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Aspirin Responsive Sticky Platelet Mediated Thrombophilia Caused by Gain of Function Mutations in the Thrombopoietin, JAK2 and MPL Genes in Hereditary and Acquired Essential Thrombocythemia

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Abstract

Sticky platelet mediated thrombophiia is featured by aspirin responsive and platelet mediated arteriolar microvascular circulation disturbances including erythromelalgia and atypical transient ischemic attacks. The spectrum of aspirin responsive sticky platelet mediated thrombophilia (SPT) in hereditary essential thrombocythemia (HET) due to germline gain of function mutations in the TPO, JAK2 and MPL genes is comparable to the spectrum of SPT in acquired essential thrombocythemia (ET) caused by somatic gain of function mutations $JAK2^{V617F}$ and MPL^{515}. Increase of large platelets in blood smears and large mature megakaryocytes with hyperploid nuclei in a normal cellular bone marrow were diagnostic for autosomal dominant HET and for acquired ET. Evolution of HET and ET into secondary myelofibrosis (MF) belong to the natural history of TPO, JAK2, MPL mutated HET and acquired mutated $JAK2^{V617F}$ and MPL^{515} mutated acquired ET. In congenital HET caused by heterozygous germline gain of function mutation in the TPO and the JAK2 gene, $JAK2^{V617I}$ and $JAK2^{R564Q}$, the responses of mutated CD33 and CD34+ cells to TPO are increased, whereas the responses to EPO were normal thereby explaining why HET caused by heterozygous germline TPO and JAK2 mutations are associated with the biological characteristics of ET without PV features. Acquired MPL^{515} mutated ET has no features of PV, whereas acquired $JAK2^{V617F}$ ET is associated with typical features of PV in blood and bone marrow including low serum EPO and spontaneous endogenous erythroid colony (EEC) formation. CALR mutated ET and BCR/ABL positive ET are associated with the production of indolent platelet with the absence of sticky platelet mediated thrombophilia and show a rather high tendency of ET evolution into myelofibrosis.

Introduction

In the 1990s it was known that thrombopoietin (TPO) stimulates megakaryocyte production both in vitro and in vivo [1-3]. Size, number, and mean geometric ploidy of megakaryocytes are much more increased by TPO as compared with other cytokines with thrombopoietic activity. Evidence for a decisive role of deregulated TPO in ET comes from observations in mice overexpressing a TPO transgene where increased TPO production resulted in a fatal myeloproliferative disorder [4]. High dose exposure to TPO, lethally irradiated mice grafted with bone marrow cells infected with a retrovirus carrying the murin TPO cDNA (TPO^{high} mice) developed a lethal myeloproliferative disorder of TPO induced megakaryocytic myeloproliferation (Figure 1 and 2) with reduced erythropoiesis in the spleen and bone marrow [5,6]. Transient myelofibrosis is observed in rats receiving recombinant TPO [5]. Mice respond to TPO treatment by increasing the number of platelets in the circulation and megakaryocytes in the spleen at day 7 to 10 and returned to pretreatment values at day 14 (Figure 2) [6]. In wild type mice, TPO treatment increases platelet counts 2.3-fold and increased number of megakaryocytes and CFU-Mks. TPO treatment had profound effects on the morphology of megakaryocytes in wild type mice. The overall morphology of the megakaryocytes in the spleen became less mature as revealed by reduced localization of P-selectin and von Willebrand factor on the alpha granules. In addition, a significant portion of these megakaryocytes had heavy-electron dense para-apoptotic morphology and contained neutrophils embedded in the cytoplasm, as confirmed by myeloperoxidase immunostaining. In wild mice, TPO treatment decreased GATA-1 content in megakaryocytes, and the development of myelofibrosis is associated with high levels of transforming growth factor-beta-1 (TGF-beta-1) expression in bone marrow and spleen (figure 1) [6]. The strict association between the occurrence of the TPO-induced disease with low GATA-1 content in megakaryocytes and high GF-beta-1 expression represent a common pathobiological pathway leading to the sequential development of essential thrombocythemia and subsequent myelofibrotic transformation of the bone marrow in mice (figure 1) and possible of myelofibrosis in thrombocythemia of various molecular etiology in humans. Continuous forced expression of TPO, (TPO^{high} mice) in mice induces megakaryocyte proliferation and differentiation and subsequently develop myelofibrosis [4-6]. TPO^{high} mice engineered to overexpress TPO in their liver and those that received transplants of marrow cells infected with a TPO containing retrovirus develop thrombocythemia due to massive hyperplasia of large megakaryocytes and granulocytes and hypoplasia of erythropoiesis in the bone marrow followed by myelofibrosis and extramedullary hematopoiesis within 2 to 3 months and die from myeloid metaplasia and myelofibrosis after subsequent follow-up (Figure 2) [5]. TGF-beta 1 has been implicated in the pathobiology of myelofibrosis by the observation that megakaryocytes from TPO^{high} rats and mice express high levels of TGF-beta1 in marrow extracellular fluids and plasma (figure 1) [6]. In wild mice TGF-beta 1 mRNA expression in bone marrow and spleen was barely detectable before TPO treatment, and significantly increased in both organs after TPO treatment and returned to basal levels at day 14 [6]. Another growth factor produced by megakaryocytes, PDGF was found to be upregulated in a fashion similar to TGF-beta 1 [6]. High levels of TGF-beta 1 mRNA in the bone marrow and spleen cells in TPO^{high} mice were associated with high levels of TGF-beta1 protein in extracellular fluids from these organs (figure 1).

In 1997 Michiels first read the Blood Abstract number 1832 on "An activating mutation in the TPO gene causes Hereditary Essential Thrombocythemia (HET)" in the Dutch HET family published in Blood supplement 1, November 15, 1997. Wiestner, Schlemper, van der Maas& Skoda demonstrated the co-segregation of G to C transversion in the splice donor site of intron 3 in the TPO gene as the cause of a germline gain of function mutation in the TPO gene and the presence of ET in the Dutch family with autosomal dominant hereditary essential thrombocythemia (HET) [7,8]. All affected ET family members showed increased TPO levels (Figure 1 pedigree Dutch HET family) [8]. Transfection studies demonstrated that the mutant TPO gene produced an increased amount of circulating TPO plasma levels as an essential step in the pathophysiology of the benign hereditary essential thrombocythemia (ET) in the Dutch family with dominant HET [8]. Another germline TPO gene gain of function mutation in familial essential thrombocythemia (ET) showed a one-base deletion in the initiation region of the TPO gene giving rise to overexpression of the TPO gene has been reported at that by Kondo during the 1997 ASH meeting in San Diego, Blood Abstract number 231and published in Blood 1998 [9].

Sticky Platelet Thrombophilia in dominant hereditary essential thrombocythemia: HET

The proband II3 of the Dutch family with TPO mutated hereditary ET presented in 1968 with typical aspirin responsive platelet mediated erythromelalgia complicated by acrocyanosis of a few toes followed by gangrene and amputation of a toe (Table 1) [7].

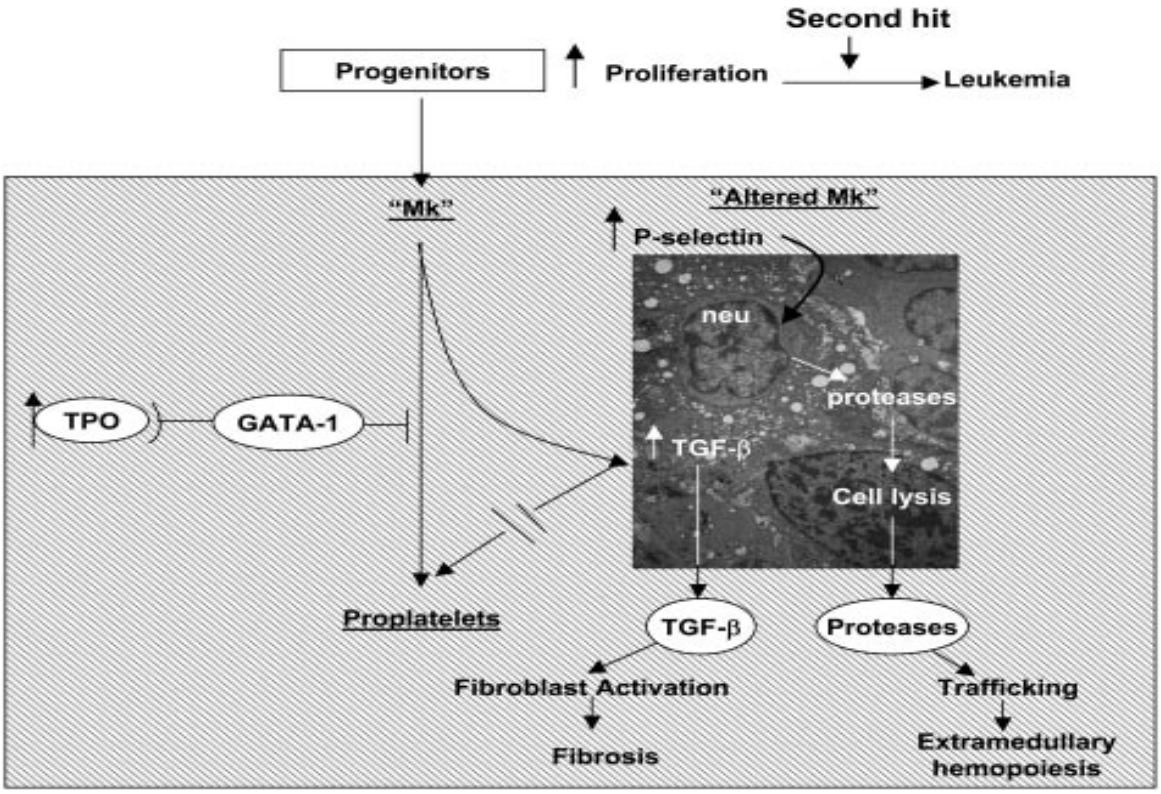

Figure 1. Pathobiologic pathway according to Vannucchi *et al* linking TPO, GATA-1, and TGF-beta-1 in the development of megakaryocytic myeloproliferation and secondary myelofibrosis in TPO^{high} mice followed by endstage myelofibrosis [6].

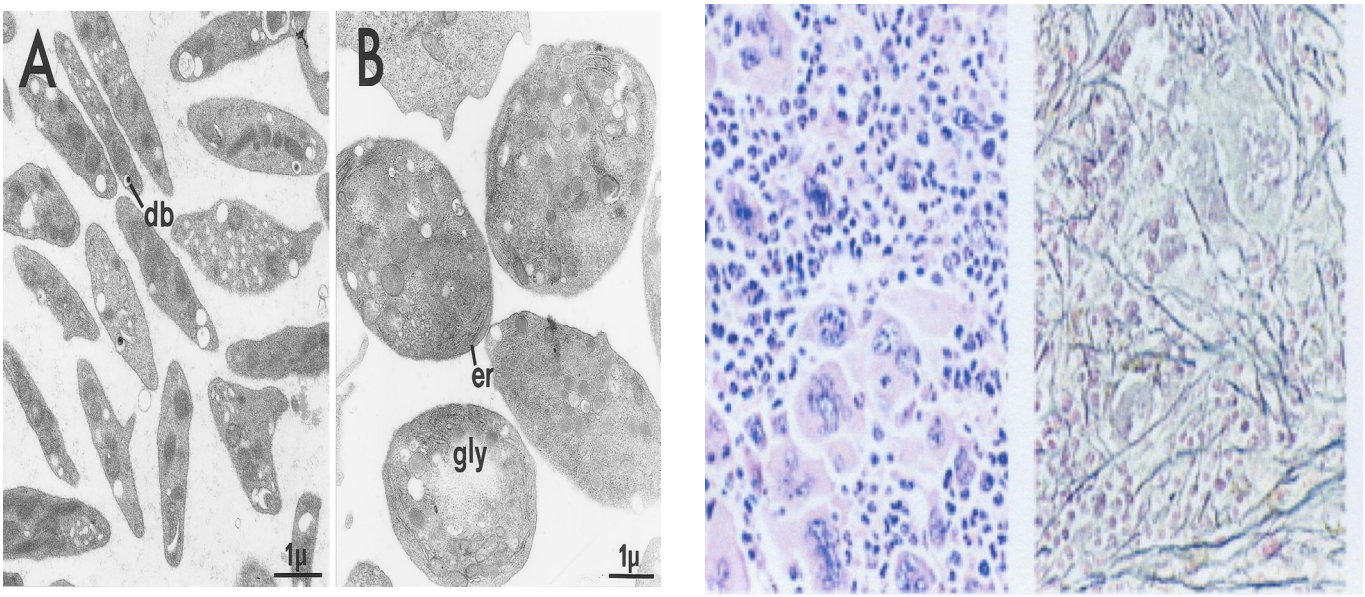

Figure 2. Normal platelets in control mice (A) and large platelets in TPO^{high} mice (B). Bone marrow histology showing a significant increase of large mature megakaryocytes and increase of reticuline fibrosis grade 2 starting after 3 months of TPO treatment in TPO ^{high} mice in the study of Villeval et al in Blood 1997 [5].

Table 1. Clinical manifestations and bone marrow histology data in the Dutch family with autosomal dominant hereditary essential thrombocythemia (Dutch HET)[7]

Clinical data of Dutch HET patients and non-HET subjects	**HET**	**Non-HET**
	(%)	**(%)**
Number of cases	10	5
Age years, range	11-58	
Vaso-occlusive symptoms: erythromelalgia: E*	50	0
Early E, tip paresthesia	30	0
Red congested erythromelalgia	10	0
E complicated by cold tip feeling-acrocyanosis/gangrene	20 / 10	0 / 0
Transient ischemic attack: TIA	30	0
Angina pectoris	20	0
Bone marrow histology data of ten HET patient [7]	**(%)**	
Megakaryopoiesis -increased	100	
Megakaryocyte clustering of 2-4 cells	100	
Erythropoiesis increased	10	
Reticulin-content increased grade 1	60	
Storage iron present	90	
Chromosome abnormalities	0	

E*. While on low dose aspirin since 1986 all TPO mutated HET patients were free of microvascular ischemic circulation disturbances at stable platelet counts above $400x10^9$/L and to around $1000x10^9$/L was associated with increased plasma TPO levels during the life long follow up without the need of myelosuppressive treatment [7,8].

Recurrence of erythromelalgia and acrocyanosis in 1986 again typically responded to low dose aspirin but not to coumadin similar as described by Michiels et al in the Annals of Internal Medicine 1985 entitled: erythromelalgia caused by platelet-mediated arteriolar inflammation and thrombosis in thrombocythemia [10]. In 1994 Dr van der Maas consulted Dr Michiels at the Hematology Department, Academic Hospital Dijkzigt, Erasmus University Rotterdam (EUR) for expert evaluation and to look into the bone marrow to address the question whether histopathologic findings in bone marrow biopsies of the proband II3 were compatible with ET as described by Dr. Michiels in patients with acquired ET and PV complicated by aspirin responsive erythromelalgia caused by platelet-mediated arteriolar inflammation and thrombosis (aspirin responsive sticky platelet arterial thrombophilia, Table 2) [10]. The first bone marrow biopsy in 1986 of case II3 is consistent with ET according to the RCP criteria proposed by the TVSG (figure 3) [11]. A second bone marrow biopsy performed in 1991 showed dense clustering of small to medium-sized megakaryocytes with hyperploid nuclei in normocellular bone marrow with reticulin stain grade 1 (figure 4). The histology picture of the third follow-up bone marrow biopsy in 1996 was dramatically changed (figure 5) and showed a hypocellular bone marrow with focal dense clustered small dysplastic megakaryocytes and reticulin grade 3 to 4 according to PVSG criteria in 1975 (Ellis et al) [12]. At time of diagnosis of Hereditary ET (HET) the histopathology from bone marrow biopsy material from the proband case II3 in 1986 and 1991 was very characteristic and diagnostic for ET (Figures 3, 4 and 5). All features according to the 1980 RCP and 1997 TVSG criteria for the diagnosis of ET [11] were present. First, the increase of platelet count in excess of 400 $x10^9$/l in normocellular bone marrow in the absence of any cause or sign of reactive thrombocytosis.

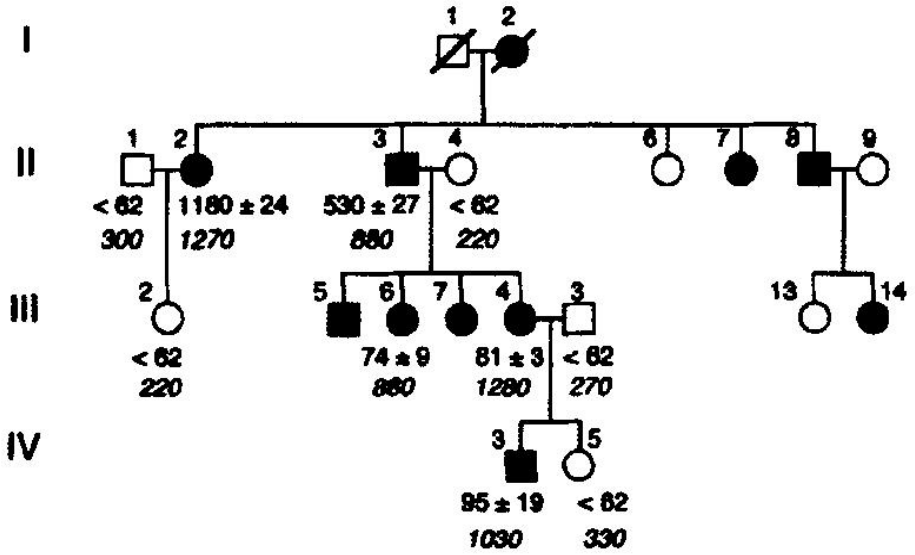

Figure 3. Pedigree of the Dutch family with hereditary essential thrombocythemia (HET). Increased plasma TPO concentrations (pg/ml) from the available Dutch family members with autosomal hereditary essential thrombocythemia (HET) caused by a gain of function mutation in the TPO gene [7,8]. Filled in symbols affected individuals with germline gain of function mutation in the TPO gene.

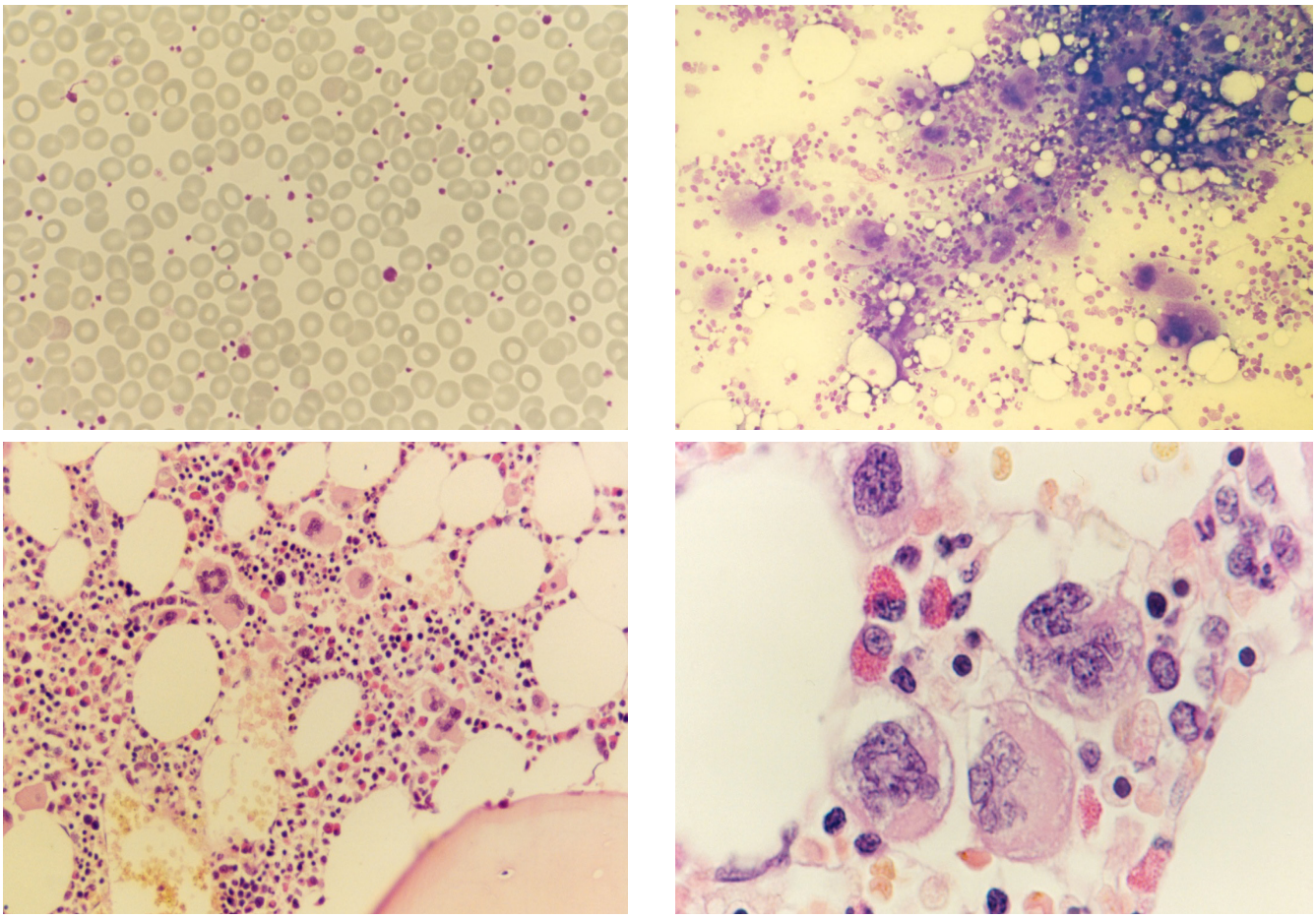

Figure 4. Peripheral blood showing some large platelets, bone marrow smear with increase of dense clustered large megakaryocytes and bone marrow histology (1986) with increase of large mature megakaryocytes in a normal cellular bone marrow and no increase of reticulin fibrosis (RF 0/1) in the proband II3 of the Dutch HET family with a gain of function mutation in the TPO gene.

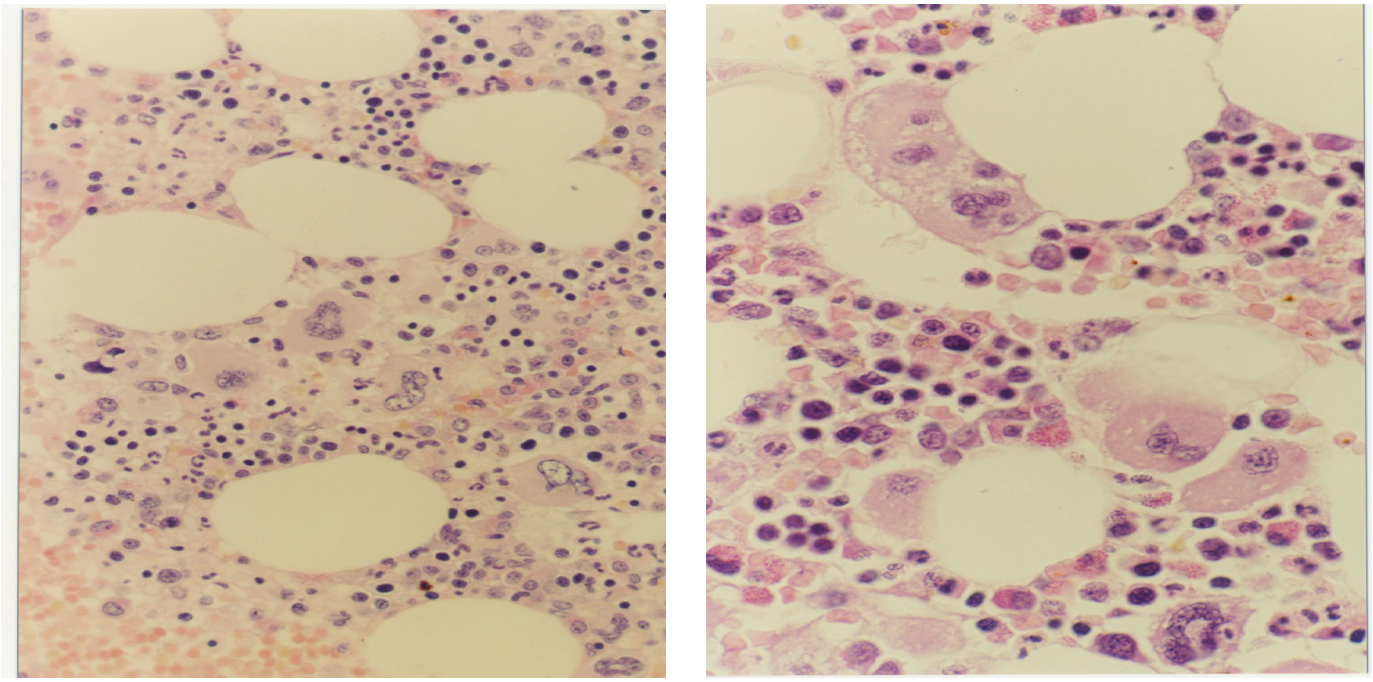

Figure 5. Bone marrow morphology (1991) showing increase and clustering of large megakaryocytes in normal cellular bone marrow and biopsy in the proband II3 of the Dutch HET family caused by a gain of function mutation in the TPO gene.

JAK2 V617F gain of function mutation in trilinear hematopoietic cells of CMPD patients is detectable in platelets, erythroblast and granulocytes

Step 1 JAK2 +	Step 1 → 2 JAK2 ++ LOH	Step 1 → 2 JAK2++ LOH
↓	↓ ↓↓	↓ ↓↓
Spontanuous	**Spontanuous**	**Leukocyte activation**
CFU-MK / EEC: ET	**EEC, CFU-MK: PV**	**PVR-1 =↑ LAF↑**
↓	↓ ↓↓	↓ ↓↓
Increase of enlarged hypersensitive platelets	Increase of hematocrit to above 0.45-0.50: PV	**Leukocythemia**/cytokines ↑↑ Fatigue, splenomegaly,
Already at platelet >400	higher platelets	unclassified MPD, PMGM 0/1
↓ ↓↓	↓ ↓↓	↓↓↓
Clinical Step 1 →	Clinical Step 2 →	Clinical Step 3. CIMF 2/3: 30%
Microvascular Thrombosis →	Macrovascular Thrombosis →	Secondary MF. Fatigue, itching constitutional symptoms
Aspirin sensitive	Aspirin/Phlebotomy	IFN / Hydroxyrea

Table 2. Molecular etiology of platelet-mediated microvascular thrombosis, increased red cell mass and secondary myelofibrosis in JAK2^{V617F}positive MPDs ET, PV, PMGM Vainchenker & Michiels 2005

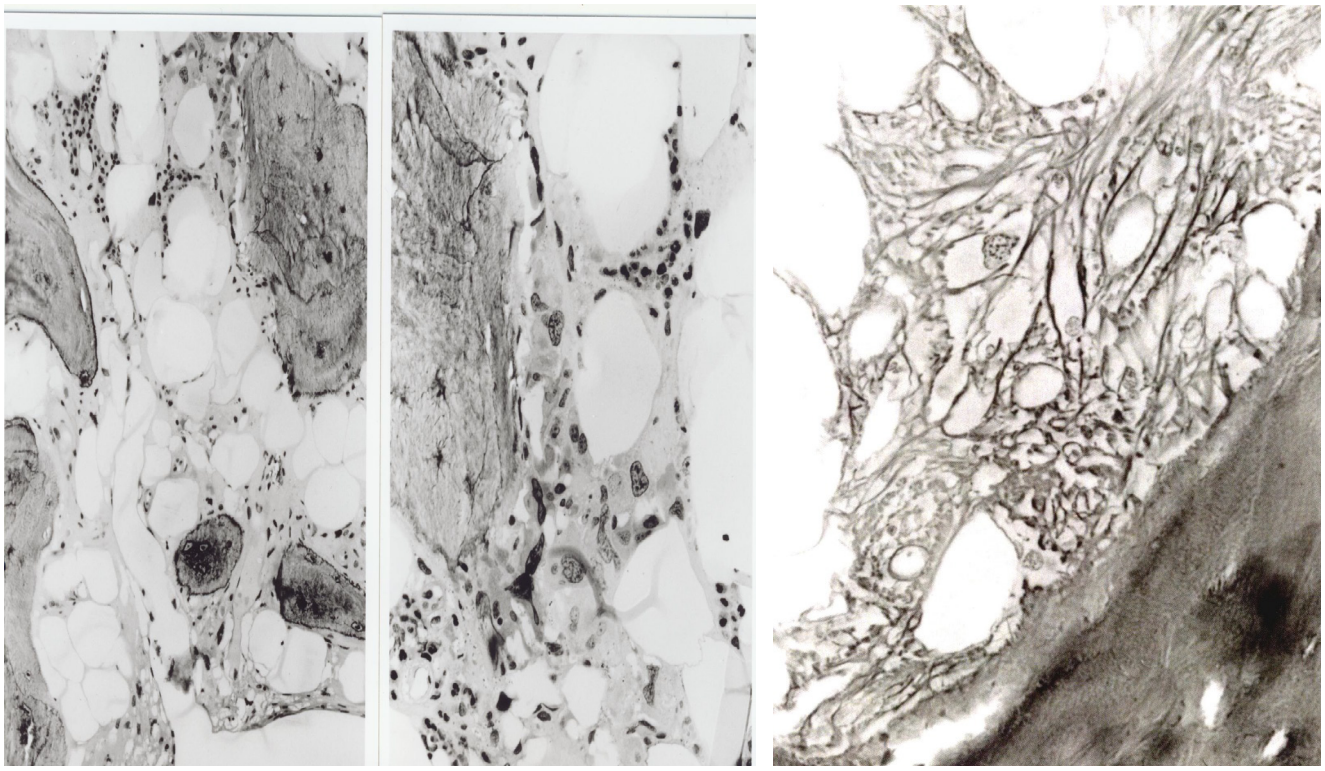

Figure 6. Bone marrow histology (1996) in the proband II3 at age 63 of the Dutch HET family caused by a gain of function mutation in the TPO gene showing a hypocellular bone marrow with the increase of reticulin fibrosis (RF 3, MF 2) and a few clustered megakaryocytes with hypolobulated nuclei.

Second, typically clustering and increase of enlarged megakaryocytes showing mature cytoplasm and hyperploid nuclei in normocellular bone marrow. Third, there was no preceding or allied another subtype of myeloproliferative disorder (MPD) or myelodysplastic syndrome (MDS).

Based on this analysis of the Dutch HET family by Michiels in 1994 and his knowledge at that time of the publications on the discovery of TPO and the experimental ET TPO^{high} mice model [1-5], Michiels suggested in October 1994 to search for a TPO gene mutation in this Dutch family with autosomal hereditary ET (Dutch HET family pedigree, Figure 1, Table 1) [7]. The Department of Medical Genetics of the Erasmus University Medical Center (EUMC) Rotterdam looked into molecular genetics and tried to detect a mutation in the TPO gene but failed. Dr van der Maas referred this question to Dr. Skoda, Basel University, Switzerland who discovered a point mutation in the TPO gene, which according to the transfection studies was very likely the cause increased plasma levels of TPO [8]. A C→G transversion in the splice donor of intron 3 co-segregated with the affected autosomal dominant hereditary ET (HET) in the family. This mutation destroys the splice donor site in intron 3 and results in exon 3 skipping. The resulting shortened 5'UTR leads to overproduction of thrombopoietin by a mechanism of increased efficiency of the TPO mRNA translation. It was already known that in vivo increased TPO levels were very likely responsible for the etiology and phenotype of hereditary ET (HET). Autosomal dominant hereditary ET (HET) due to a gain of function mutation in the TPO gene in the Dutch family appeared to cause marked increased TPO levels, with typical clinical manifestations of microvascular circulation disturbances including erythromelalgia and atypical transient ischemic attacks similar to acquired ET [10]. Peripheral blood and bone marrow morphology of the proband (case II3, man born in 1934, showed the combined increase of platelet count 880 $x10^9$/l) and plasma thrombopoietin (TPO) of 530 ± 27 as compared to controls <62 pg/ml due to a germline gain of function mutation in the TPO gene [8,13]. The presence of large platelets in a peripheral blood smear, enlarged megakaryocytes in a bone marrow smear and a typical WHO-defined ET picture in bone marrow biopsy specimens is diagnostic for ET (figures 4, and 5). In the study of Kralovics *et al,* the patients in the Dutch HET family showed no endogenous erythroid colony (EEC) formation in the absence of EPO in all affected members with hereditary ET [13]. The proband of the Dutch HET family with the gain of function mutation in the TPO gene showed no further increase of platelet counts, no features of PV, no splenomegaly, but developed myelofibrosis at the age of 73 years (Figure 6) during life-long follow up (personal observations Dr. Michiels).

Skoda, van der Maas, Kralovics & Liu described a second Polish family with HET caused by the identical mutation C→G transversion in the splice donor of intron 3 of the THPO gene in 11 affected family members with autosomal dominant HET (Figure 7, Table 3) [14]. The frequencies of aspirin responsive platelet-mediated microvascular circulation disturbances were similar in the Dutch and Polish HET families did occur at platelet counts were between 400x10^9/L and around 1000x10^9/L (Table 3). This was associated with normal leukocyte and erythrocyte counts, no or slight splenomegaly but an increase of clustered large megakaryocytes in bone marrow with normal myeloid / erythroid ratio and absence of EEC [13,14]. The megakaryocytes were medium-sized and strikingly compact and clustered in the Polnish HET family (Figure 7) similar to the Dutch HET family caused by the same gain of function mutation in the TPO gene (Figures 4 and 5). The size of megakaryocytes and the compactness for megakaryocyte nuclei was significantly higher in the affected members of the Polnish HET family (Figure 7) similar to our bone marrow histology findings in acquired ET carrying the JAK2^{V617F} mutation (Figure 8). The majority of affected patients from the two HET families suffered from episodic attacks of aspirin-responsive erythromelalgia microvascular symptoms (Tables 1 and 3) similar as observed in proven JAK2^{V617F} mutated ET patients (Table 3) [10,15,16]. In 2010 Posthuma et al updated the natural history of the affected members of the Dutch HET family during follow-up 15 years later [17]. Case II2 of the Dutch HET family died at the age of 71 due to myelofibrosis with severe pancytopenia. The bone marrow of Case II2 showed myelofibrosis with dysplastic megakaryopoiesis, granulopoiesis and erythropoiesis, 10% blasts and increased LDH. The peripheral blood showed leukoerythroblastosis, macrothrombocytes, and teardrop erythrocytes. She died 3 months after diagnosis of myelofibrosis. Case II8 of the Dutch HET pedigree had a history of diabetes, hypertension and transient ischemic attack in 1989. Case II8 was referred in 2008 because of fatigue, anemia and fever (hemoglobin 6.1 mmol/L, leukocytes 4.8x10^9/L, platelets 90x10^9/L, 4% blasts and increased LDH, 1509 U/L [17]. Bone marrow cytology was consistent with unclassifiable AML. Complex cytogenetic abnormalities were detected. The AML was refractory to treatment and the patient died at the age of 71 years [17]. Patients II2, II8 and III3 were treated with low dose aspirin and had not received cytoreductive agents [17].

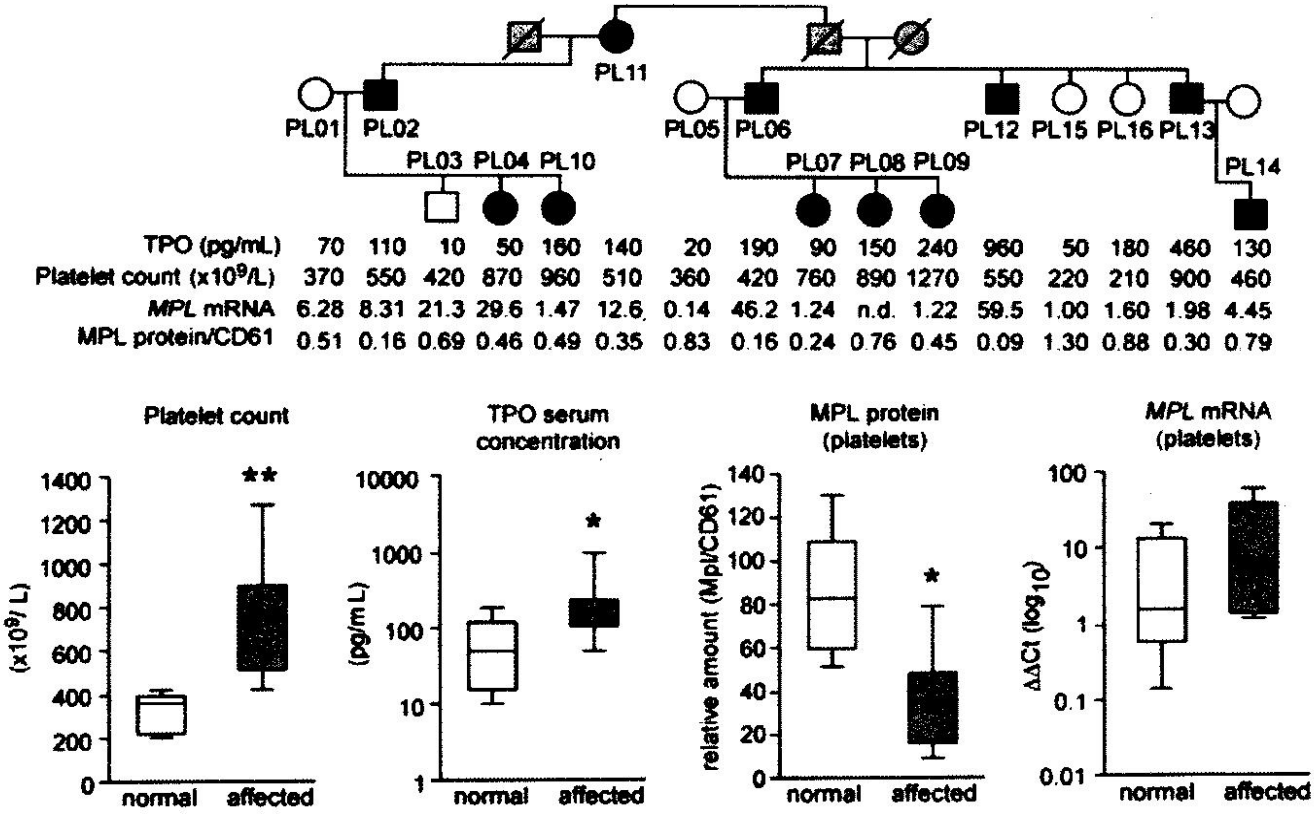

Figure 7. Pedigree of the Polish family with hereditary essential thrombocythemia (HET) due to germline gain of function mutation in the TPO gene (black) and related increased plasma TPO levels and platelet counts in affected as compared to non-affected family members [14]. The figures in the lower part show platelet count, serum TPO, platelet MPL protein (TPO-R) content and MPL mRNA in the affected and non-affected members of the Polnish HET family.

Case HET PL Family	Age Years	Hb g/dL	RBC $x10^{12}$/L	WBC $x10^{9}$/L	Platelets $x10^{9}$/L	TPO HET associated symptoms
PL11	84	15.0	5.0	10.6	550	Acrocyanosis gangrene foot
PL12	59 M	15.6	5.5	5.3	550 - 560	not available
PL13	58 M	15.0	5.0	7.7	510	not available
PL06	56 M	14.5	5.0	6.5	408 - 410	None
PL02	50 F	13.1	4.5	5.9	545 - 560	None
PL04	30 F	12.3	4.7	5.9	595 - 1300	headaches, hypertension
PL07	28 F	13.2	4.7	6.1	760 - 960	TIA, miscarriage, erythromelalgia
PL08	24 F	13.5	4.7	7.1	750 - 890	erythromelalgia venous thrombosis
PL09	24 F	12.7	4.1	6.7	740-1340	Transient ischemic attacks (TIA)
PL10	15 F	14.2	4.1	10.6	960	None
PL14	14 M	12.3	4.6	6.2	460	None

Table 3. Clinical and laboratory features in 11 affected members of the Polish (PL) family with autosomal dominant hereditary essential thrombocythemia (HET) caused by germline gain of function mutation in the TPO gene[14].

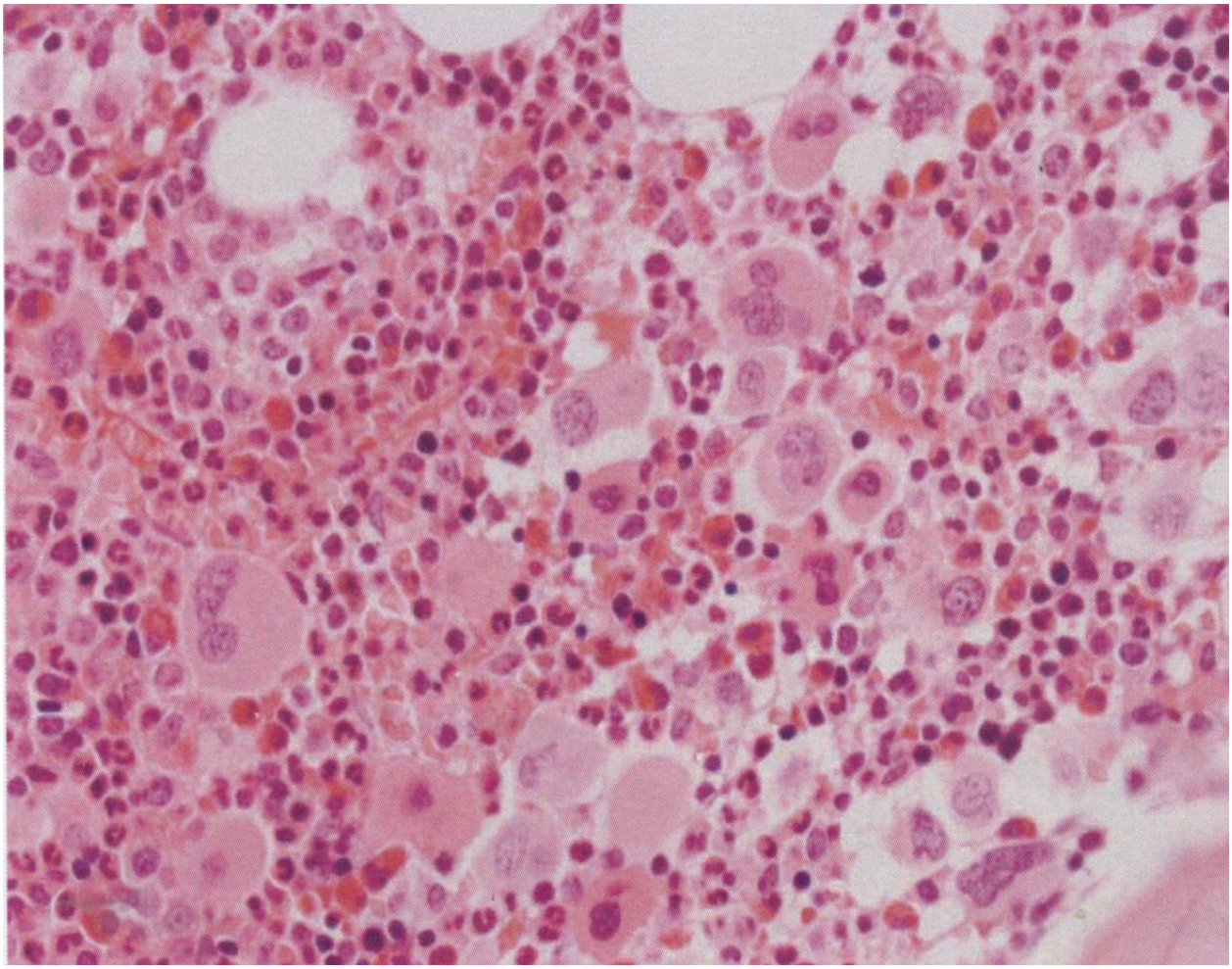

Figure 8. Bone marrow histology showed the increase and loose to dense clustering of medium-sized to large mature megakaryocytes, and normal myeloid to the erythroid ratio of bone marrow nucleated cells, no to slight increased cellularity according to age, no increase of erythropoiesis, and no increase of reticulin fibrosis (RF grade 0 to 1). As compared to controls, the clustered mature normal and medium-sized megakaryocytes were more compact with hyperlobulated nuclei [14].

Sticky platelet mediated thrombophilia in JAK2^{V617F} mutated acquired ET

The exciting findings in 1998 by Skoda in the TPO induced Dutch HET family did have an enormous impact not only on our understanding since 2005 when the molecular defects and pathophysiologic mechanisms in the etiology of acquired ET due to the somatic JAK2^{V617F} mutation was discovered by Constantinescu & Vainchenker (personal communications). On the basis of that discovery we could delineate both TPO induced HET and acquired JAK2^{V617F} mutated ET as a distinct myeloproliferative disorder of megakaryocytes in the bone marrow with the similar clinical presentation of sticky platelet thrombophilia (Table 2) [8,15,16]. The megakaryocytes morphology in symptomatic HET patients due to the gain of function mutation in the TPO gene appeared to be identical in appearance as in acquired heterozygous JAK2^{V617F}mutated ET patients with aspirin responsive platelet mediated erythromelalgia and microvascular complications (sticky platelet mediated thrombophilia (Table 2) [10,15,18,19]. Heterozygous JAK2^{V617F} mutated ET patients show spontaneous endogenous erythroid colony formation (EEC) and have low serum EPO levels[15,16]. In contrast, spontaneous EEC is absent and levels of serum EPO are normal in TPO induced HET. Bone marrow histology in acquired JAK2^{V617F} mutated ET complicated by erythromelalgia is typical features by the increase of clustered large mature pleomorphic megakaryocytes with normal (<60%) to increased celllarity (60-80%) due to increased erythropoiesis in ET and prodromal PV[15,16,18-20]. In 2006 Michiels produced very good evidence that acquired JAK2^{V617F} mutated thrombocythemia in ET and PV patients appeared to be characterized by sticky platelet mediated thrombophilia of aspirin responsive microvascular circulations disturbances caused by platelet mediated arteriolar inflammation and thrombosis in JAK2^{V617F} thrombocythemia in ET and PV patients (Table 2) [15,16]. Besides, acquired ET caused by a somatic gain of function mutation in the MPL gene, MPL^{515}also result in activation of megakaryopoiesis with the presence of large to giant megakaryocytes with hyperlobulated staghorn-like nuclei in the completely normocellular bone marrow. MPL^{515}mutated ET shows no increase of erythropoiesis and absence of PV features of PV [15,18-20] similar as in normocellular ET bone marrow histology observed in the Dutch and Polnish TPO / HET caused by the gain of a function germline mutation in the TPO gene. All affected members in the Polish TPO / HET family had normal myeloid/erythroid ratios of nucleated bone marrow cells [14], whereas the myeloid/erythroid ratio is decreased in acquired ET caused by the JAK2^{V617F} mutation in ET and prodromal PV patients [15,16]. Importantly, expression of thrombopoietin receptor/myeloproliferative leukemia (TPO-R=MPL) protein in platelets were decreased reflecting down-regulated TPO-R (MPL receptor). The decreased TPO-R cq MPL receptor expression was associated with increased TPO-R = MPL mRNA expression in platelets indicating an increased TPO-R = MPL receptor protein turn-over metabolism. From these basic research findings it can be concluded that increased levels of TPO in HET patients are caused by a gain of function mutation in the TPO gene that activates the TPO-R MPL receptor pathway thereby causing the typical ET features of increased platelet counts and increase of abnormal polyclonal megakaryocytes arising from normal polyclonal hematopoietic stem cells (Figure 7). JAK2^{V617F} mutated acquired is characterized by proliferation of large mature megakaryocytes associated with a low serum EPO and the presence spontaneous EEC mimicking early-stage PV not meeting the PVSG-WHO criteria of classical PV [15,16,18-20]. Bone marrow histology of acquired MPL^{515} mutated ET is featured by proliferation of large mature megakaryocytes in a normal cellular bone marrow, whereas in CALR mutated ET associated with primary megakaryocytic granulocytic myeloproliferation (PMGM) the megakaryocytes are large, dysmorphic and immature with cloud-like nuclei, which are never seen in JAK2^{V617F} mutated ET and PV [16,18-23].

HET due to germline JAK2 gain of function mutations and sticky platelet mediated thrombophilia

The germline gain of function mutation JAK2^{V617I} is the sole driver mutation in JAK2^{V617I}-positive individuals in a family with dominant hereditary essential thrombocythemia (HET) associated with clinical manifestations consistent with platelet mediated thrombophilia without features of PV (Figure 9, Table 4) [24,25]. Phenotypic hematopoietic stem cells (HSCs) were increased in the blood and bone marrow of JAK2^{V617I}-positive individuals and were sustained at higher levels than controls after xenotransplantation. In signaling and transcriptional assays, JAK2^{V617I} demonstrated more activity than wild-type JAK2 but substantially less than JAK2^{V617F} (Figure 10). After cytokine stimulation, JAK2^{V617I} resulted in markedly increased downstream signaling compared with wild-type JAK2 and comparable with JAK2^{V617F} [25]. These findings demonstrate that JAK2^{V617I} induces sufficient cytokine hyperresponsiveness in the absence of other molecular events to induce a homogeneous ET phenotype without features of PV. The authors also provide evidence that the JAK2^{V617I} mutation may expand the HSC pool, providing insights into both JAK2 mutation biology and MPN disease pathogenesis [25].

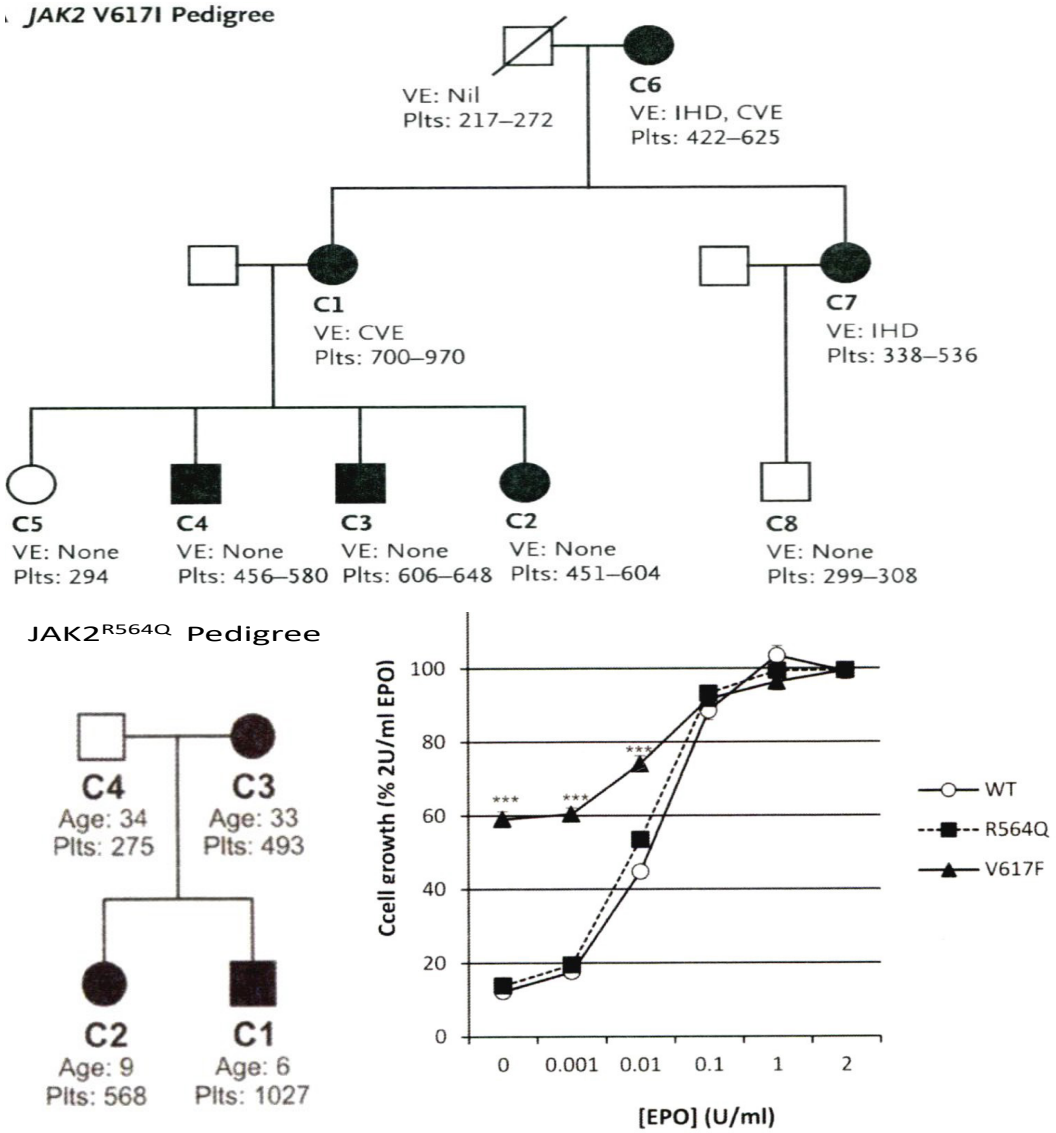

Figure 9. Upper. Pedigree of the UK family with dominant hereditary essential thrombocythemia (HET) due to germline gain of function mutation JAK^{V617I} showing the presenting vascular events (VE) IHD = ischemic heart disease and CVE = ischemic cerebrovascular event related to increased platelet counts in the affected JAK2^{V617I} mutated HET patients (Table 4) [24,25].

Lower. Pedigree of the UK family with dominant hereditary essential thrombocythemia (HET) due to germline gain of function mutation JAK2^{R564Q} (Table 4) [26]. Affected JAK2^{R564Q} mutated individuals do need EPO to induce spontaneous endogenous erythroid (EEC) colony formation in JAK2^{R564Q} HET as compared to spontaneous EEC in acquired JAK2^{V617F} ET [26].

JAK2 ^{V617I} case	C1	C2	C3	C4	C6	C7
Age at diagnosis	53	34	36	38	79	61
Gender	F	F	M	M	F	F
Hemoglobin g/dL	15.7	14.7	15.6	14.1	15.4	14.9
Erythrocytes x 10^{12}/L	4.6	4.7	4.9	4.9	4.6	4.7
MCV fl	100	92	97	89	100	93
WBC x10^9/L	10.6	9.6	8.3	8.9	7.2	6.8
Neutrophils x10^9/L	5.9	5.5	4.5	4.3	3.9	4.7
Platelets x 10^9/L	**750**	**600**	**648**	**526**	**645**	**445**
EPO IU/L	nt	9.1	5.9	10.6	nt	nt
TPO pg/mL	nt	99	113	79	nt	nt
Ferritin ug/L	nt	127	146	290	nt	nt
V617I allelic level						
MCN %	51	52	49	50	51	51
$CD66^+$ %	51	51	50	50	nt	nt

Table 4. Laboratory characteristics of six affected members of dominant JAK2^{V617I} positive Hereditary Essential Thrombocythemia (HET). Platelet counts in 6 affected family members ranged from 445 to 750x10^9/L [24,25].

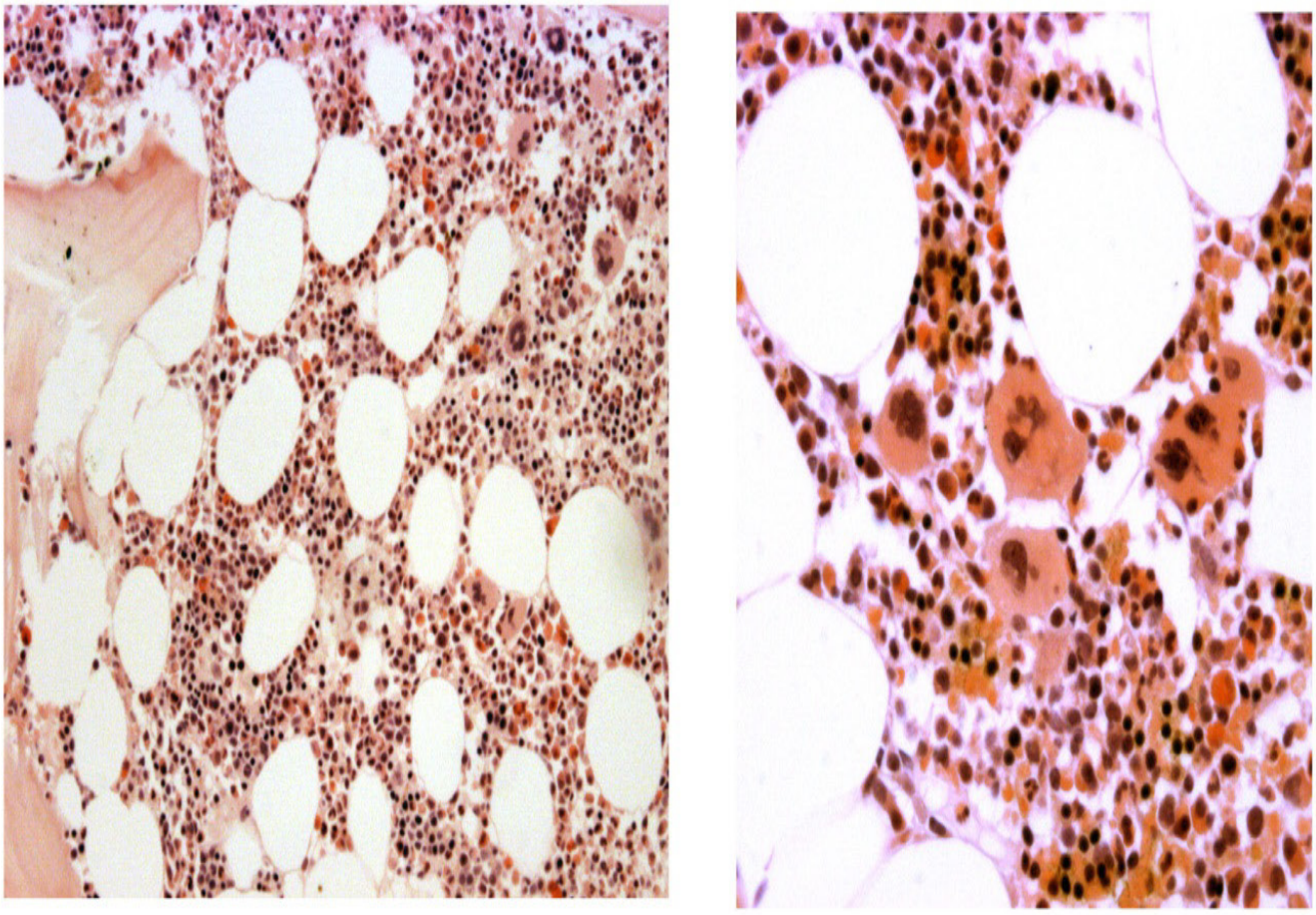

Figure 10. Bone marrow histology from case C1 (left) and case C4 (right) of the family with JAK2^{V617I} mutated HET showing a normal cellular bone marrow with increase and clustering of large megakaryocytes with some lobulation of the nuclei consistent with ET (Table 4) [24.25].

The germline mutation JAK2^{R564Q} was identified in a family with autosomal dominant hereditary essential thrombocythemia (HET, Figure 9) [26], JAK2^{R564Q} expressing mutated cells show increased cell growth due to suppression of apoptosis in Ba/F3-MPL cells. Although JAK2^{R564Q} and JAK2^{V617F} have similar levels of increased kinase activity, the growth-promoting effects of JAK2^{R564Q} cells are much milder than those of JAK2^{V617F}. The investigators also found higher levels of pSTAT1 and pSTAT3 in cells expressing JAK2^{V617F}, compared to JAK2^{R564Q} and total STAT1 levels were also increased with JAK2^{R564Q} expression, compared to wild type JAK2 and this effect was even more prominent with JAK2^{V617F} [26]. An overall increase in downstream signaling in mutant JAK2 cells under starved conditions was further demonstrated. Tyrosine-phosphorylation of proteins was also up-regulated in JAK2^{R564Q}-expressing cells, compared to wild type *JAK2*, and this was even more robust in the JAK2^{V617F}-expressing mutants. Similar increased signaling was observed in JAK2R^{564Q}-positive patients. The growth characteristics in response to TPO-treatment of cells expressing JAK2^{V617F}, either with or without JAK2^{R564Q}, were significantly increased as compared to wild type JAK2-expressing cells at all concentrations of TPO. JAK2^{R564Q}-expressing cells also showed significantly increased proliferation in response to TPO, compared to wild type JAK2 cells (Figure 11). Baseline pSTAT3 activity was not different from that of normal controls and was supported by a lack of cytokine-independent colonies clearly distinct from the presence of JAK2^{V617F} cytokine-independent colony endogenous erythroid colony (EEC) formation. After stimulation with granulocyte colony-stimulating factor, JAK2^{V617I} positive peripheral blood CD33+ myeloid and CD34+ stem and progenitor cells showed a marked increase in pSTAT3 levels, particularly in response to low concentrations of granulocyte colony-stimulating factor. These findings suggest that JAK2^{V617I} causes limited constitutive activation but results in a considerably reduced threshold for cytokine-induced activation thereby explaining why HET caused by the germline gain of function mutation JAK2^{V6171} is associated with an ET phenotype without features of PV (Figure 11) [26].

Sticky platelet mediated thrombophilia in MPL^{Ser505Asn} germline mutated HET

In 2004 Ding et al described the first case of congenital ET in the pedigree of a Japanese family caused by a G to A nucleotide substitution at position 1073 in exon 10 of the MPL gene leading to the exchange of serine for asparagine position 505 (MPL^{S505N}) [27]. The presence of the germline MPL^{S505N} mutation is associated with autonomous activation in the MPL downstream signaling pathways, both in vitro in cells transfected with the mutant and in vivo in platelets obtained from affected individuals. Hematopoietic cells expressing MPL^{S505N} showed autonomous phosphorylation of both Mek1/2 and Stat5 down signaling transduction pathways. The clinical course of the disease in terms of vascular complications was not reported in affected members of this family with HET carrying the MPL^{S505N} mutation. The 2010 study of Teofili et al on eight Italian families with autosomal dominant HET positive for the MPL^{S505N} mutation reported the clinical manifestations and hematological features in 41 affected patients: 21 HET patients with a proven MPL^{S505N} mutation and 20 HET patients with clinical HET (Figure 12) [28]. The family history of 41 individuals reported 15 major thrombotic episodes in 14 members (34%): Budd-Chiari syndrome age 17 in 1, deep vein thrombosis leg age 41 in 1, ecclampsia and fetal death in1, stroke at ages 43,72, 76 and 80 in 4 and myocardial infarction in 1. The major thrombotic events occurred at ages between 31-81 years (median 52). These patients were not on aspirin at the time of the major thrombotic event. After a major thrombotic event HET patients were over-treated with hydroxyurea or pegylated interferon (IFN) according to Italian guidelines [28]. The overall survival and thrombosis-free survival in 41 affected HET family members carrying the MPL^{S505N} mutation was compromised as compared to normal overall and thrombosis-free survival in non-affected family members. Clinical manifestations of aspirin responsive microvascular disturbances including erythromelalgia, migraine-like atypical cerebral transient ischemic attacks (MIAs) and visual ischemic disturbances (sticky platelet mediated thrombophilia) usually precede the occurrence of major thrombosis in congenital and acquired in ET of various molecular etiology when not on aspirin[10,15]. The indication of aspirin in 15 out of 21 MPL^{S505N} mutated ET cases was a migraine-like headache and none experienced bleeding complications during follow-up. Fourteen out of 21 documented MPL^{S505N} carriers were free of major thrombosis during follow-up and did not develop splenomegaly in 13 of them at ages between 2 and 76 years (2, 4, 7, 20, 25, 28, 31, 34, 42, 69 and 76 years) [28].

Three stages of atypical evolution megakaryopoiesis and increase of reticulin fibrosis (RF) in bone marrow biopsies reflect the natural history of MPL^{S505N} ET from normocelluar or hypercellular ET in adolescent into hypocellular MF in elderly patients[28]. At age 16 years a hypercellular ET with increased neutrophils and loose clusters of atypical megakaryocytes was associated with normal erythropoiesis and increase of RF grade 0/1. At age 43 years moderate hypercellularity (70%) in ET and dense clusters of atypical megakaryocytes was associated with an increase of RF

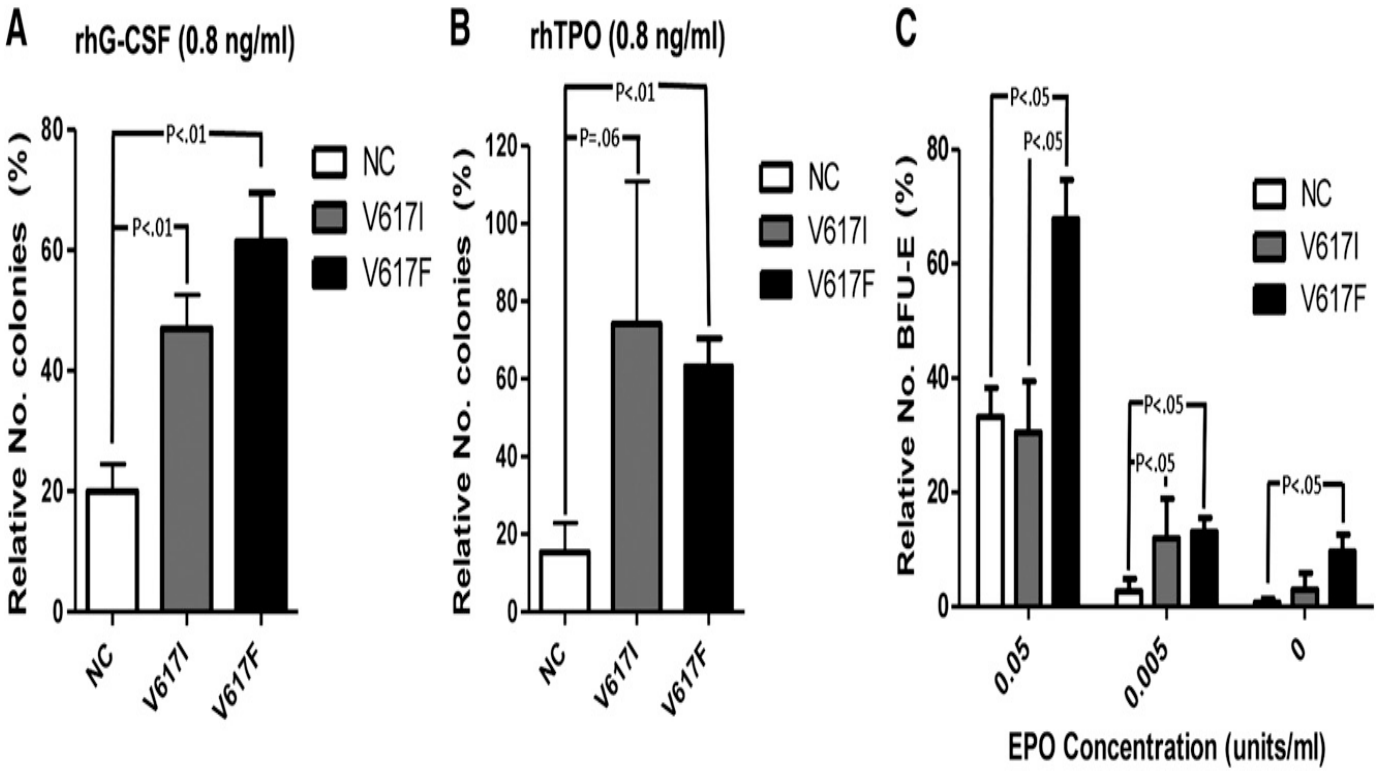

Figure 11. Response (number of colonies) of CD33+ myeloid and CD34+ hematopoietic bone marrow progenitor cells to recombinant TPO (0.8 ng/ml, left) and to EPO concentration (units/ml, right) in normal controls (NC), in congenital JAK2^{V617I} mutated HET patients and in JAK2^{V617F} mutated ET patients. The responses to TPO are equally increased in JAK2^{V671I} and JAK2^{V617F} mutated CD33 and CD34+ cells, but the response to EPO is normal in JAK2^{V671I} and increased in JAK2^{V617F} mutated hematopoietic progenitor cells in the study of Mead et al [25].

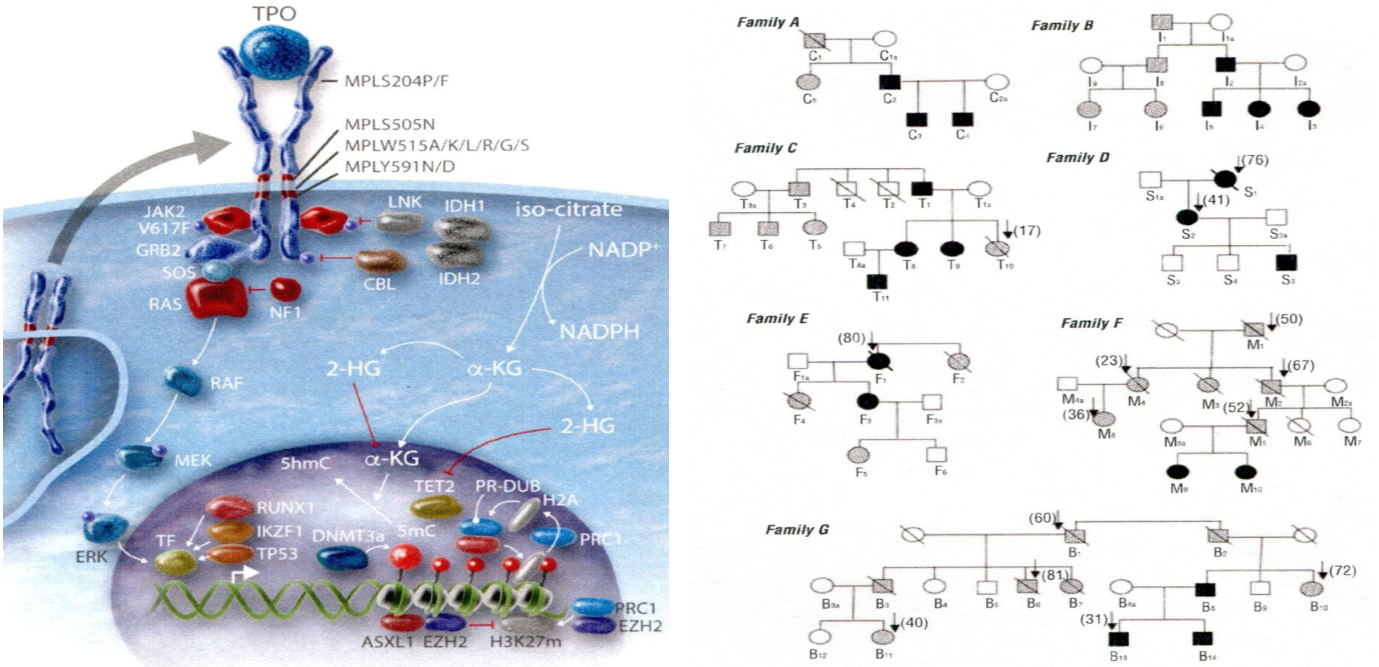

Figure 12. Left. Molecular etiology of dominant TPO mediated hereditary essential thrombocythemia (HET), MPL^{S505N} mutated HET, JAK2^{V671I} and JAK2^{R564Q} mutated HET and MPL^{515} and JAK2^{V617F} mutated acquired essential thrombocythemia (ET) according to Vainchenker & Kralovics in Blood 2017 [40]. **Right.** Dominant Hereditary Essential Thrombocythemia (HET) caused by germline MPL^{S505N} mutation in 21 affected members from seven families pedigrees and the molecular etiology of congenital and acquired MPL mutated thrombocythemias without features of PV [28].

2 = MF 1. At age 69 years a hypocellular bone marrow with numerous distorted megakaryocytes was associated with a diffuse increase in RF grade 3 to 4 and focal bundles of collagen myelofibrosis MF-3 with osteosclerosis [28].

In the cohort of 21 HET patents with a documented MPL^{S505N} mutation four cases presented with major thrombosis (19%); stroke at age 76 and 80 in 2, DVT/TIA at age 41/43 in 1 and myocardial infraction at age 31 in 1. Eight MPL^{S505N} patients had myelofibrosis (MF) [28] grade MF1 in 5 and grade MF2 in 3 at ages 76, 55, 80, 28, 23, 33, 67, which was associated with mild to moderate splenomegaly (spleen length diameter 14,5 to18 cm). Five of these MF patients were treated for several years with hydroxyurea (HU) in 3, interferon (IFN) in 1 and HU/IFN in 1, and four of these five MF cases had anemia as a side effect of hydroxyurea and/or myelofibrotic transformation. All MF1 or MF2 patients had normal leukocyte counts except one female at age 72 years. Six anemic cases at hemoglobin levels between 10 and 11.9 g/dL had platelet counts between 317 and $963x10^9$/L. At hemoglobin levels above 12 g/dl platelet counts ranged from 605 to $1726x10^9$/L. Leukocyte counts were completely normal except leucocytosis above 10 in 3 young affected children (age 4 to 7 years) and one female at age 72. Among 9 deceived family members 3 died prematurely of major thrombosis, 3 from anemia with hypocellular myelofibrosis (2 cases at age 76 and 80 years), and 3 from liver cirrhosis, gastric cancer or undefined. The overall survival and thrombosis-free survival was significantly shortened in MPL^{S505N} mutated HET patients. The maximum life expectancy of MPL^{S505N} family members with HET was 50% at 80 years, as compared to 90% at 80 years of non-affected family members without HET [28]. The loss of life expectancy is mainly due to major thrombosis and myelofibrosis. Two of 3 MPL^{S505N} cases died from endstage hypocellular myelofibrosis at ages of 76 and 80 years.

Sticky platelet mediated thrombophilia in acquired MPL^{515} mutated ET

The frequency of the $MPL^{W515L/K}$ mutation in the two large studies was 5.3% in the $JAK2^{V617F}$ wild type (WT) ET and 9.6% in $JAK2^{V617}$ wild type PMF patients [29,30]. In the GIMEMA population of 952 ET patients, subdivided into 546 $JAK2^{V617F}$ mutated (57%) and 418 JAK2 wild type (43%), Vannucchi *et al* found 30 (3% of total ET and 7.2% of JAK2 wild type ET) carrying the MPLW515WL/K mutation. $MPL^{W515L/K}$ and $JAK2^{V617F}$ coexisted in 3 patients with MPL^{W515L} and 5 with MPL^{W515K} allele [31]. Splenomegaly on palpation was only present in 5 (17%) of 30 MPL^{515} mutated ET cases as compared to 21% in $JAK2^{V617F}$ mutated ET and 20% in JAK2/MPL wild type ET (in retrospect mainly CALR mutated). Mutation allele burden was greater than 50% in half of the MPL^{W515K} patients compared to 17% of MPL^{W515L} mutated ET patients [31]. The 30 MPL^{515} mutated ET patients in the study of Vannucchi *et al* had mean platelet count of 956 ± 331 $x10^9$/L and presented with arterial events at diagnosis in 10% and at follow-up in 13% venous thrombosis at diagnosis in 3% and at follow-up in 7% and microcirculatory disturbances consistent with sticky platelet mediated thrombophilia in 60% [31].

The laboratory data in 35 cases of acquired $MPL^{W515L/K}$ mutated ET and ET with various degrees of myelofibrosis (MF) in the German study of Schnittger et al were diagnosed according to WHO criteria as ET in 24 and MF in 10 patients [32]. Platelet count in 24 $MPL^{W515L/K}$ mutated ET patients ranged from 420 to $1500x10^9$/L and from 22 to $400x10^9$/L in nine $MPL^{W515L/K}$ mutated MF cases with one exception of $726x10^9$/L in one MF case. MPL^{515} mutated ET have normal serum EPO and ferritin levels at diagnosis and follow-up. Serum EPO levels in $MPL^{W515L/K}$ mutated ET patients were normal but significantly lower to decreased in $JAK2^{V617F}$ positive ET (prodromal PV) [27,28]. Two patients with the MPL^{W515L} and MPL^{W515K} mutations in the study of Chaligne *et al* showed a spontaneous megakaryocyte growth in culture with an overall normal response to thrombopoietin (TPO), but the erythroid progenitors remained EPO dependent and did not show spontaneous erythroid colony (EEC) formation [33].

As compared to acquired $JAK2^{V617F}$ positive ET both erythroid and granulocytic cellularity were reduced in the MPL^{515} mutant group (P<.001) with the absence of PV features [19,20]. We investigated bone marrow histology in a case of MPL^{515} mutated acquired ET with a high platelet count of $1243x10^9$/L as the only presentation in an asymptomatic 73-year-old woman (**Figure 13**). Laboratory features at time of diagnosis were, hemoglobin 12.5 g/L, hematocrit 0.39, erythrocytes $4.2x10^{12}$/L, MCV 82 fL, leukocytes $6.9x10^9$/L, normal LDH and spleen size on echogram 12.6 cm (normal value <12 cm). This case of acquired MPL^{515} mutated ET typically shows large to giant megakaryocytes with hyperlobulated nuclei in a completely normocellular bone marrow with no increase of erythropoiesis (Figure 13). Clustering of large mature large to giant megakaryocytes with staghorn-like hyperlobulated in a normocellular bone marrow appears to be the hallmark of acquired JAK2-wild type MPL^{515} mutated thrombocythemia [34,35]. As compared to $JAK2^{V617F}$ mutated ET [34], bone marrow histology in MPL^{515} mutated ET patients revealed

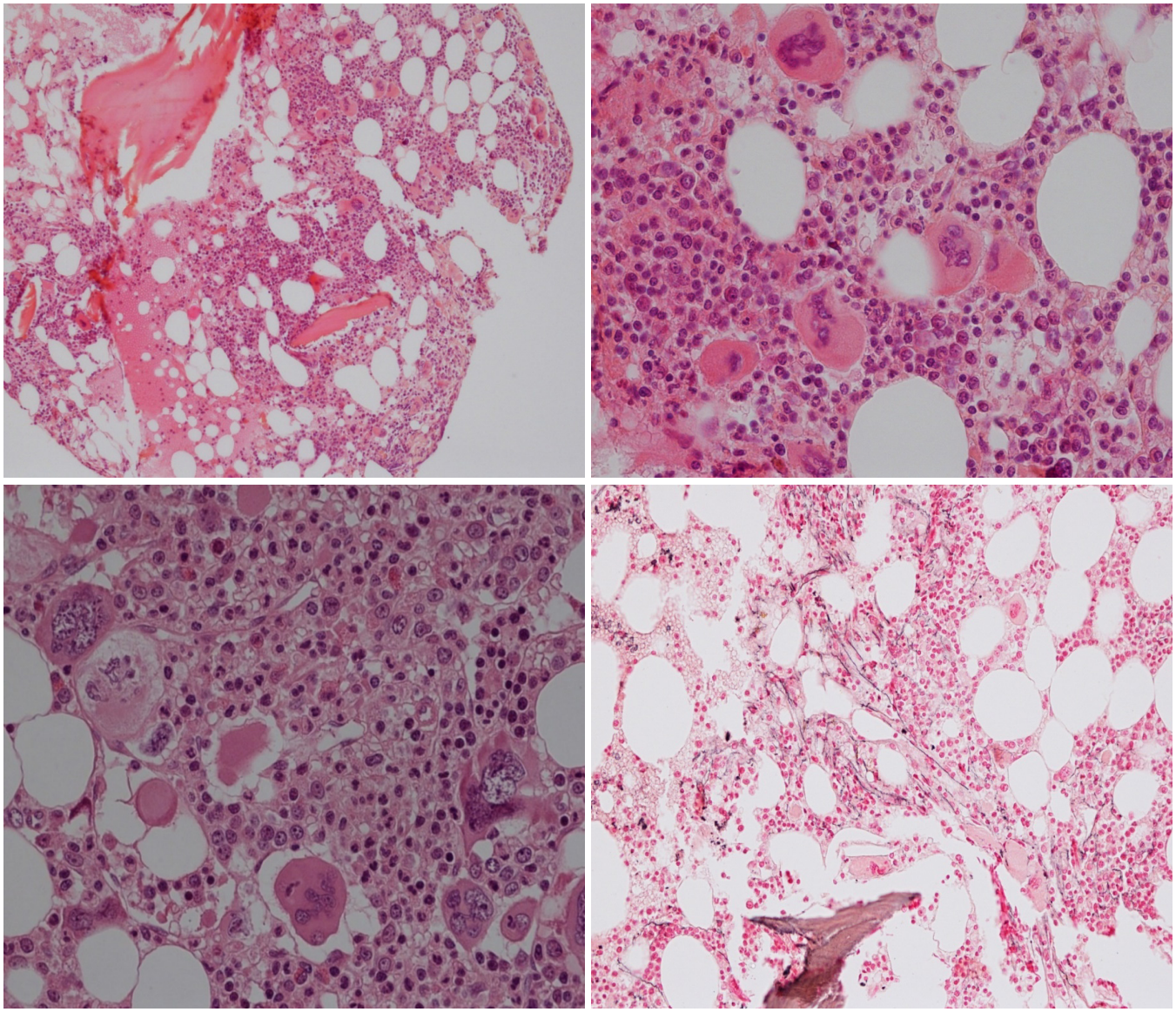

Figure 13. Bone marrow histology from a woman, age 73 at time of diagnosis of MPL^{515} mutated ET featured by platelet count of $1273x10^{9}$/L, normal red and white blood cells, no splenomegaly and bone marrow histology showing increase and clustering of large and giant mature megakaryocytes with hyperlobulated staghorn-like in a normocellular bone marrow with increased reticulin fibrosis (RF 2).

more isolated megakaryocytic proliferation in normocellular bone marrow at diagnosis with a reduction of erythropoiesis during follow-up and increase of reticulin fibrosis in hypocellular bone marrow [19,20,34,35]. Table 5 summarises the characteristic laboratory findings in TPO, $JAK2^{V617I}$ and MPL^{S505N} mutated hereditary essential thrombocythemia (HET) versus $JAK2^{V617F}$ and MPL^{515} mutated acquired essential thrombocythemia (ET).

Absence of sticky platelet mediated thrombophilia in CALR mutated ET

By targeted whole-exome sequencing in 6 JAK2/MPL negative primary myelofibrosis (PMF) patients Kralovics and his team discovered somatic mutations of a 52-bp deletion in 1, of 1bp deletion in 1 and recurrent 5-bp insertion in 4 MF patients [36]. Subsequent CALR mutation screening and sequencing in a large Austrian Italian cohort of 896 MPN patients, Kralovics detected CALR mutations in 78 of 311 (25%) ET, in 72 of 203 (35%) PMF but in none of 382 PV patients. A total of 36 types of somatic CALR mutations (insertions and deletions) appeared to be caused by a frameshift reading frame with the resulting mutant CALR protein that shares a novel sequence at the C-terminal with positively charged amino acids, whereas the c-terminal of normal CALR protein is negatively charged. The 52-bp deletions (CALR type 1 in 53%) eliminate almost all negatively charged amino acids, whereas the 5-bp insertions (CALR type 2 in 31.7%) retain approximately half of the negatively charged amino acids [31]. The other mutations including type 3 (1.7%),

Category HET vs ET	Number patients	Platelet x10^9/L Range		Plasma TPO pg/mL	EEC	Serum EPO
Dutch TPO HET	10	880	1280	**increased**	neg	normal
Polish TOP HET	11	701	1340	**increased**	neg	normal
JAK2 ^{V617I} HET	**6**	**445**	**750**	normal	neg	normal
JAK2 ^{V617F} ET	**6**	**425**	**814**	normal	**Pos**	**decreased**
MPL ^{S505N} HET				normal	neg	normal
No splenomegaly	9	627	1726			
Splenomegaly (S)	11	317	1060			
MPL $^{W515L/K}$ET	23	380	1500	normal	neg	normal

Table 5. Characteristic laboratory findings in TPO, JAK2^{V617I} and MPL^{S505N}mutated hereditary essential thrombocythemia (HET) versus JAK2^{V617F} and MPL^{515} mutated acquired essential thrombocythemia (ET).

type 4 (1.0%), types 5 to10 (0.7% each), types 11 to 36 (0.3% each) were observed in 15.3% at low frequencies for each type only in a single patient [36].

The clinical and laboratory findings in our series of 13 consecutive cases of CALR mutated thrombocythemia seen between 2014 and 2019 presented with the diagnosis of prefibrotic ET associated with a typical PMGM: ET/PMGM bone marrow histology in 11 cases (85%) and MF/PMGM bone marrow histology in 2 cases (15%). ET/PMGM patients presented with fatigue only not suffering from constitutional symptoms. Platelet mediated erythromelalgia microvascular ischemic events were not recorded at increased platelet counts of between 400 and 1000x10^9/L. Two ET/PMGM cases presented with hemorrhagic manifestation at a platelet count above 1000x10^9/L. The two MF/PMGM cases presented with anemia and splenomegaly had platelet counts of 265 and 347x10^9/L respectively. CALR mutated ET/PMGM showed dense clusters of large immature megakaryocytes with immature bulky, cloud-like nuclei in anormocellular bone marrow with normal erythropoiesis and no increase of reticuline fibrosis grade 0 (RF 0) (Figure 14) or with a typical hypercellular PMGM bone marrow due to increase of dual megakaryopoiesis and granulopoiesis with relative reduction of erythropoiesis and the presence of fine reticuline fibrosis grade 1 (RF 1) [19,20]. Two cases of CALR mutated ET/PMGM showed increase of reticulin fibrosis grade 2 (RF 2) and clusters of large megakaryocytes with dysmature, bulky, cloud-like nuclei in a normocellular to slightly increased cellular bone marrow. One case of MF/PMGM showed dense clustered large dysmature megakaryocytes with hyper-lobulated nuclei in a hypercellular MG bone marrow with increased reticulin fibrosis grade 2(RF 2) and the second case of CALR mutated MF/PMGM presented with asymptomatic splenomegaly, no constitutional symptoms and symptomatic transfusion-dependent anemia associated with advanced myelofibrosis (RF 4, MF 3)) in a hypocellular bone marrow showing dysmorphic immature megakaryocytes in fibrotic parts of the bone marrow (Michiels et al 2019,manuscript in preparation).

Absence of sticky platelet thrombophilia in BCR/ABL positive ET

The clinical symptoms and hematological features in 23 cases of Philadelphia chromosome-positive (Ph+) BCR/ABL positive ET are featured by the increase of small mononuclear megakaryocytes in bone marrow smears and biopsy material and no evidence of chronic myeloid leukemia (CML) in peripheral blood [38,39]. As compared to cases of reactive thrombocytosis, the megakaryocytes in BCR/ABL positive ET are smaller than normal ones and typically have hypolobulated round nuclei. This contrasts with the finding of clustered and large megakaryocytes in JAK2^{V617F}, MPL^{515} and CALR mutated ET [19,20,34,35]. Patients diagnosed as BCR/ABL ET may progress to CML and show a high tendency to myelofibrosis and blastic transformation indicating that BCR/ABL positive ET belong to the early manifestations of the chronic stable phase of BCR/ABL positive CML. I n BCR/ABL-positive ET the platelets are small, indolent and non-reactive produced by small megakaryocytes with hypolobulated nuclei [38,39]. BCR/ABL-positive ET patients do not present aspirin responsive platelet mediated erythromelalgic thrombotic or bleedings manifestations at increased platelet count in excess of 400 to 1500 × 10^9/L [10,39]. The platelets and megakaryocytes in JAK2^{V617F} and MPL^{515} mutated ET are large, hyperactive caused by constitutively activated large and mature megakaryocytes and associated with a high risk on aspirin re-

sponsive platelet-mediated inflammation and thrombosis in the end-arterial circulation [10] or sticky platelet mediated thrombophilia in ET and PV patients (Table 2) [15] also seen in TPO, JAK2 and MPL mutated HET and in acquired MPL^{515} mutated ET but not in CALR mutated and BCR/ABL positive acquired ET.

Contributions of authors: JJM and HDR designed the study and wrote the manuscript thanks to significant contributions by all authors between 1980 and 2018.

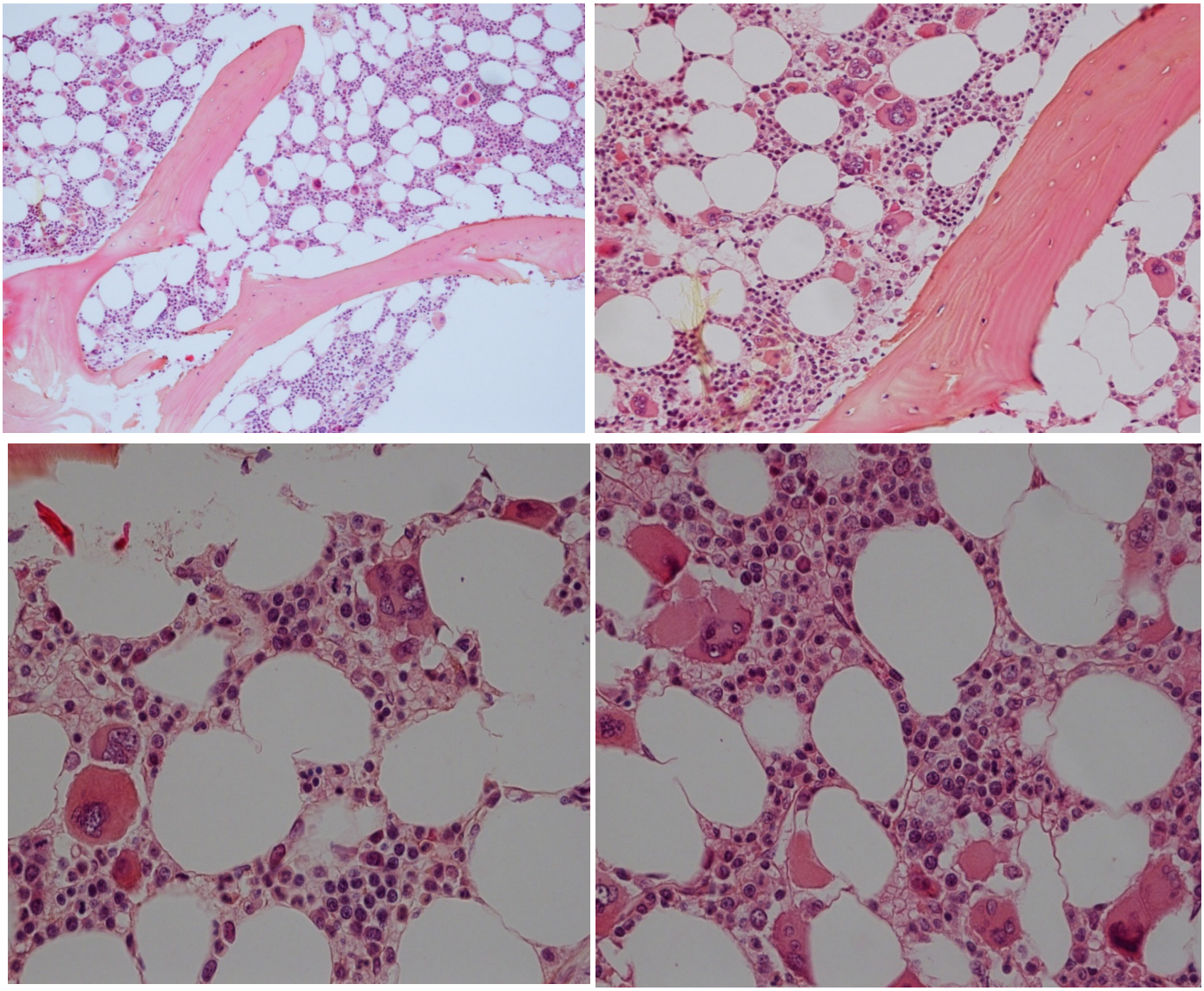

Figure 14. Bone marrow histology of CALR mutated ET in an asymptomatic man, age 39, normally red and white blood cells, no splenomegaly and bone marrow histology showing dense clusters of large immature megakaryocytes with immature bulky, cloud-like nuclei in the normocellular bone marrow and no increase of reticuline fibrosis (RF 0).

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