ET in 40 and MF in 16 cases. Six MPL cases were diagnosed as ET in 3 and MF in 3. The mean age of CALR mutated MPN patients (57.5 years) was 8.5 years younger than in JAK2 mutated MPN patients (66 years). JAK2 mutated MPN had significantly higher values for leukocytes (11.9 x10^9/L) compared to CALR MPN (8.6x10^9/L) and lower values for platelets (643x10^9/L) compared to CALR MPN (898x10^9/L). CALR mutated MPN patients presented with decreased to normal values for hemoglobin, hematocrit and erthrocyte counts not exceeding the upper limit of normal. The bone marrow lineage proliferation profile in 285 cases of JAK2 mutated MPN featured dual increased proliferation of erythropoiesis and megakaryopoiesis (EM) in 13.5%, trilinear increased proliferation of erythropoiesis, granulopoiesis and megakaryopoiesis in 31.3%, monolinear megakaryocytic proliferation (M) consistent with WHO define ET in 29,1% and dual granulocytic megakaryocytic myeloproliferation mimicking PMGM in 26.2% [49]. Bone marrow histology in 56 cases of CALR mutated MPN typically featured predominant increased megakaryopoiesis in two thirds and increased granulopoiesis and megakaryopoiesis in one third. The overall bone marrow histology findings of erythroid, granulocytic and/or megakaryocytic hyperplasia in $JAK2^{V617F}$ mutated MPN, and of granulocytic and/or megakaryocytic hyperplasia in CALR mutated MPN, are completely in line with the 2018 CLMP classification of at least four distinct MPN disease entities and transitional states including $JAK2^{V617F}$, JAK2 exon 12, MPL and CALR mutated MPNs (Figure 10).

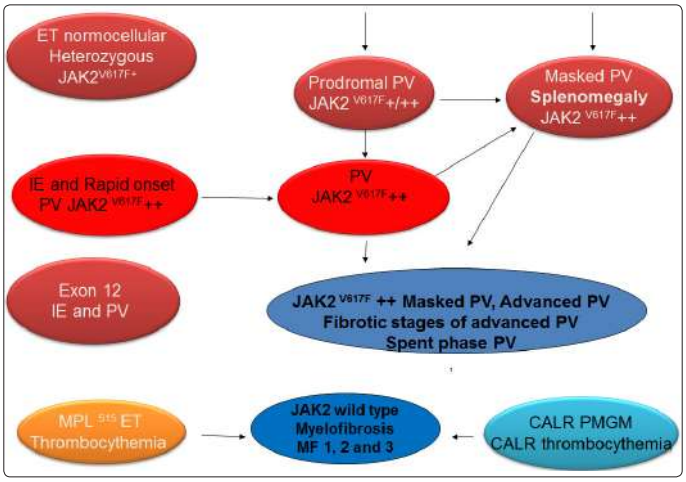

Figure 10: $JAK2^{V617F,}$ JAK2 exon 12, CALR and MPL^{515} myeloproliferative neoplasms (MPN) mutually exclude each other. The novel clinical, laboratory, molecular and pathological (2018 CLMP) classification defines a broad spectrum of $JAK2^{V617F}$ positive translational states of ET, prodromal PV, classical and masked PV, advanced PV and post PV myelofibrosis that has significant prognostic and therapeutic implications (Table 6). $JAK2^{V617F}$ PV is clearly distinct from JAK2 exon 12 erythrocythemia. The two ML variants of CALR and MPL^{515} mutated thrombocythemias and myelofibrosis have no features of PV.

First line treatment options of newly diagnosed MPNs in 2018 and beyond

A primary rigid venesection regimen in PV patients aiming at a hematocrit of 0.40 in both males and females appears to us superior for the relief of hypervolemic symptoms than the WHO recommendation of keeping the haematocrit around 0.45 in males and 0.42 in females [50]. Low risk PV patients have a normal life expectancy since phlebotomy on top of low dose aspirin in early and overt PV significantly reduces the cumulative incidence of minor and major thrombosis from above 50% to less than 2% per patient/year during long-term follow-up [50]. ET and PV patients in the hypercellular prefibrotic stage featured by platelet count above 1000x10/^{9}/L, leukocytes above 15x10^9/L and or splenomegaly (more than 14 cm length diameter) are candidate for low dose pegylated interferon (IFN) [45]. IFN-induced complete hematological responses (CHR) do occur within one year, and major molecular responses (MMR) were frequently seen after a follow-up of 2 to 3 years in PV and ET patients in two prospective clinical and basic research studies [51,52]. The cumulative incidence of MMR was 14% at 2 years and 30% at 4 years follow-up in one study [54]. Peglyated IFN α-2a (Pegasys^R) reduced the median JAK2-allele burden from 45% to 5% in 37 PV patients in one study and from 64% to 12% in a second study of 79 PV and ET patients [51,52]. A complete molecular response (CMR) with normalization of bone marrow histology may be reached, but cure of MPN (ET or PV) in the very long term is unlikely [53]. Kiladjian and his team of clinical investigators produced very good responses to pegylated IFN in 31 CALR mutated ET patients during a mean follow-up of 11.8 years [54,55]. A hematological response was achieved in all CALR mutated patients and the median CALR mutation allele burden significantly decreased from 41% at baseline to 26% after treatment but only 2 CALR ET patients (6%) achieved a complete molecular response, whereas the percentage of CALR mutation was not significantly modified in CALR ET patients previously treated with hydroxyurea or aspirin only [55]. The presence of additional mutations (TET2, ASXL1, IDH2 and TP53) was associated with only minor or no molecular responses on IFN treatment [48,56]. MPN patients resistant to IFN or not responsive to IFN showing with a progressive myeloproliferative disease, splenomegaly and constitutional symptoms are candidates for myelosuppressive

therapy with hydroxyurea or myeloreductive JAK2 inhibitor as described in great detail elsewhere (Table 6) [56].

Table 6: 2018 CLMP staging of JAK2^{V617F} positive prodromal PV, erythrocythemic PV, classical PV, early MF, inapparent PV, spent phase PV and post-PV myelofibrosis (MF)

PV: CMP stage	0	1	2	3	4	5	6
Clinical Diagnosis	Prodromal PV	Erythrocythemia	Early PV	Classical PV	Masked advanced PV	Inapparent PV: IPV Advanced	Post-PV MF
LAP-score, CD11B	↑	↑	↑	↑	↑/↑↑	↑	Variable
EEC	+	+	+	+	+	+	+
Red Cell Mass	**N**	**N**	**↑**	**↑/↑↑**	**↑/↑↑**	**↓ or ↑**	**Variable**
Erythrocytes x10^{12}/l	**<5.8**	**>5.8**	**>5.8**	**>5.8**	**N**	**N**	**↓**
Leukocytes x10^{9}/l	<12	<12	<or >12	< or->15	>15	N or ↑	>20
Platelets x10^{9}/l	>400	400	< or >400	>400	+1000	N or ↑	Variable
CLMP bone marrow histology	EM	EM	EM	EMG	EMG	MG-MF	MF
BM cellularity (%)	50-80	50-80	60-100	80-100	80-100	60-100	↓
Grading RF	RF 0-1	RF 0-1	RF 0-1	RF 0/1,	RCF2/3	RCF 2/3	RCF 3/ 4
Grading MF	MF 0	MF 0	MF 0	MF 0	MF 1 2	MF 1 2	MF 2/3
Spleen size:							
On echogram	<12-15	<13	12-15	12-16	18->20	16 >20	>20 cm
Below MCL	0-3	NP	0-3	4-6	>6	>6	>8 cm
JAK2^{V617F} load	Low	Low	Moderate	Mod/High	High >50%	High	High
Granulocytes %	+(++)	+(++)	<50% +	+ / ++	++	>50% ++	>50% ++
Risk stratification							
→Therapeutic implications	Low	Low	Low	Inter- mediate	High	High	Post-PV MF
	Aspirin	Phlebot Aspirin	Phlebot Aspirin	IFN	IFN if non Responsive HU-JAK2 inh	JAK2 inhibitor	

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