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Myelofibrosis is a Secondary Event in JAK2 Trilinear Myeloproliferative Neoplasm (MPN) and in CALR and MPL Thrombocythemia: Implications for Novel Treatment Options of Prefibrotic MPN

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Abstract

JAK2^{V617F} PV is a trilinear myeloproliferative neoplasm preceded by erythromelalgic thrombocythemia followed by myeloproliferative myeloid metaplasia of spleen and bone marrow and secondary myelofibrosis. The CALR and MPL mutated JAK2 wild type thrombocythemia complicated by myelofibrosis (MF) and agnogenic myeloid metaplasia (AMM) have no features of polycythemia vera (PV) are not primary or agnogenic anymore. The natural history of CALR and MPL thrombocythemia and secondary bone marrow fibrosis clearly differ fromJAK2^{V617F} trilinear essential thrombocythemia (ET), PV, post-ET and post-PV secondary myelofibrosis. Evolution of anemia, splenomegaly and myelofibrosis in MPL, CALR thrombocythemia and JAK2^{V617F} trilinear thrombocythemia and polycythemia vera (TPV) should be evaluated separately simple because treatment options differ.

Keywords: Agnogenic myeloid metaplasia; Primary myelofibrosis; Polycythemia vera; Essential thrombocythemia; Myelofibrosis; JAK2 mutation; CALR mutation; MPL mutation; Myeloproliferative neoplasms

Introduction

The one cause hypothesis of one stimulatory or the lack of one inhibitory factor of bone marrow hematopoiesis for trilinear erythrocythemic (E), megakaryocytic (M) and granulocytic (G) benign myeloneoproliferation in PV proposed by Dameshek in 1950 has been confirmed by Constantinescu & Vainchenker by their discovery in 2005 of the acquired somatic JAK2^{V617F} mutation as the driver cause of erythrocytic, megakaryocytic and granulocytic (EMG) trilinear myeloproliferative neoplasms (MPN) [1-4].

JAK2^{V617F} trilinear PV runs through three sequential clinical phenotypes of JAK2^{V617F} mutated ET, PV and myelofibrosis (MF) during lifelong follow-up [4-7]. Advanced PV is complicated by extra medullary myeloid metaplasia of the spleen with increasing splenomegaly, myelofibrosis and the development of anemia in about one fourth of the cases after long-term follow-up of about 15 to 30 years [1,4-7].

The evolution of the heterozygous into homozygous JAK2^{V617F} mutation can readily explain the three sequential phenotypes of ET, PV and MF [3,4,8]. Slight increase in the JAK2^{V617F} kinase activity of less than 10% in heterozygous mutated MPN is enough to produce the clinical phenotype of essential thrombocythemia (ET). Increasing JAK2^{V617F} kinase activity in combined hetero/homozygous or predominantly homozygous JAK2^{V617F} mutated MPN is associated with classical and advanced trilinear PV due to mitotic recombination of the JAK2^{V617F} mutation on chromosome 9p (change from heterozygosity for 9p into loss of heterozygosity for 9p: LOH 9p) indicating homozygosity for the JAK2^{V617F} mutation) [3,4-8].

The natural history JAK2^{V617F} MPN disease runs a broad continuous spectrum ranging from normocellular ET, prodromal PV mimicking ET and the definitive increase in red cells (>5.7 × 10^{12}/l) in classical PV followed by masked PV and advanced PV complicated by fibrosis and splenomegaly spent phase PV and blastic [2,4-8].

The initial stage of JAK2^{V617F} mutated ET and prodromal PV with no or minor splenomegaly have normal red cell mass (RCM) and erythrocyte counts of less than 5.7 × 10^{12}/l whereas manifest PV is featured by definitive increased RCM and erythrocytes above 5.7 × 10^{12}/l [7,8]. Patients with masked PV (Inapparent PV) are typically featured by normal values for hemoglobin; hematocrit and erythrocytes pronounced splenomegaly and increased RCM related to splenomegaly [4-8].

In this report we demonstrate that primary myelofibrosis (PMF) in the PVSG and WHO classifications is not a distinct disease anymore because myelofibrosis in JAK2^{V617F} trilinear MPN and in CALR and MPL thrombocythemia is a secondary event of progressive myeloproliferative disease stages (Figure 1).

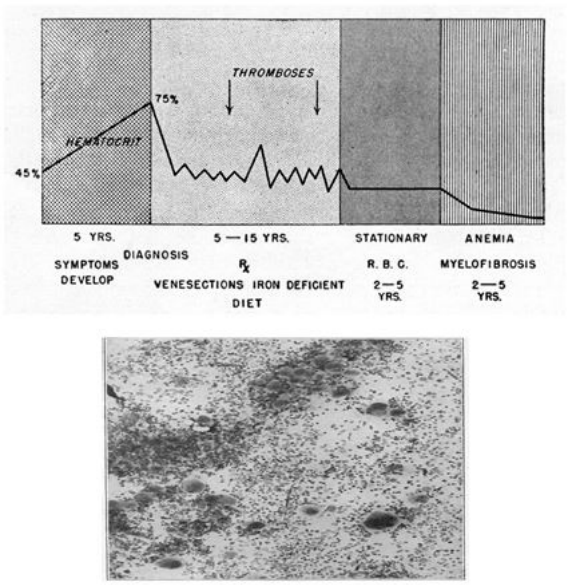


Figure 1: The natural history of PV defined as a trilinear myeloproliferative disorder with marked erythrocytic, megakaryocytic, granulocytic (EMG) hyperplasia in the bone marrow smear or biopsy with increased erythrocytes >6 × 10^{12}/l and blood volume. The effect of venesections reduce blood volume due to low MCV (mirco-erythrocytosis) and normalization of haemoglobin and haematocrit but increased erythrocyte counts above 6 × 10^{12}/l; so called complete remission by venesections. During the course of PV 'complete' remission by venesections has no effect on increased platelets, leukocytes and splenomegaly indicating the need of myeloreductive treatment in advanced stage of PV. After 20 to 30 years for those who survived thromboses the marrow PV patients become "burnt out" and will develop anemia and myelofibrosis in about 2 of 10 cases.

PVSG *vs.* RCP and ECP Classification of MPDs

Dameshek described in 1951 in fact different and distinct myeloproliferative disorders (MPD) showing trilinear bone marrow features in PV, dual increase of megakaryocytes and fibroblasts in agnogenic myeloid metaplasia (AMM) and unilinear megakaryopoiesis megakaryocytic leukemia (ML) [9].

Bone marrow in overt and advanced stages of PV shows trilinear hypercellularity (panmyelosis). There is a subgroup of PV patients who transform or evolve into a clinical and laboratory picture mimicking AMM.

AMM or PMF has been classified by the PVSG in 1975 as a clinicopathological entity not proceeded by PV, CML or MDS. PMF or AMM patients have no features of PV have large spleens, leukoerythroblastosis, striking teardrop erythrocytes, poikilocytosis and dry tap on bone marrow aspiration (Figure 2) [10].

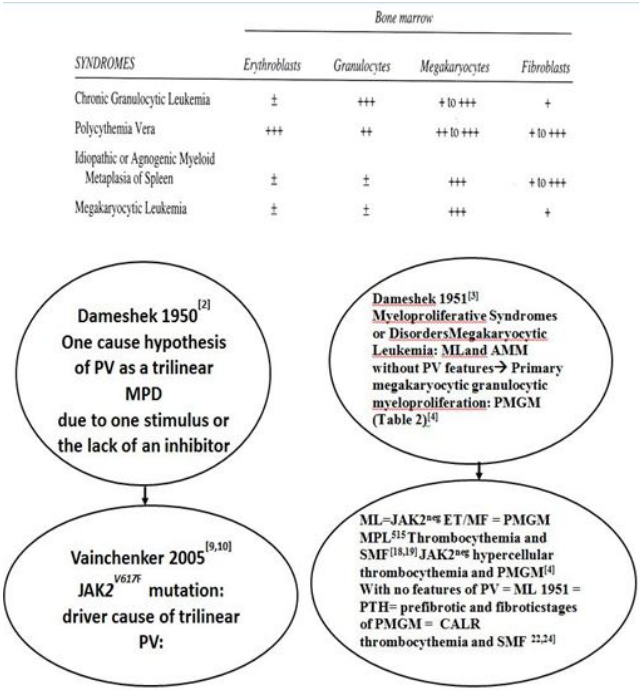


Figure 2: The 1950 Dameshek one cause hypothesis of trilinear polycythemia vera (PV) and Vainchenker's discovery in 2005 of the $JAK2^{V617F}$ mutation as the driver of trilinear myeloproliferative neoplasms (MPNs), essential thrombocythemia (ET), PV and myelofibrosis (MF). Dameshek (1951) recognized megakaryocytic leukemia (ML) as a distinct entity without features of PV. The original description of ML without PV features has been desribed as primary thrombocythemia hemorrhagica (PTH) by Thiele et al in 1988 and as JAK2 wild type hyercellular ET by the 2008 WHO classification. JAK2 wild type ET has been recognized and separated by Michiels in 2014-2015 as MPL515-mutated normocellular ET and hypercellular ET calreticulin (CALR) mutated thrombocythemia as two distinct MPN disease entities without features of PV.

The presence of tear drop erythrocytes in the peripheral blood reflects extramedullary erythropoiesis in an enlarged spleen. A typical bone marrow biopsy section in PMF shows stranding considerable fibrosis and a few scattered megakaryocytes at low magnification and a dense fibrotic reaction is usually apparent at higher magnification. The bone marrow in the study of Silverstein tends to be hypocellular in about 85%, normocellular in about 5% and hypercellular in about 10% of AMM patients [10].

Anemia of ineffective erythropoiesis develops in about 60% of PMF and AMM patients within 5 to 10 years. Thrombocytopenia and leucopenia related to hypersplenism and myelofibrosis are seen in 30% and 14% of PMF/AMM patients respectively. PVSG defined PMF or AMM is a clinicopathological entity characterized by various degrees of anemia, splenomegaly, leukoerythroblastosis, tear drop-shaped erythrocytes and dry tap on BM aspiration due to various degrees of myelofibrosis (MF) or osteosclerosis [11,12].

A small but significant group of hypercellular ET without features of PV develops a similar picture with myelofibrosis. Thrombocytosis with

either increased bleeding, or thrombosis and asymptomatic splenomegalgy are the most common presentations of megakaryocytic leukemia (ML) or primary thrombocythemia hemorrhagica (PTH) at platelet counts around or above 1000 × 10^9/l without features of PV [13-15].

In the book Polycythemia vera and the Myeloproliferative Disorders edited by Wassserman, Berk and Berlin the chapter by Rosenthal et al. stated that the pathology and etiology of AMM and PMF showing various degrees of the trias anemie, splenomegaly and myelofibrosis (MF) involving more than 1/3 of the sectional area of the biopsy, leukoerythroblastosis with tear drop erythrocytes, extramedullary hematopoiesis (myeloid metaplasia) remained completely unknown (Figure 3) [16].

Laboratory data	PTH	P. vera
Thrombocytes (× 10^9/L)	1,421±488	615±365
Erythrocytes (× 10^{12}/L)	4.7±0.6	7.2±1.1
Hemoglobin (g/dL)	13.4±1.6	18.6±2.7
Hematocrit (%)	41.3±5.3	56.8±8.2
Leukocytes (× 10^9/L)	12.9±4.6	14.7±6.0
Neutrophils (%)	69.2	71.1
Basophils (%)	0.9	0.9
Promyelocytes (%)	—	—
Myeloblasts (%)	—	—
Normoblasts (%)	—	—
LAP score	67.4±43	162.4±69.5
LDH (U/L)	305.3±100	351.6±103
Spleen size*	0.6±1	2.6±2.7
Liver size*	1.1±2.5	3.6±11

*One centimeter below costal margin.

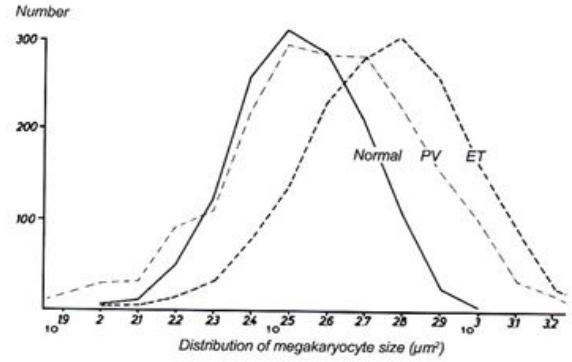


Figure 3: Prefibrotic bone marrow histology of PVSG defined primary thrombocythemia hemorrhagica (PTH) versus polycythemia vera (PV). PTH is characterized by uniformly large to giant megakaryocytes with large normal/hypolubulated nuclei dispersed among normal erythropoiesis and granulopoiesis and the production of large megakaryocytes. PV is characterized by marked pleiomorphism of medium sized to large megakaryocytes with hyperlobulated nuclei and a left shifted erythropoiesis with numerous erythroblasts and normoblasts. The figure shows the laboratories features of PTH and PV.

AMM is not preceded by PVSG defined ET, PV, CML or MDS and the Ph-chromosome is absent. The 1975 PVSG criteria for the diagnosis of primary hemorrhagic thrombocythemia (PTH) was a minimum platelet count of 1000 × 10^9/l. PTH has been defined as ML by Dameshek with platelet count above 1000 × 10^9/l, no PV features as documented by normal erythrocyte counts (4.7+0.6 × 10^{12}/l) and absence of myeloid metaplasia of the spleen Thiele et al. directly compared bone marrow (BM) histology in 25 PVSG defined PV patients and in 25 PVSG defined PTH patients (Figure 2).

PTH or ML bone marrow histology is characterized by the uniform appearance of large to giant megakaryocytes with normal erythropoiesis and low to decreased leukocyte alkaline phosphatase (LAP) score (Figure 4) [15]. PV in this study of Thiele et al revealed increased erythrocyte counts (7.2+1.1 × 10^{12}/l), increased LAP score and marked pleiomorphism of megakaryocytic growth with medium sized to large forms and increase of left shifted increase of erythropoiesis with numerous erythroblasts and normoblasts.

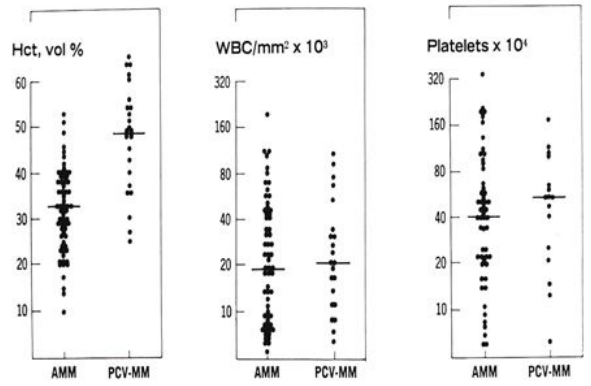


Figure 4: Laboratory findings in 98 patients with PVSG defined agnogenic myeloid metaplasia (AMM) and post-PV myeloid metaplasia (PCV/MM) show overlap in hematocrit values but white blood cell counts (WBC) and platelet counts were similar.

Between 1975 and 1980 Michiels discovered the early stage ET at platelet count between 400 and 1000 × 10^9/l with increase of large mature megakaryocytes in a normocellular bone marrow but complicated by erythromelalgia as an early myeloproliferative thrombocythemia stage preceding PV [4-8]. ET mimicking PV with increase of erythropoiesis is clearly in between ET and PV (prodromal PV).

Prodromal PV is typically featured by increased LAP score, low serum EPO, pleiomorphic megakaryocytes and increased erythropoiesis at platelet count above 400 × 10^9/l but normal erythrocyte count of less than 5.7 × 10^{12}/l [5-7]. The PVSG (1986) reduced the minimum number for the diagnosis of ET from 1000 to 600 × 10^9/l.

The majority of symptomatic ET and prodromal PV patients in the landmark study of Michiels et al. had platelet counts between 400 and 1000 × 10^9/l [17-19]. Georgii and Michiels recognized between 1987 and 1997 three distinct MPD entities of prefibrotic ET, PV and chronic megakaryocytic granulocytic myeloproliferation (CMGM) at the bone marrow and clinical level respectively [17-26].

CMGM became the third benign MPN of prefibrotic and fibrotic stages of clonal myeloproliferation of the hematopoietic cell lines with secondary bone marrow fibrosis [17,20,21].

In the 1980s, it became evident from bone histology studies that the megakaryocytes in ET and trilinear PV are large with increased, clustered and enlarged with dark and deeply lobulated nuclei but large to giant megakaryocytes with immature hypolobulated cloud-like nuclei in PTH according to Thiele et al. (Table 1) [15,22].

European Clinical Molecular and Pathological {EMCP) criteria for the diagnosis and staging of PMF or PMGM			
J.J. Michiels	**Clinical criteria 2005**	**J.Thiele**	**Pathological criteria 2005-2008**
A1	No preceding or allied other subtype of myeloproliferative neoplasms, CML or MDS. Main presenting feature is pronounced thrombocythemia and no dry tap on bone marrow aspiration JAK2 and MPL wild type	B1	Primary megakaryocytic and granulocytic proliferation (PMGM) and no or relative reduction of erythroid precursor. Abnormal clustering and increase in atypical giant to medium sized megakaryocytes containing bulbous (cloud-like) hypolobulated nuclei and definitive maturation defects.
C	Clinical stages of PMF or PMGM	MF	Staging of myelofibrosis (MF)
C1	**Early Clinical Stages**		
	Normal hemoglobin or slight anemia, grade I: Hemoglobin>12 g/dl	MF-0	Prefibrotic stage PMF or PMGM: No reticulin fibrosis
	Slight or moderate splenomegaly on ultrasound scan or CT thrombocytosis, platelets in excess of 400, 600 or even 1,000 × 10^{9-1}	MF-1	Early PMF or PMGM slight reticulin fibrosis
	Normal or increased LAF-score No Leuko-erythroblastose	-	-
C2	**Intermediate Clinical Stage**		
	Anemia grade II: Hemoglobin>10 g/dl	MF-1	PMF or PMGM: Slight reticulin fibrosis
	Definitive leuko-erythroblastic blood picture and/or tear drop erythrocytes increased LDH	MF-2	Fibrotic PMGM or PME: Marked increase in reticulin and slight to moderate collagen fibrosis
	Splenomegaly	-	-
C3	**Advanced Clinical Stage**		
	Anemia Grade III: Hemoglobin<10 g/dl	MF-3	Fibrotic PMGM or PMF: Advanced collagen fibrosis with optional osteosclerosis
	Definitive leuko-erythroblastic blood picture and/or tear drop erythrocytes	-	-
	Splenomegaly, thrombocytopenia, leukocytosis, lweukopenia	-	-

Table 1: The combination of A1+B1 establish PMF or PMGM; any other criterion C or MF contributes to staging.

In retrospect PTH is consistent with hypercellular ET in CMGM first described as chronic megakaryocytic granulocytic myeloproliferation (CMGM) as the third distinct MPN entity by Georgii et al. in the Hannover Bone Marrow Classification [23-25].

Applying the Rotterdam Clinical and Pathological (RCP) and the European Clinical and Pathological (ECP) criteria of Michiels & Thiele the platelet count cut-off for the diagnosis of ET should be reduced to 400 × 10^9/l as demonstrated in the landmark ET study of Lengfelder et al. showing that one third of RCP and ECP defined ET patients had platelet counts between 400 and 600 × 10^9/l and therefore overlooked by the 1986 PVSG criteria for ET [20,23,24,26].

The clinical and pathological description of prefibrotic megakaryocytic leukemia (ML) and agnogenic myeloid metaplasia of the spleen defined by Dameshek clearly fit with the prefibrotic and fibrotic stages of JAK2 wild type PMGM which according to Michiels in 2014-2015 is consistent with the CALR mutated thrombocythemia and myelofibrosis without features of PV (Figure 2 and Table 1) [5-7].

The PVSG criteria (1975) for PMF and AMM without a history of PV features in fact reflect JAK2 wild type CALR or MPL mutated thrombocythemia or myelofibrosis in the 2008 and 2016 WHO classifications clearly distinct from $JAK2^{V617F}$ post-ET end post-PV myelofibrosis [27-30].

Myelofibrosis is not a Primary Disease but a Secondary Event in Advanced MPD

The main complaints of AMM patients according to the Dutch Internist Snapper (1960) are fatigue and lassitude due to anemia [31]. AMM mainly occurs in older adults and only rarely is bone pain an outstanding symptom. The presenting signs consist of a tremendous spleen, large liver, severe anemia and leukopenia, tear drop erythrocytes, myelocytes, myeloblasts and normoblasts.

AMM has been labeled by Snapper as aleukemic megakaryocytic myelosis characterized by hematopoietic proliferation of

megakaryocytes in bone marrow, spleen and liver in which proliferating reticulum cells of the bone marrow do not originate from megakaryocytes but transform into fibrocytes and osteocytes.

The original understanding of Snapper was that the megakaryocytic leukemia (ML) or aleukemic megakaryocytic myelosis with normal white blood cells was frequently complicated by various degrees of 'secondary' myelofibrosis and myelosclerosis [31].

Aleukemic megakaryocytic myeloproliferation (myelosis) followed by myelofibrosis originating from fibrocytes are distinct entities in the natural history of AMM [31]. Using G6PD (glucose 6 phosphodiesterase isoenzyme) in a heterozygous N-ras mutation, and x-linked restriction-length polycymorfism demonstrated that myeloproliferation in AMM, PV and ET resulted from a clonal amplification of primitive hematopoietic progenitor cells [8,32-35].

WHO defined advanced stages of AMM including PMF and post ET/PV MF patients is characterized by an increased number (up to 200-fold) of circulating clonal dedifferentiated CD34+ hematopietic progenitor cells (metaplasia).

This increase of clonal CD34 hematopoietic cell population included colony forming units (CFU) of granulocyte, erythrocyte, megakaryocyte, macrophage (CFU-granulocyte-macrophage), erythroid (burst-forming unit-erythroid) and CFU-megakaryocytic progenitors. The clonal cell expansion clonal progenitor cells in the bone marrow and inflow from the bone marrow into spleen (metaplasia) is associated with myeloid metaplasia of the spleen with anemia and splenomegaly [16].

Jacobsen et al. produced very good evidence that fibroblast proliferation is polyclonal reactive process of myelofibrosis in AMM with clonal myloproliferation of hematopoietic stem cells [32]. Cases of Ph^+ chromosome positive CML shows progressive myelofibrosis which appeared to be secondary to the primary neoplastic proliferation because the metaphases of bone marrow fibroblast lacks the Ph^+ cytogenetic aberration of the hematopoietic cells of the same patient [33].

Castro-Malaspina et al. developed a liquid culture system for growing bone marrow fibroblasts and demonstrated that the marrow collagen producing cells in PMF/AMM and in PV and ET with or without fibrosis behave in vitro as do fibroblasts from normal individuals and are nonclonal in origin.

The myelofibrosis (MF) in PMF, post-PV MF and post-ET MF results from a reactive process of non-hematopoietic fibrocytes resulting in reticulin and collagen-producing cells [34].

These results implied that the fibroblast in ET, PV and CML was a reactive cell thereby indicating that PMF or MMM is not a disease and that prefibrotic and fibrotic stages of myeloproliferative thrombocythemia in CMGM or PMGM without features of PV indeed proved to be the third distinct MPD entity (Table 1).

According to Ward & Block essential thrombocythemia (ET) or ML *vs.* PV with agnogenic myeloid metaplasia are characterized by benign proliferation of hematopoietic cells in the bone marrow and spleen [35]. Ward & Block demonstrated significant correlation between increased megakaryocyte density in the marrow hematopoietic compartment and the degree of myelofibrosis in MPD patients (Figure 5).

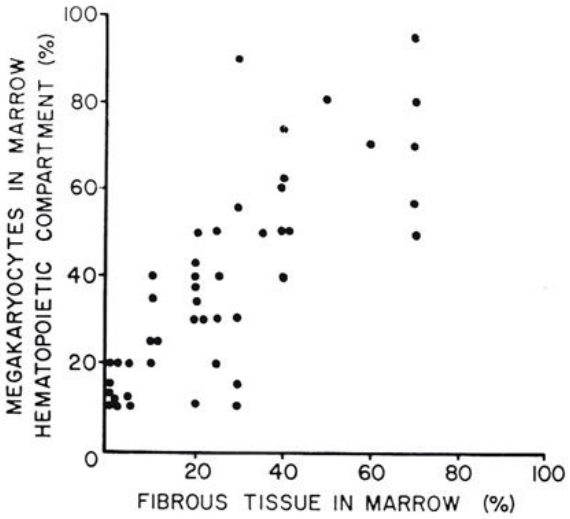


Figure 5: Relationship between megakaryocyte density in hematopoietic bone marrow compartment and the degree of bone marrow fibrous tissue in ET, PV and AMM patients.

The increase of platelet and megakaryocytes, reticulin fiber and collagen production is correlated with high levels of platelet derived growth factor (PDGF) which stimulates fibroblasts to divide and secrete fibers and collagen [16]. Simultaneous release of platelet factor 4 (PF4) from the megakaryocytes may inhibit breakdown of fibrosis competing with collagenase activity creating an imbalance between fiber production and degradation and thus excessive deposition of marrow reticulin and collagen.

Recently, expert investigators on PMF and clinical molecular biologists Vainchenker, Constantinescu and Plo on the etiology of secondary myelofibrosis in clonal mutated JAK2, CALR, MPL and TPO MPNs concluded that megakaryocytes (MKs) play a central role in the pathogenesis of clinical manifestations and clonal bone marrow neoproliferation and the evolution of secondary myelofibrosis [36-41].

The MPL/JAK2 pathway is activated by the 4 MPN-restricted mutations ($JAK2^{V617F}$, CALR, MPL and TPO mutants) placing MK hyperplasia and eventually dysplasia as a central determinant in the myeloneoproliferative manifestations and epiphenomena (Figure 6).

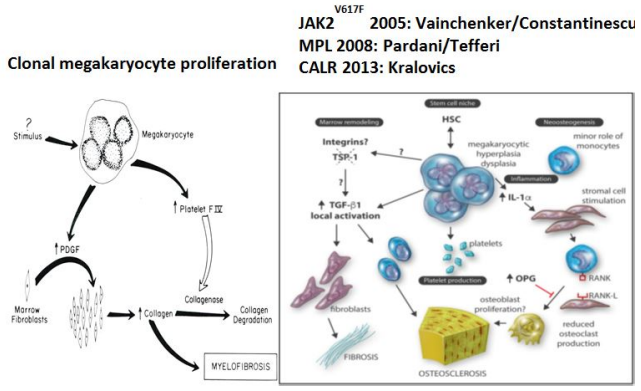


Figure 6: Left: Megakaryocytic myeloproliferation as the driver cause of secondary myelofibrosis in the bone marrow mainly mediated by platelet derived growth factor secreted by the proliferating megakaryocytes Right: Megakaryocytic hyperplasia and dysplasia as a central determinant player in the hematopoietic niche regulating hematopietic stem cells (HSCs) in JAK2, CALR and MPL myeloproliferative neoplasms, remodeling the marrow by secretion of TGF-b1 (transfoming growth factor-beta 1), PDGF (platelet-derived growth factor and other cytkines VEGF (vascular endothelial growth factor) for the induction of secondary reticulin and collagen fibrosis and induction of neo-osteogenesis by inducing an osteoblastic differentiation through TGF-b1 and inhibiting osteoclast differentiation through osteoprotegerin. The role of clonal megakaryocytic hyperplasia and dysplasia in MPN and secretion numerous inflammatory cytokines such as IL1a, TGF-b1 and other cytokines are poorly known. OPG, osteoprotegerin; ANK, receptor activator of NF-kB; RANKL, RANK ligand; TGF, transforming growth factor; TSP, thrombospondin still remain elusive.

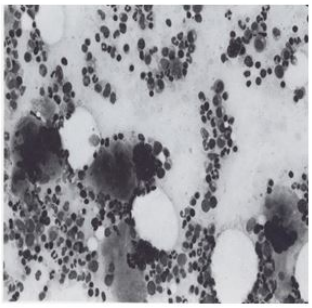
Clonal megakaryocytes (MKs) are mainly involved in overproduction of constitutively activated (sticky) platelets responsible for aspirin responsive erythromelalgic microascular (Sticky Platelet Syndrome) and increased of activated leukocytes as main risk factor for major arterial and venous thrombosis but also play an important role in the hematopoietic niche by regulating HSCs, remodeling the marrow by secretion of transforming growth factor-beta-1 (TGF-b1) and other cytokines platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) which ultimately lead to secondary MF and also inducing a neo-osteogenesis by inducing an osteoblastic differentiation through TGF-b1 and inhibiting osteoclast differentiation through osteoprotegerin [8,36,37,40]. Furthermore, MKs secretes numerous inflammatory cytokines such as IL1a.

The mechanisms of local activation of TGF-b1 and the contributions of OPG, osteoprotegerin; RANK, receptor activator of NF-kB; RANKL, RANK ligand; TGF, transforming growth factor; TSP, thrombospondin in the etiology of secondary myelofibrosis are not yet well understood [16,36,37].

Personal Observations 1975-2017

Natural history of PMF or PMGM without features of PV in the 1990s

Blood and bone marrow characteristic of case 1 shows a typical case of primary prefibrotic MPD featured by three sequential stages of splenomegaly, erythromelalgic thrombocythemia and anemia with fibrotic stages of AMM or PMF (Figure 7) [42].



	Case 1				
	1971	1978	1985	1988	1989
Erythromelalgia	–	–	+	+	+
Peripheral blood					
Hemoglobin mmol/l	9.8	8.0	7.1	5.9	5.7
Platelets × 10^9/l	285	265	421	484	811
Leukocytes × 10^9/l	6.9	10.8	21.4	21.9	24
Bone Marrow					
Cellularity	N	↑	↑		↑
Erythropoiesis	N	N	N		N
Ringed sideroblasts	–	–	–		–
Megakaryocytes	↑	↑	↑↑		↑↑
Reticulin	↑	↑↑	↑↑↑		↑↑↑
Collagen	–	–	↑		↑↑
Osteoid	–	–	–		+
Myelodysplasia	–	–	–		–
Karyotype of bone marrow cells					
Spleen size on scan					
Length diameter cm		18		23	

Figure 7: Bone marrow aspirates and biopsies in 1971 and 1978 at time of prefibrotic PMF in the table showed normal cellularity, no increase of erythropoiesis, selective increase of large dysmorphic megakaryocytes in a bone marrow smears and fine reticulin fibers (RF) grade 1 in bone marrow biopsy followed by PVSG defined primary myelofibrosis (PMF) indicating that PMF is usually preceded by of long history of latent MPD.

Prefibrotic MF with increased reticulin fibers grade 1 was diagnosed in 1971 at age of 61 years. The spleen had progressed to 7 cm and 13 cm below the costal margin in 1978 and 1988, respectively. Since 1985 he suffered from aspirin responsive burning pain and red swelling of the right hand fingers and left foot toes (erythromelalgia) at platelet counts between 400 and 500 × 10^9/l after correction of increased platelet counts (800 × 10^9/l) by hydroxyurea to 200 × 10^9/l, there was no further need for aspirin because erythromelalgia did not recur.

Sequential bone marrow aspirates and biopsies in 1971 and 1978 showed normal cellularity, no increase of erythropoiesis, selective increase of large dysmorphic megakaryocytes in a bone marrow smears and fine reticulin fibers in a bone marrow biopsy. In 1978, diagnosis of primary MPD with reticulin fibrosis grade 2 associated with significant splenemegaly was associated with normal platelet counts and no anemia. The PVSG diagnosis of agnogenic myeloid metaplasia (AMM) or primary myelofibrosis (PMF) was based on anemia and splenomegaly complicated by erythromelalgic thrombocythemia.

Bone marrow biopsies in 1985 and 1989 showed normal cellularity, coarse reticulin fibers, collagen fibrosis (with dry tap on aspiration) and increase of clustered large dysmorphic megakaryocytes. Such cases are labeled as PMF by the PVSG in 1975 as chronic megakaryocytic granulocytic myelosis (CMGM) by Georgii et al. of the Hannover Bone Marrow Classification and as PMF by the WHO in 2008 and 2016 WHO criteria.

Presentation of CALR mutated PMF as advanced secondary MF

In 2015 we observed a typical case of aleukemic megakaryocytic myelosis complicated by fibrosis of the bone marrow in a 67 year old man who first presented with AMM or PMF according to Silverstein with a clinical picture of normocytic anemia (hemoglobin 5.2 mol/l, hematocrit 0.25 and erythrocytes 3.1 × 10^{12}/l, platelets 265 × 10^9/l, leukocytes 6.5 × 10^9/l) weight loss from 84Kg to 81 kg in the last 2 years, minor fatigue, no sweating, asymptomatic splenomegaly and dense clustered dysmorphic megakaryocytes with reticulin fibrosis grade 3 to 4 (Figure 8) [10,31].

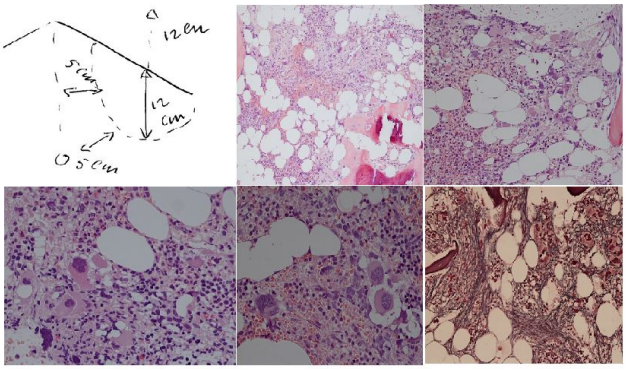


Figure 8: 68 year Man: Presentation with symptomatic anemia, CALR advanced myelofibrosis (RF 3) of the bone marrow and asymptomatic splenomegaly (WHO-PMF). transfusion dependent anemia→and EPO induced independent anemia (Hb 4.2 →Hb 5,7 mmol/l 68 year Man: Presentation with symptomatic anemia, CALR advanced reticulin fibrosis (RF 3) of the bone marrow and asymptomatic splenomegaly (WHO-PMF), transfusion dependent anemia and EPO induced transfusion independent anemia (Hb 4.2 →Hb 5,7 mmol/l).

This case was diagnosed as JAK2 wild type calreticulin (CALR) mutated myelofibrosis (MF) in the bone marrow, no bone pain, significant splenomegaly and anemia without a history of PV or allied MPN. Despite the presence of splenomegaly this patients was asymptomatic according to classical MPN-SAF assessment except fatigue related to severe anemia [41].

PMGM diagnosed as JAK2 wild ET in 2006 and CALR thrombocythemia in 2014

A 37 years old woman (asymptomatic except fatigue) with hypercellular ET: platelets 1205 × 10^9/l, Hb 12.5 g/dl, erythrocytes 4.9 × 10^{12}/l, leukocytes 18 × 10^9/l, no splenomegaly on palpation and first diagnosed in 2004 as WHO defined PMF abd ECP defined. This early fibrotic stage of PMGM proved to be JAK2 wild type in 2006.

This case of JAK2 wild type ET associated with progressive PMGM developed anemia significant splenomegaly and myelofibrosis during 10 years of follow-up and appeared to be CALR positive when tested in 2014.

Hydroxyurea induced anemia in JAK2 wild type ET associated with PMGM8.

Hypercellular ET associated with primary megakaryocytic granulocytic myeloproliferation (PMGM) was diagnosed as JAK2 wild ET-MGM (hypercellular ET with a PMGM bone marrow) and labeled as PMF according to PVSG/WHO criteria in 2006; hypercellular bone marrow histology with the presence of abnormal clustering and increase in atypical giant to medium sized dysmorphic megakaryocytes containing bulky/clumsy (cloud-like) hypolobulated nuclei and definitive maturation defects (Table 1) (Figures 9 and 10).

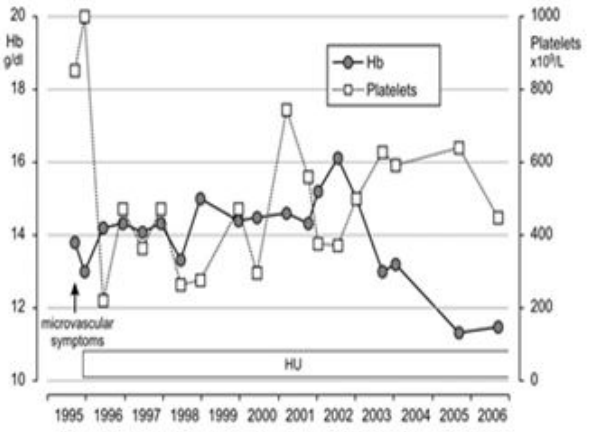


Figure 9: A case with JAK2 wild type PMGM treated for 10 years with hydroxyurea for reduction of platelet counts because of microvascular symptoms was complicated by anemia after six years and partial control of platelet counts to values around 600 × 10^9/l (hydroxyurea burn-out phenomenon).

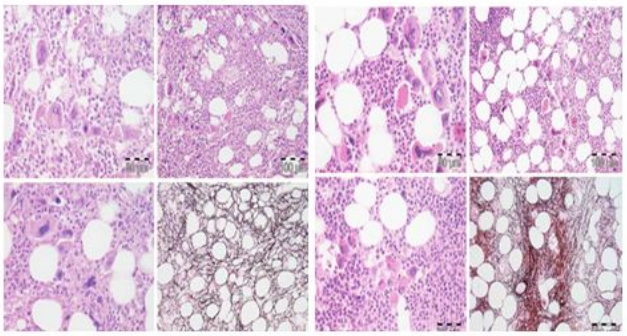


Figure 10: Bone marrow histology in a case of hypercellur essential thrombocythemia associated with a PMGM in Figure 9 and reticulin fibrosis grade 3 in Table 2 before treatment with hydroxyurea (left) and 'normocellular' PMGM bone marrow with bundles of reticulin fibers after (right) 6 years after treatment with hydroxyurea complicated by anemia and partial control of platelet counts (hydroxyurea burn-out phenomenon).

This case of hypercellular ET associated with PMGM presented in 1995 with micro vascular circulation disturbances and was treated with hydroxyurea for 11 years complicated by mild anemia at platelet counts of 600 mm^3/l after 10 years of hydroxyurea (HU) for 10 years and was complicated by anemia and increased bundles of reticulin fibrosis grade 2 after 10 years of hydroxurea treatment.

Bone marrow histology findings in 2006 show tightly clustered immature megakaryocytes with low degree of dysmegakaryopoiesis and cloud-like nuclei and increase in reticulin fibrosis with many cross-sections grade 3 reticulin fibrosis.

A similar case of normocellular with increase of clustered large, mature megakaryocytes (WHO-ET) presented with microcirculation disturbances and low MPD burden and no splenomegaly in 1995. Bone marrow histology before and after treatment with hydroxyurea is shown below. Hydroxyurea treatment resulted in partial remission of thrombocythemia and anemia within 7 years whereas the bone marrow persisted to show a lower cellularity and no increase of reticilune fibrosis as compared to pre-HU treatment (Figures 11 and 12).

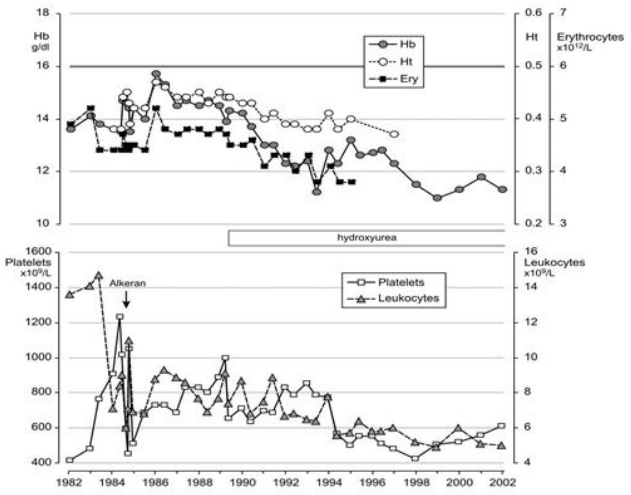


Figure 11: A case with essential thrombocythemia (ET) featured by increased clustered large mature megakaryocytes and increased cellularity (65%) due to increased erythropoiesis with increased reticulin fibrosis grade 2 consistent with the diagnosis of WHO-ET. After initial treatment with alkeran a second treatment with hydroxyurea for reduction of platelet counts from around 1000 × 10^9/l to normal was complicated by anemia within 8 years (hydroxyurea burn-out phenomenon).

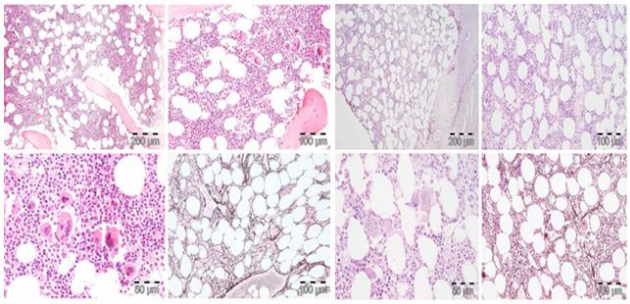


Figure 12: Bone marrow histology in essential thrombocythemia (ET) featured by increased clustered large mature megakaryocytes and increased cellularity (65%) due to increased erythropiesis with increased reticulin fibrosis grade 2 consistent with the diagnosis of WHO-ET before treatment with hydroxyurea (left) and hydroxyurea induced control of platelet counts complicated by anemia and a hypocellular bone marrow within 8 years (hydroxyurea burn-out phenomenon).

Effectiveness of anagrelide in JAK2 wild thrombocythemia and PMGM

A 9 year old Caucasian boy presented in 1994 with severe headache, attacks of migraine, aggressive behavior and minor bleeding symptoms. Initial abnormal laboratory data were a platelet count of 1596 × 10^9/l and slight splenomegaly on echogram. Low-dose aspirin 100 mg/day relieved the cerebral symptoms but a pronounced spontaneous bleeding tendency became evident. Severe epistaxis, bruises, hematomas and gum bleedings resulted in an iron deficiency state (hemoglobin 5.7 mmol/l, hematocrit 0.30, MCV 77 fl, ferritine 6 µg/l) in November 1995. The combination of mucocutaneous bleeding, high platelet counts (1946 × 10^9/l) and increase of enlarged megakaryocytes in a bone marrow smear was consistent with the diagnosis of hemorrhagic thrombocythemia due to acquired von Willebrand syndrome. The peripheral blood film showed red cells with slight anisocytosis and microcytosis, a few schistocytes, a few ovalocytes and a sporadic tear drop cell, absence of normoblasts, a normal white blood differential count and pronounced increase and clumps of platelets. The leucocyte alkaline phosphatase (LAP) score was low 14 (normal score 10-100). Molecular studies using southern blot analysis of extracted DNA revealed the absence of a rearrangement within the bcr on chromosome 22 in 1995 and the absence of the $JAK2^{V617F}$ mutation in 2006.

Bone marrow biopsy specimens showed a hypercellular bone marrow with predominant megakaryocytic and granulocytic myeloproliferation and the absence of reticulin fibers. Several megakaryocyte show definite abnormalities of maturation with bulky (bulbous) hyperchromatic nuclei and some disturbances of the nuclear cytoplasmic ratio (arrows) consistent with WHO defined PMF and WHO-CMP defined JAK2 wild type PMGM (Figure 13).

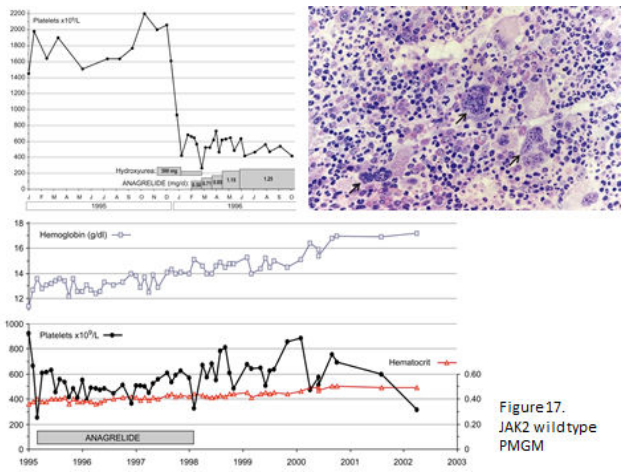


Figure 17. JAK2 wildtype PMGM

Figure 13: Platelet counts in a 6 year old boy with hemorrhagic thrombocythemia before and after short-term treatment with hydroxyurea followed by long-term treatment with anagrelide of hypercellular ET diagnosed as JAK2 wild type prefibrotic PMGM with no splenomegaly on palpation and stable MPN disease during long-term follow-up.

Initial treatment with hydroxyurea (500 mg daily) followed by anagrelide resulted in correction of platelet count from 2000 × 10^9/l to near normal (400-600 × 10^9/l) which was associated with relief of bleeding symptoms, and correction of plasma VWF values and VWF multimeric pattern to normal. Reduction of platelet count by treatment of hydroxyurea to near normal values between 400 and 600 × 10^9/l was taken over by long-term treatment with anagrelide. The subsequent natural history was featured by spontaneous reduction of platelet count to near normal, absence of splenomegaly on palpation and no progression of myeloproliferative disease for more than 13 years follow-up. After two years of treatment with anagrelide the dose to control platelet number could be decreased. Anagrelide could be discontinued at the end of 1997 without significant increase of the platelet counts. There was a slight increase of both hemoglobin and hematocrit from low normal to high normal levels during subsequent follow-up of 12 years. During that period up to 2003 the spleen was not palpable. Further follow-up was uneventful but the patient was not retrievable since 2008 for MPL and CALR screening.

Effectiviness of IFN α-2a (Pegasys^R) in prefibrotic CALR thrombocythemia

A 24 year old man presented with transient facialis paresis and easy bruisability caused by von Willebrand factor (VWF) ristocetine deficiency (VWF:RCo 29%) and normal VWF antigen concentration with the presence of typical increased of low VWF multimers with protelolytic VWF characteristic for Acquired Willebrand disease (AVWD) type 2A at platelet count of 1306 × 10^9/l due to PMGM caused by the CALR driver mutation (JAK2 and MPL wild type) (Table 2).

Laboratory features of 10 consecutive PMGM cases with CALR mutated ET or MF (bold)										
MPN case	1	2	3	4	5	6	7	8	9	10
Gender F/M	F	M	M	M	M	F	F	M	M	F
Age Diagnosis	34	24	40→52	66	68	63	64	45	73	73
Year birth	1970	1990	1962	1949	1947	1949	1950	1967	1940	1940
Year diagnosis	2004	2013	2001→2014	2015	2015	2012	2014	2012	2014	2014
Hb mmol/l	7.8	10/12	8.0→7.6	7.4	4.2/5.2	7.3	7.5	8.56	6.95	7.75
Hematocrit	0.37	0.47	0.37→0.36	0.37	0.26	0.33	0.40	0.1	0.34	0.39
Erys 1012/l	4.2	5.0	4.4→4.3	4.2	3.3	3.32	4.3	5.2	4.1	4.2
MCV fL	-	93	84→83	89	78	103	92.6	84.3	81.5	92.4
Platelets 109/l	1230	1306	852→536	781	265	768	1039	707	347	1243
Leuko's 109/l	18.3	9.7	7.8→7.4	22.7	5.9	4.3	6.1	6.3	9.2	6.9
LDH U/l	1369	390	N→ N	N	452	556	475	568	1519	475
Spleen cm	18	16	13→13	20	24	11.3	12	13	14	12
Symptomatic	yes	no	no→ no	no	yes	no	no	yes	no	no
WHO diagnosis	MF	ET	ET→ ET	ET	MF	ET	ET	ET	MF	ET

Table 2: Laboratory features of 10 consecutive PMGM cases with CALR mutated ET or MF (bold)

Values for hemoglobin (10 mmol/l), leukocytes (9.7 × 10^9/l) and erythrocytes (5.0 × 10^9/l) were normal. Bone marrow histology showed a hypercellular marrow (70%) due to primary megakaryocytic granulocytic myeloproliferation (PMGM) without features of PV and

with the presence of clustered large immature megakaryocytes with hypo or hyperlobulated cloud-like nuclei consistent with CALR prefibrotic thrombocythemia (Table 2). Treatment with pegylated interferon (Pegasys^{R}) 90 µg once per 2 weeks for 16 months the platelet counts dropped from 1306 to 450 × 10^9/l, leukocytes from 10 to 4 × 10^9/l and spleen size on echogram reduced from 16 to 14 cm length diameter (Figure 14).

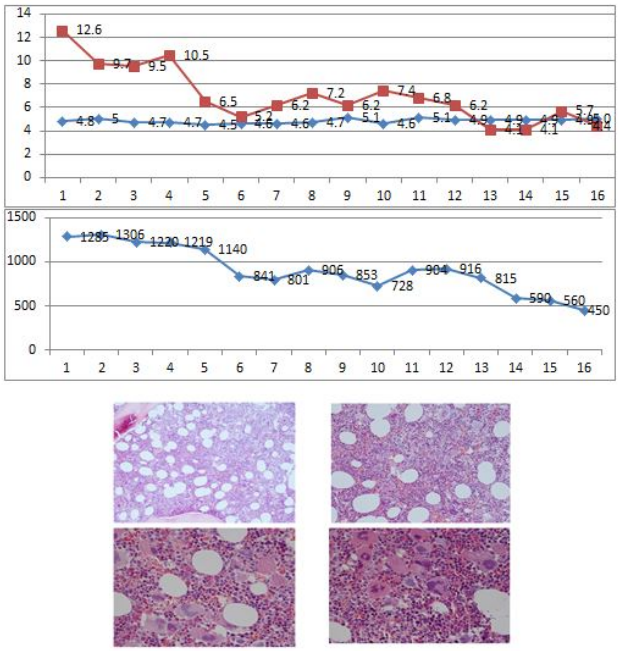


Figure 14: 24 year old man with aspirin sensitive thrombohemorhagic hypercellular CALR mutated Thrombocythemia and PMGM (Table 2) bone marrow complicated by acquired von Willebrand syndrome (AVWS type 2A, VWF 29%) showing increased triplet structure of each von Willebrand factor (VWF) band (left). Pegylated interferon (IFN, Pegasys^{R}) induced a complete hematological response (CHR) and corrected the AVWS by reduction of platelets from 1306 to 450 × 10^9/l (blue) and reduced leukocytes from around 10 to 4 × 10^9/l (red) and decreased spleen size from 16 cm to 14 cm during 16 months follow-up.

Effectiviness of IFN α-2a in prefibrotic PV peceded by a 10 years history of ET

A 66 year old women diagnosed in 2006 as ET with features of PV (prodromal PV) suffered since the age of 56 from attacks of transient blindness frequently followed by nausea and associated with vertigo but no headache. The attack starts with sudden partial blindness of the left under quadrant which after a few minutes is followed by white-yellow colored scotomas for about one hour. The attacks vary in frequency from a few per week or per month. The type of attacks did not change during the years. From 1997 to 2005 increase of platelet counts 508, 575, 544, 714, 566, usually below 600 × 10^9/l were documented. In 2005 the patient was seen by a neurologist and an internist and first diagnosed by the authors as ET type 2 with features of early PV (prodromal PV) (Figure 15).

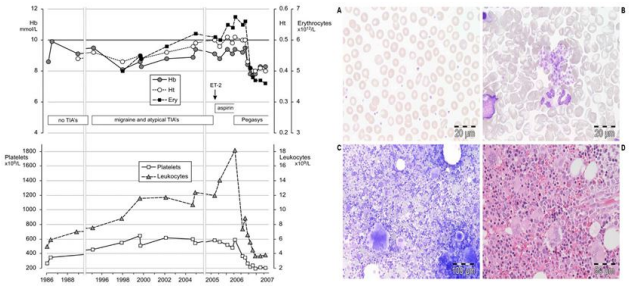


Figure 15: Woman 56 years with latent ET and attacks of migraine-like atypical cerebral ischemic attacks for 10 years 1995-2005. Diagnosis ET with features of PV was diagnosed in 2005 at age 66 years and pronounced PV in 2006 at age 67 years. This JAK2^{V617F} mutated trilinear PV had a 10 year history of latent ET and ET with PV features (prodromal PV). At time of PV diagnosis low dose IFN (Pegasys) 45 µg every ten days or 2 weeks induced a complete hematological response within one year (left). This case is in maintained complete hematological remission and major molecular remission for 7 years using low dose pegylated interferon (Pegasys^{R}) 30 µg every 3 to 4 weeks. IFN could be stopped in 2012 because of a complete hematological and major molecular response but within three year there was a slow relapse of PV for which low dose peylated IFN (Pegasys^{R}) was prescribed again until the end 2017 reaching the age of 78 years.

At that time she had a 5 years history fatigue and aquagenic pruritis and a 10 year history of ET related migraine attacks. Since the use of low aspirin (80 mg/day) in 2005 the atypical TIAs did not recur. The bone marrow was hyper cellular due to increased erythropoiesis with slight increase of granulopoiesis and cluster of large pleiomorphic megakaryocytes consistent with the diagnosis of PV. In 2006, overt PV developed (hb 9.4, ht 0.51 erythrocytes 6.75 × 10^9/l, MCV 75 fl, increased RCM, leukocytes 18.2 × 10^9/l, platelets 646 × 10^9/l, low serum EPO) for which she was phlebotomized. According to WHO recommendations there was a clear indication of hydroxyurea because of high thrombotic risk in a symptomatic patient with PV at the age of 66 years in 2006. After full informed consent, this PV patient was treated with low dose Pegasys of 45 µg/week for 6 months and a subsequent maintenance dose of 30 µg/week) was enough to keep the PV complete hematologic remission of the PV for several years. The PV remained in maintained complete hematological remission and major molecular remission for 7 years was achieved using low dose Pegasys 30 µg every 3 to 4 weeks at her age of 73 years being rather healthy for a PV patient. Because of maintained complete hematological remission and major molecular remission IFN could be discontinued in 2012 but within three year there was a slow relapse of PV for which low dose Pegasys was prescribed again until 2017 and the patient is doing well at age of 78 years.

Pegylated interferon (Pegasys^{R}) induced complete hematological responses (CHR) within one year and major molecular responses (MMR) were reached after a follow-up of 2 to 3 years in PV and ET patients in two prospective clinical and basic research studies. The cumulative incidence of MMR was reached in 14% at 2 years and 30% at 4 years follow-up in one study. 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 [43,44]. A complete molecular response (CMR) with normalization of bone marrow histology may be reached in two studies but cure of MPN (ET or PV) in the very long term is unlikely [45].

Kiladjian and his team of clinical investigators reported in 2015 good responses to pegylated IFN (Pegasys^{R}) in 31 CALR mutated ET patients during a mean follow-up of 11.8 years [46,47]. 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 [46]. Only 2 CALR ET patients (6%) achieved a complete molecular response (CMR). The percentage of CALR mutation was not significantly modified in CALR ET patients treated with hydroxyurea or aspirin only. The presence of additional mutations (TET2, ASXL1, IDH2 and TP53) was associated with only minor or no molecular responses on CALR mutant clones. Early stage prodromal and overt PV are candidate low dose aspirin on top of phlebotomy to keep the hematocrit around 0.40 and IFN is the first line treatment option in symptomatic JAK2, CALR and MPL thrombocythemia patients to postpone the use of hydroxyurea as long as possible [48]. JAK2 PV patients not responsive to IFN with progressive myeloproliferative disease, splenomegaly and constitutional symptoms are candidates for myelosuppressive therapy with hydroxyurea or with a JAK2 inhibitor according to the decision of the physician and his patient [5-7,48].

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