REVIEW ARTICLE

Bone Marrow Histology is a Pathognomonic Clue to Each of the JAK2^{V617F}, MPL,^{515} and Calreticulin Mutated Thrombocythemia in Myeloproliferative Neoplasms

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ABSTRACT

According to the World Health Organization and Clinical Laboratory Molecular and Pathological criteria bone marrow pathology in JAK2^{V617F} mutated trilinear myeloproliferative neoplasm (MPN) patients essential thrombocythemia (ET) and polycythemia vera are indistinguishably featured by clustered medium to large pleomorphic megakaryocytes and increased cellularity (60–90%) due to increased erythropoiesis and megakaryopoiesis. MPL^{515} mutated ET is the second distinct clonal MPN characterized by thrombocythemia in a normocellular bone marrow showing clustered increased large to giant mature megakaryocytes with staghorn-like hyperlobulated nuclei. Calreticulin (CALR) mutated hypercellular thrombocythemia associated with prefibrotic megakaryocytic, granulocytic myeloproliferation (MGM) recently became the third distinct MPN featured by dense clusters of immature megakaryocytes with cloud-like nuclei. Bone marrow pathology in newly diagnosed MPN patients appears to be a pathognomonic clue for diagnostic differentiation between JAK2^{V617F} mutated trilinear MPN, MPL^{515} normocellular thrombocythemia, and CALR thrombocythemia with MGM characteristics followed by secondary reticulin fibrosis. Their natural histories clearly differ featured by an increase of erythro/granulopoiesis and cellularity in JAK2^{V617F}, decrease of erythropoiesis and cellularity in MPL^{515} and increase of dual megakaryo/granulopoiesis and cellularity in CALR mutated MPN.

Key words: Bone marrow pathology, calreticulin mutation, essential thrombocythemia, JAK2 mutation, MPL mutation, myelofibrosis, polycythemia vera, reticulin fibrosis

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INTRODUCTION

The clinical and pathological features for prodromal erythrocythemia and classical polycythemic stages of polycythemia vera (PV) are variable and featured by increased erythrocytes above 6×10^{12}/L, increased leukocyte alkaline phosphatase (LAP) score (increased CD11b expression), normal or increased platelets, leukocytes, and spleen size, and by characteristic bone marrow features with increased pleomorphic large megakaryocytes and erythropoiesis [Table 1].[1,2] The peripheral blood findings in "true" essential thrombocythemia (ET) without features of

Table 1: The 2000–2015 ECMP criteria[3,6] for the diagnosis of PV,[1,2,6] "true" ET[3-6] and PMGM.[5-8] Modified according to Michiels *et al*., Georgii *et al*.[1-8]

Clinical criteria PV
A1 Erythrocyte count below and above 6×10^{12}/L in ET and PV respectively. Levels below or above hemoglobin 18.5 g/dL male and>16.5 g/dL female in ET and PV resp.
A2 Persistent increase of platelet count Grade I between 400 and 1000×109/L, Grade II above (>) 1000×10^{9}/L
A3 Splenomegaly on echogram or computed tomography (>12 cm) or splenomegaly on palpation
A4 Granulocytes>10×10^{9}/L or leukocytes>12×10^{9}/L and raised LAP score>100 in the absence of fever and no increase of ESR
A5 Presence of JAK2V617F mutation
A6 Low plasma or serum EPO level
Clinical criteria "true" normocellular ET
A1 Persistent increase of platelet count Grade 1 between 400 and 1000×10^{9}/, Grade II above 1000×10^{9}/L
A2 Normal spleen or only minor splenomegaly on echogram
A3 Normal LAP score, normal plasma or serum EPO, normal ESR and increased MPV
A4 Spontaneous megakaryocyte colony formation (CFU-Meg)
A5 Presence of MPL^{515} mutation
A6 No preceding or allied other subtype of MPN, PV, MDS, or CML
Clinical criteria PMGM
A1 Thrombocythemia with no preceding or allied other subtype of MPN, PV, CML, or MDS
A2 Presence of CALR mutation
Early clinical-stage: Platelets Grade I between 400 and 1000×10^{9}/and Grade II above 1000×10^{9}/L, normal hemoglobin, or anemia
Grade I: hemoglobin>12 g/dL, slight or moderate splenomegaly on palpation or>11 cm on ultrasound or CT. Platelets around or above 1000×10^{9}/L
Intermediate clinical stage: Anemia
Grade II, hemoglobin>10 g/dL, definitive leukoerythroblastic blood picture and/or tear-drop erythrocytes. Splenomegaly on palpation, no adverse signs
Advance clinical stage: Anemia Grade III, hemoglobin<10 g/dL, significant splenomegaly, and adverse signs

Pathological criteria $JAK2^{V617F}$ mutated PV
B1 Normal to increased cellularity due to increased erythropoiesis or due to trilinear myeloproliferation of megakaryopoiesis, erythropoiesis, and granulopoiesis (e.g., panmyelosis). The proliferation of small medium-sized and large (pleomorphic) megakaryocytes. The absence of stainable iron, No or slight increase of reticulin fibers.
B2 Spontaneous EEC formation
A1+B1 and none of the others are $JAK2^{V617F}$ mutated idiopathic erythrocythemia: IE not meeting the WHO criteria
A2+B1 and none of the others are $JAK2^{V617F}$ mutated ET without features of PV or ET with features of PV (prodromal PV) with erythrocytes below 6×10^{9}/l and Hb and Ht in the upper normal level.
A3 and B1 and none of the other are primary $JAK2^{V617F}$ mutated MPN or latent PV
A1+B1 plus one of A2 to A6 or B2 is classical $JAK2^{V617F}$ PV
Pathological criteria "true" MPL515 mutated ET
B1 Predominant proliferation of enlarged to giant mature megakaryocytes wit hyperlobulated staghorn-like nuclei and mature cytoplasm, lacking conspicuous cytological abnormalities
B2 Normal cellularity, granulopoiesis and erythropoiesis
B3 No or increase in RF
The combination of A1 and B1+B2 establishes "true" normocellular ET. Any other criterion confirms MPL^{515} ET.
Abbreviations: LAP: Leukocyte alkaline phosphatase; ESR: Erythrocyte sedimentation rate; MPV: Mean platelet volume; MPN: Myeloproliferative neoplasm; PV: Polycythemia vera; MDS: Myelodysplastic syndrome; CML: Chronic myeloid leukemia
Pathological criteria CALR mutated PMGM
B1 Primary megakaryocytic or megakaryocytic and granulocytic myeloproliferation (PMGM) and relative or absolute reduction of erythropoiesis erythroid precursors Abnormal clustering and increase of atypical immature medium-sized large to giant megakaryocyte containing (cloud-like) hypolobulated nuclei and definitive maturation defects
Staging of myelofibrosis: MF in ET, PV, and PMGM
MF 0 No reticulin fibrosis RF 0/1
MF 1 Slight reticulin fibrosis RF 2
MF 2 Marked increase RF Grade 3 and slight to moderate collagen fibrosis
MF 3 Advanced RF Grade 3–4 and collagen fibrosis-osteosclerosis (endophytic bone formation)

ECMP: European clinical, molecular, and pathological, ET: Essential thrombocythemia, PMGM: Primary megakaryocytic granulocytic myeloproliferative, EEC: Erythroid colony, WHO: World Health Organization, RF: Reticuline fibers

PV are featured by high platelet counts, normal values for hemoglobin, hematocrit, erythrocyte and white blood cells, and normal values for LAP score, LDH and no or minor splenomegaly despite platelet counts above 1000×10^9/L at time of first presentation.[3-5] The megakaryocytes in "true" ET are larger than in ET preceding PV and classical PV [Table 1].[3-6] The Hannover Bone Marrow Classification of the MPDs distinguished three primary prefibrotic MPDs ET, PV, and primary megakaryocytic granulocytic myeloproliferative (PMGM) from the advanced fibrotic stages of MPD.[7] Bone marrow pathology in PV is typically featured by large pleomorphic megakaryocytes with hyperploid nuclei in a hypercellular bone marrow due to increased erythropoiesis or increased trilinear erythrocytic, megakaryocytic, granulocytic myeloproliferation (EMGM)[6] Michiels and Thiele defined normocellular "true" ET as a distinct myeloproliferative neoplasm (MPN) entity different from classical PV and hypercellular ET associated with prefibrotic primary myelofibrosis (pPMF), which is consistent with primary megakaryocytic granulocytic myeloproliferation [PMGM, Table 1].[5,6] Georgii *et al*. labeled pPMF as the third MPD entity of chronic or primary MGM (CMGM/PMGM) in the absence of reticulin or collagen fibrosis in bone marrow biopsy material.[7,8] Michiels *et al*. replaced the term CMGM by primary MGM (PMGM) as the third JAK2 wild-type MPN without features of PV or CML.[6] Hypercellular JAK2 wild-type ET associated with PMGM is dominated by an increase of clustered atypical dysmorphic megakaryocytes due to increases of cellular and nuclear size and bulky nuclei with clumsy lobuli and irregular roundish shaped forms (so-called cloud-like nuclei), which are never described in $JAK2^{V617F}$ mutated ET and PV [Table 1].[4-6] The natural history of $JAK2^{V617F}$ thrombocythemia and PV is best reflected by the increase of erythropoiesis, granulopoiesis, and cellularity followed by the degree of anemia, splenomegaly, bone marrow cellularity, and an increase of reticulin and collagen fibrosis.[7,8]

BONE MARROW PATHOLOGY IN MPL^{515} MUTATED NORMOCELLULAR THROMBOCYTHEMIA

Bone marrow histology from a patient with thrombocythemia carrying the MPL^{W515L} mutation displayed clusters of large megakaryocytes with a greater number of giant megakaryocytes with hyperlobulated stag-horn nuclei in normal cellular bone marrow and no increase of erythropoiesis [Figures 1-4].[9-13] We here extend our previous descriptions on the differential diagnostic significance between patients with MPL^{515} mutated (n = 12) versus $JAK2^{V617F}$ mutated trilinear MPN[6]. The presence of clustered small and giant megakaryocytes with deeply lobulated staghorn like hyperlobulated nuclei [Figures 1-4] in MPL^{515} mutated

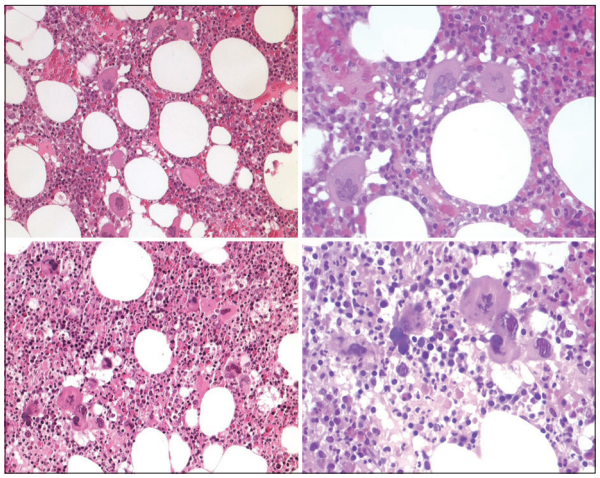

Figure 1: JAK2 wild-type essential thrombocythemia (ET) carrying the MPL^{515} mutation with loose or dense clustered small, large, and giant mature megakaryocytes with the presence of hyperlobulated, "stag-horn" hyperlobulated nuclei. Case 1, upper panel and Case 2, lower panel provided by Dr. Vannucchi, Florence, Italy[9] are consistent with World Health Organization defined normocellular ET carrying the MPL^{W515L} mutation showing large, giant mature megakaryocytes with hyperlobulated stag-horn like nuclei [Table 1]. Source Michiels *et al.* 2015[6]

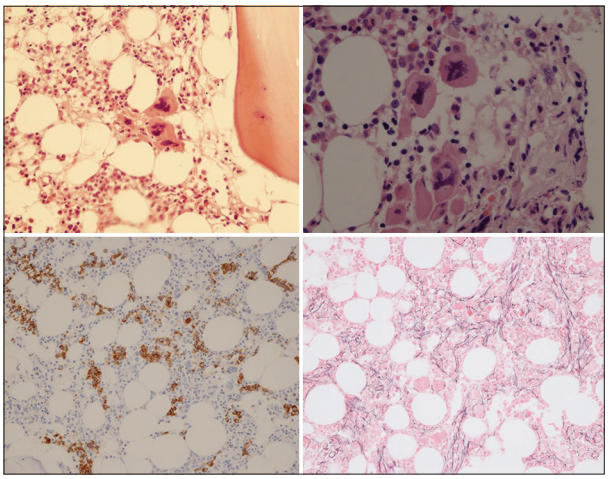

Figure 2: Standardized set of bone marrow histology in a case of MPL^{515} mutated essential thrombocythemia (ET) (hemoglobin 13.1 g/dL, hematoctit 0.40, erythrocytes unknown, leukocytes 8.2×10^9/L, platelets 802×10^9/L, no splenomegaly showing normocellular bone marrow, loosely clustered large megakaryocytes with hypersegmented nuclei, and normal erythropoiesis in the CD71 immunostain), normal granulopoiesis and slight increase in reticulin fibers with a few crossing-overs reticuline fibers Grade 2

thrombocythemia is not seen in $JAK2^{V617F}$ mutated ET and PV [Figures 5 and 6] and calreticulin (CALR) thrombocythemia. The increase of erythropoiesis is not seen in MPL^{515} mutated ET [Figures 1-4].[6] MPL^{515} mutated ET have no clinical, laboratory and bone marrow features of prodromal PV at

diagnosis [Table 1], do not evolve into PV during follow-up. JAK2^{V617F} mutated ET [Figure 5] show local increase of erythropoiesis in areas of loose clustered pleiomorphic megakaryocytes in normocelluar JAK2^{V617F} mutated ET [Figure 5], whereas bone marrow is hypercellular due to increased erythropoiesis and megakaryopoiesis (EM) [12,13] JAK2^{V617F} mutated ET, prodromal PV, and classical PV have increased score for LAP stain, low serum EPO and pleomorphic medium-sized to large mature megakaryocyte morphology [Figures 5 and 6]. In contrast, MPL^{515} mutated normocellular ET has normal values for LAP score, serum EPO and ferritin levels.[3,6,9-11] As demonstrated in Figures 2-4, the natural history of MPL^{515} normocellular ET is best reflected by decreased cellularity due to decreased erythropoiesis and increase of reticulin fibrosis (RF) from Grade 0 to Grades 1, 2, and 3.

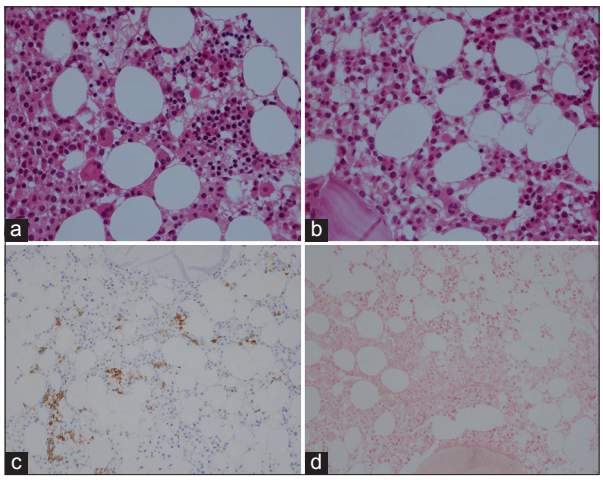

Figure 3: (a-d) Standardized set of bone marrow histology in a case of MPL^{515} mutated essential thrombocythemia (ET) (hemoglobin 11.3 g/dL, hematocrit 0.34, erythrocytes 3.6×10^{12}/L, leukocytes 7.8×10/L, platelets 678×10^{9}/L, no splenomegaly showing a normocellular bone marrow, enlarged megakaryocytes, and decreased erythropoiesis in the CD71 immunostain), normal granulopoiesis and no increase in reticulin fibers (reticuline fibers Grade 0)

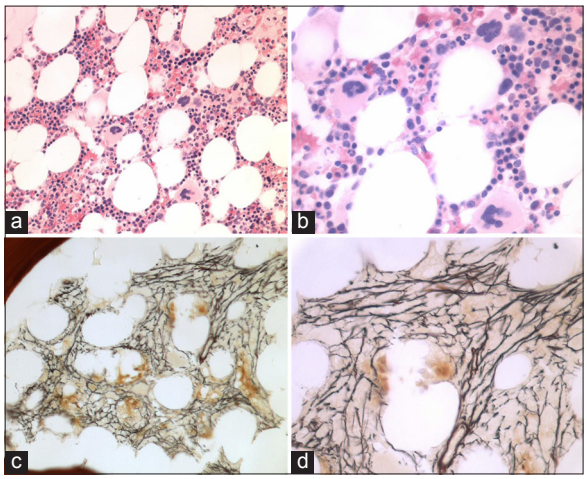

Figure 4: (a-d) JAK2 wild-type essential thrombocythemia (ET) carrying the MPL^{515} mutation in case 3 provided by Dr. Vannucchi, Florence, Italy[9] showed the presence of clustered large and giant megakaryocytes, decreased cellularity (45%) and increase of reticulin fibrosis Grade 2/3 (reticuline fibers (RF) 2/3). Such increase of RF Grade 3 in low cellular bone marrow has never been observed in JAK2^{V617F} mutated ET, and PV [Figures 5 and 6] and also not in CALR mutated hypercellular thrombocythemia associated with PMGM [Figure 7]

BONE MARROW HISTOLOGY IN CALR MUTATED ET AND MF

We found consistent bone marrow characteristics of hypercellular ET as the presenting feature of prefibrotic and early fibrotic or even advanced stages of PMGM in 13 consecutive newly diagnosed CALR positive ET cases (manuscript in preparation). 13 CALR mutated PMGM patients do not present present with ET complicated by aspirin-sensitive microvascular disturbances of erythromelalgic, cerebral and ocular ischemic manifestations (Sticky Platelet Syndrome) as the specific presenting manifestations of JAK2^{V617F} and MPL^{515} mutated myeloproliferative thrombocythemia. Bone marrow histology in prefibrotic CALR Thrombocythemia in Figure 7 show dysmorphic megakaryocytes with definite abnormalities of maturation with bulky (bulbous) hyperchromatic nuclei and some disturbances of the nuclear-cytoplasmic ratio consistent

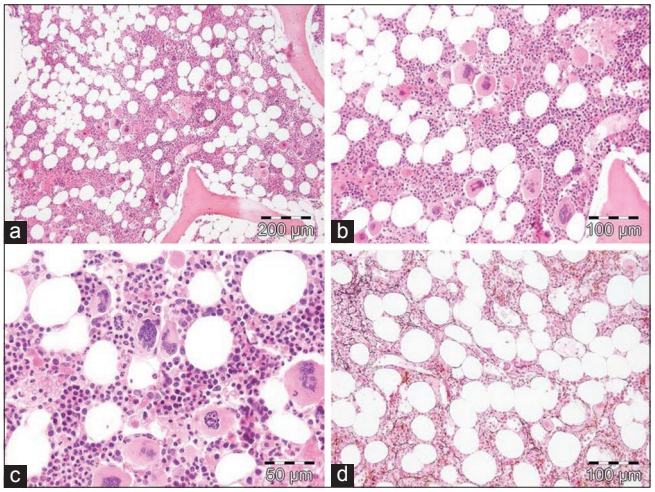

Figure 5: (a-d) Heterozygous mutated JAK2^{V617F} positive ET featured by hemoglobin 10.5 mmol/L, hematocrit 0.48, erythrocytes 5.4x10^{12}/L (normal value <5.8×10^{12}/L), leukocytes 8.2×10^{9}/L, platelets 820×10^{9}/L, and no splenomegaly at time of diagnosis in 1995 showed a typical ET bone marrow picture with slightly increased cellularity (60%) due to increased erythropoiesis and reticulin fibrosis reticuline fibers Grade 1. This ET case was on maintained low dose aspirin for >20 years with stable asymptomatic heterozygous JAK2^{V617F} mutated ET disease between 1995 and 2018. Case described by Dr. Michiels and Dr. De Raeve, University Hospital Brussels, Belgium[13]

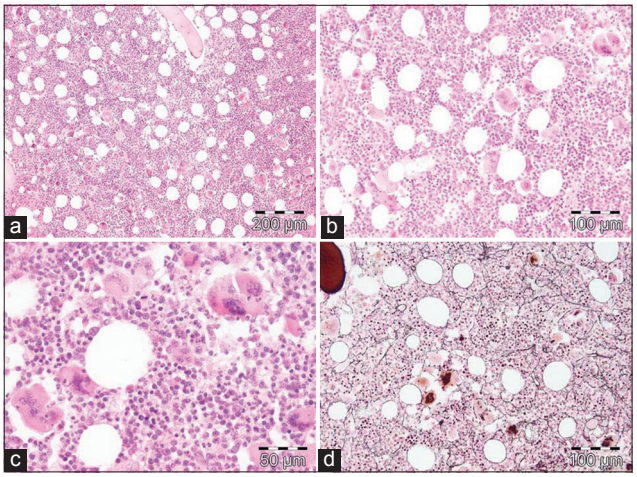

Figure 6: (a-d) Homozygous mutated JAK2^{V617F} positive polycythemia vera (PV) featured by hemoglobin 11.8 mmol/L, hematocrit 0.58, erythrocytes 6.9×10^{12}/L (normal value <5.8×10^{12}/L), leukocytes 9.1×10^{9}/L, platelets 1023×10^{9}/L, no splenomegaly at time of diagnosis in 2001 showed a typical PV bone marrow picture with increased cellularity (90%) due to increased erythropoiesis and megakaryopoiesis and reticulin fibrosis reticuline fibers Grade 1. This case has been successfully treated by hydroxyurea followed by pegylated interferon for >15 years (manuscript in preparation). Case described by Dr. Michiels and Dr. De Raeve, University Hospital Brussels, Belgium[13]

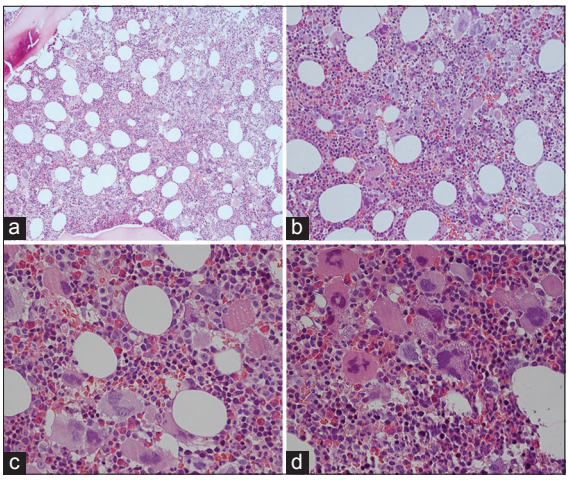

Figure 7: (a-d) Clinical case of a 24 years man diagnosed in 2014 as calreticulin (CALR) mutated essential thrombocythemia (ET), who presented with aspirin responsive platelet thrombophilia (transient microvascular ischemic disturbances), normal values for hemoglobin, hematocrit and erythrocytes, increased platelet count of 1832×10^{9}/L and slight splenomegaly (16 cm length diameter on echogram). Bone marrow histology showed increased cellularity (65%) due to megakaryocytic granulocytic myeloproliferation with normal erythropoiesis, dense cluster of immature megakaryocytes with hypolobulated nuclei and no increase of reticulin fibrosis (reticuline fibers Grade 0) consistent with hypercellular ET associated with a typical PMGM bone marrow [Table 1]. Case described by Dr. Valster and Dr. Potters, Bravis Hospital Bergen op Zoom, Netherlands[20]

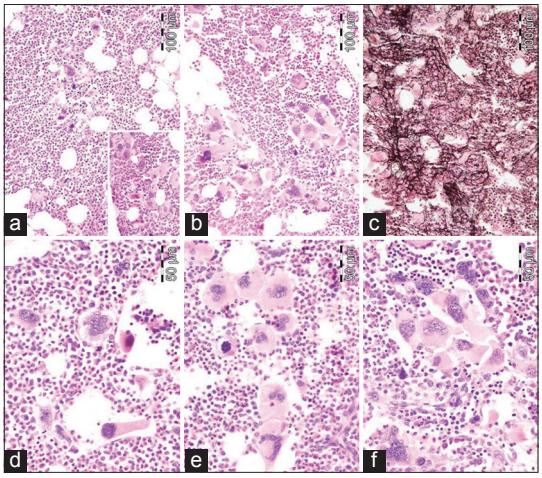

Figure 8: (a-f) Clinical case of calreticulin (CALR) positive thrombocythemia in a 37-year-old woman (asymptomatic except fatigue) with JAK2/MPL wild-type hypercelllular essential thrombocythemia in 2006/2008: Platelets 1205×10/^{9}/L, Hb 12.5 g/dl, leukocytes 18×10^{9}/L, borderline LDH, and spleen size 13 cm on echogram (normal value <12 cm) as the presenting of primary megakaryocytic granulocytic myeloproliferative with reticulin fibrosis Grade 2 and diagnosed according to World Health Organization – Clinical Laboratory Molecular and Pathological criteria as CALR mutated thrombocythemia with secondary myelofibrosis in 2014 [Table 1]. Case described by Michiels *et al*. 2015[6]

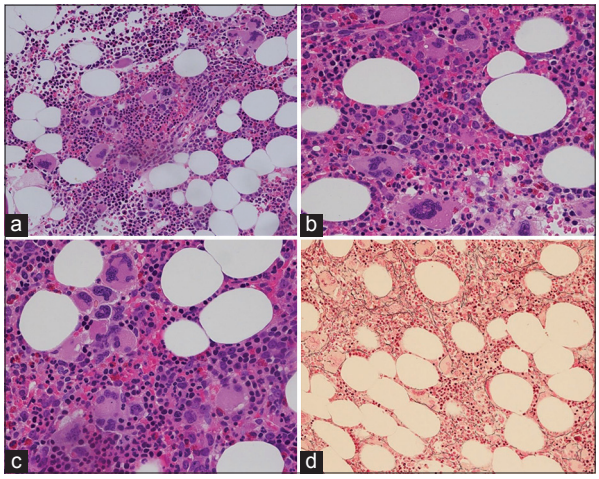

Figure 9: (a-d) Standardized set of bone marrow histology in a case of calreticulin mutated thrombocythemia and myelofibrosis (formerly diagnosed as primary megakaryocytic granulocytic myeloproiferation and myelofibrosis) with symptomatic anemia and splenomegaly featured by dense clustered immature large megakaryocytes with immature cloud-like nuclei and increased reticulin fibrosis reticuline fibers Grade 2

with CALR mutated PMGM, which are not seen in MPL^{515} mutated ET [Figures 1-4] and also not in JAK2^{V617F} mutated ET [Figure 5], prodromal PV and classical PV [Figure 5]. A 37-year-old woman (asymptomatic except fatigue) presented in 2004 with JAK2/MPL wild-type hypercellular

ET associated with PMGM featured by platelets 1205×10^9/L, Hb 12.5 g/dl, leukocytes 18 × 10^9/L, borderline LDH, spleen size 13 cm on echogram (normal value <12 cm) and PMGM bone marrow features with RF Grade 1/2 diagnosed according to the WHO - European clinical, molecular, and pathological criteria[6] [Figure 8 and Table 1]. 10 years later, this case was diagnosed as end-stage CALR mutated MF and treated by allogenic bone marrow transplantation in 2014. The three PMGM cases in Figures 7-9 clearly demonstrate that the natural history of CALR mutated PMGM, as the third distinct MPN entity, is featured by progressive anemia related to myeloid metaplasia of the spleen with splenomegaly, reduction of bone marrow erythropoiesis and progressive increase of RF Grade 1 to Grade 4.[14-18] The natural history of CALR thrombocythemia and MF is best reflected by the degree of anemia, splenomegaly, bone marrow cellularity, and an increase of RF [Figures 7-10].

DISCUSSION

WHO-defined JAK2^{V617F} mutated ET patients have PV-like morphological bone marrow changes of medium-sized to large pleomorphic megakaryocytes similar to our findings in newly diagnosed JAK2^{V617F} mutated ET, prodromal PV patients, and PV patients.[6,12,13] JAK2^{V617F} positive ET and prodromal PV patients usually have low serum EPO, increased LAP score, and slight to moderate increased bone marrow cellularity due to increased erythropoiesis. Increase of bone marrow EMG is the hallmark of classical prefibrotic PV with no or minor splenomegaly, whereas

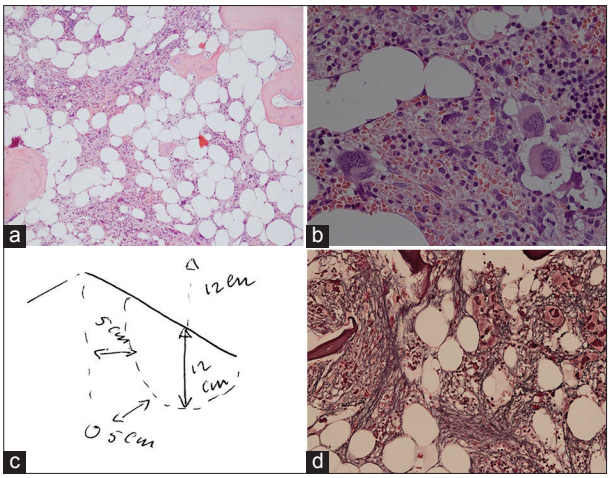

Figure 10: (a-d) Bone marrow histology in a 68-year-old man who presented with fatigue, transfusion-dependent anemia (hemoglobin 4.2 mmol/L), asymptomatic splenomegaly, and calreticulin (CALR) mutated advanced stage myelofibrosis (CALR mutated myelofibrosis) featured by a hypocellular bone marrow, with clusters of immature dysmorphic megakaryocytes and advanced reticulin fibrosis (RF). The 2008/2016 WHO defined diagnosis at the time of presentation was end-stage myelofibrosis, anemia, and splenomegaly with bundles of RF Grade 4. Case described by Michiels *et al*.[20]

serum LDH levels, CD34$^+$ circulating cells and spleen size are more pronounced in advanced JAK2^{V617F} mutated ET (masked PV) or post-PV MF at high JAK2^{V617F} mutation allele burden up to 90–100%.[6,13] Clustered large and giant megakaryocytes with hyperlobulated "staghorn" nuclei are rare in JAK2^{V617F} mutated MPN, but typically present in MPL^{515} mutated ET patients with no features of PV in the bone marrow and normal values for serum EPO, ferritin levels and LAP score.[9,10] The prevalence of MPL^{515} mutated ET or MF patients ranges from 5% to 10% of the JAK2 wild-type MPN population.[9-11]

Dr. Kralovics and his team in Vienna Austria first discovered the occurrence of CALR mutation in 78 of 311 (25%) ET patients and in 72 of 203 (35%) MF patients and none of 382 PV patients.[14] Since 2013 CALR mutated thrombocythemia and MF became the third distinct MPN entity with no features of PV, which was rapidly confirmed by Nangalia *et al*.[15] and by Rumi *et al*.[16,17] Nangalia *et al*. found somatic CALR mutations in 110 of 158 JAK2/MPL wild-type MPN, including 80 of 112 (70%) ET patients and 18 of 32 (56%) MF patients.[15] CALR mutations in the study of Nangalia *et al*. were identified in 10 of 120 (8%) MDS patients (RA in 5 of 53, RARS in 3 of 27, and RAEB-T in 2 of 27), and in one patient each with CMML and atypical CML.[15] The bone marrow pathological findings in the present study of CALR mutated thrombocythemia and MF in Figures 7-10 show dense clusters of large immature megakaryocytes with immature cloud-like nuclei, which clearly differ from the pleomorphic megakaryocytes in JAK2^{V617F} mutated ET and PV, which indeed are clearly distinct from the giant megakaryocytes with hyperlobulated staghorn-like nuclei in MPL^{515} mutated ET [Figures 1-4].[6] Since 2014 Michiels *et al*. demonstrated that cases of hypercellular ET associated with PMGM belong to the third MPN of CALR-mutated thrombocythemia and MF without features of PV at time

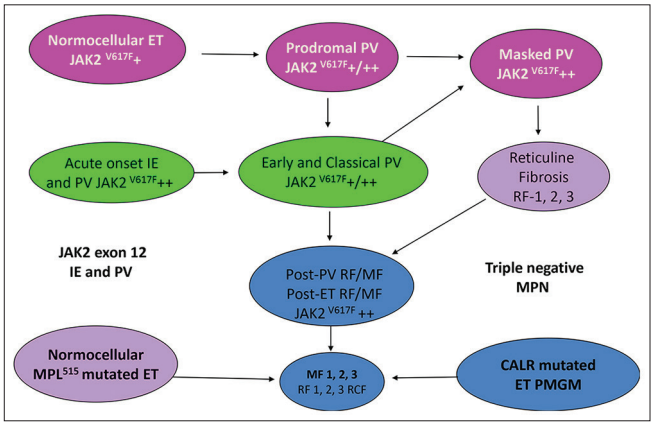

Figure 11: 2018 update of the World Health Organization and Clinical Laboratory Molecular and Pathological Classification and translational states of three distinct JAK2^{V617F}, MPL,^{515} and Calreticulin mutated clonal myeloproliferative neoplasms (MPNs), JAK2 exon 12 mutated erythrocytosis and polycythemia vera and triple negative MPN[6,14-20]

of diagnosis and during follow-up.[6,14-20] The updated 2018 WHO - Clinical Laboratory Molecular and Pathological (CLMP) criteria define five clonal mutually exclusive MPNs and transitional states at the laboratory and bone marrow level [Figure 11]: $JAK2^{V617F}$ mutated ET, prodromal PV, classical and advanced PV; JAK2 exon 12 mutated idiopathic erythrocytosis and PV; MPL^{515} mutated thrombocythemia and MF; and CALR mutated thrombocythemia and MF and triple negative ET and MF.[6,14-20] The $JAK2^{V617F}$ positive trilinear MPDs, MPL^{515} normocellular thrombocythemia, and CALR mutated thrombocythemia and MF mutually exclude each other [Table 1]. The natural history and MPN disease burden of each of the clonal MPNs is best reflected by the degree of anemia and splenomegaly on top of mutation allele burden, bone marrow cellularity and an increase of RF.[6,15-20] Prospective validation studies are warranted to confirm and amend the proposed WHO-CLMP classification.

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How to cite this article: De Raeve H, Fostier K, Valster F, Potters V, Kim Y, Kim M, Berneman Z, Schroyens W, Michiels JJ. Bone Marrow Histology is a Pathognomonic Clue to Each of the $JAK2^{V617F}$, MPL^{515} and Calreticulin Mutated Thrombocythemias in Myeloproliferative Neoplasms. Clin Res Hematol 2018;1(2):1-7.