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REVIEW

Aspirin cures erythromelalgia and cerebrovascular disturbances in JAK2-thrombocythemia through platelet-cycloxygenase inhibition

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Abstract

Hypersensitive (sticky) platelets in JAK2-mutated essential thrombocythemia (ET) and polycythemia vera (PV) with thrombocythemia spontaneously activate at high shear in arterioles, secrete their inflammatory prostaglandin endoperoxides and induce platelet-mediated arteriolar fibromuscular intimal proliferation. Constitutively activated JAK2 mutated hypersensitive (sticky) platelets spontaneously aggregate at high shear in the endarteriolar circulation as the cause of aspirin responsive erythromelalgia and platelet arterial thrombophilia in JAK2-mutated thrombocythemia patients. Increased production of prostglandin endoperoxides E2 and thromboxane A2 released by activated sticky platelets in arterioles account for redness warmth and swelling of erythromelalgia and platelet derived growth factor can readily explain the arteriolar fibromuscular intimal proliferation. Von Willebrand factor (VWF) platelet rich occlusive thrombi in arterioles are the underlying pathobiology of erythromelalgic acrocyanosis, migraine-like transient cerebral attacks (MIAs), acute coronary syndromes and abdominal microvscular ischemic events. Irreversible platelet cyco-oxygenase inhibition by aspirin cures the erythromelalgia, MIAs and microvascular events, corrects shortened platelet survival to normal, and returns increased plasma levels of beta-TG, platelet factor 4, thrombomoduline and urinary thromboxane B2 excretion to normal in symptomatic JAK2-thrombocythemia patients. *In vivo* activation of sticky platelets and VWF-platelet aggregates account for endothelial cell activation to secrete thrombomoduline and sVCAM followed by occlusion of arterioles by VWF-rich platelet thrombi in patients with erythromelalgic thrombotic thrombocythemia (ETT) in ET and PV patients. ETT is complicated by spontaneous hemorrhagic thrombocythemia (HT) or paradoxical ETT/HT due to acquired von Willebrand disease type 2A at platelet counts above 1000 × 10^9/L and disappears by cytoreduction of platelets to normal (< 400 × 10^9/L).

Key words: Aspirin; Wonder drug; Erythromelalgia; Cerebral vascular disturbances; Platelet cyclooxygenase;

Migraine

Core tip: About seventy years after the synthesis by Hoffmann, acetyl salicylic acid (aspirin) has been discovered in the late 1970s as a wunder drug that cures erythromelalgia and migraine-like cerebral microvascular disturbances by irreversible blockage of platelet cyclo-oxygenase mediated arteriolar inflammation and thrombosis in JAK2-mutated thrombocythemia of patients with essential thrombocythemia (ET) and polycythemia vera (PV). The ADP (P2Y12) receptor inhibtors ticlopedin and clopidogrel, other platelet inhibitors that do not affect platelet cycooxygenase, and coumarin are ineffective in the treatment of erythromelalgia and cerebral vascular thrombotic complications in ET and PV.

Michiels JJ. Aspirin cures erythromelalgia and cerebrovascular disturbances in JAK2-thrombocythemia through platelet-cycloxygenase inhibition. *World J Hematol* 2017; 6(3): 32-54 Available from: URL: http://www.wjgnet.com/2218-6204/full/v6/i3/32.htm DOI: http://dx.doi.org/10.5315/wjh.v6.i3.32

PLATELET AND ENDOTHELIAL CELL PROSTAGLANDIN METABOLISM IN THE 1970s

Aspirin (acetyl salicylic acid) and aspirin like drugs inhibited prostaglandin biosynthesis (Vane 1971)[1] leading to impaired prostaglandin E2 and thromboxane A2 (TxA2) synthesis in platelets (Smith and Willies 1970)[2,3]. Human platelets do form and release prostaglandin (PG) E2 and PGF2α from the precursor arachidonic acid (AA) in platelets released from the platelet membrane phopholipids in thrombin stimulated platelets[2,3]. AA (0.5 mmol/L) causes aggregation of platelets in platelet-rich plasma (Silver *et al*[3] 1973). AA in low amounts (0.1 mmol/L) enhance platelet aggregation induced by ADP, collagen or epinephrin was observed[4]. AA (0.5 mmol/L), thrombin (1 μ/mL), collagen (80 μL), epinephrin (50 μmol/L) or ADP (50 μmol/L) induced equally amounts of radioactivity released from platelets preincubated with C14-serotinine or C14-adenine (Figure 1)[4]. Large amounts of PGE2 and PGF2α are formed in platelets in response to AA, but small amounts of PGE2 and PGF2α are releasd from platelets in response to thrombin, collagen and epinephrin (Figure 1) indicating that shear stress induce spontaneous platelet activation produces large amount of platelet prostaglandin endoperoxides G2, H2, D2 and E2, which in retrospect proved to induce the inflammatory signs of erythromelalgia in thrombocythemia patients (Figures 2 and 3)[4]. The inhibiting effect of aspirin on platelet aggregation persisted a few days due to its irreversible inhibition of platelet cyclooxygenase activity[4]). We used the method of Smith *et al*[5] (1976) and measured the production of malodialdehyde (MDA) in platelet rich plasma after incubation with N-ethylmaleimide (NEM) as a measure for the degree of inhibition of cyclo-oxygenase activity and prostaglandin production in platelet (Figure 3). At that time in 1976 we discovered that aspirin cures erythromelalgia in thrombocythemia of ET and PV patients by irreversible inhibtion of platelet cyclo-oxygenase activity as measured by the degree MDA inhibition in platelet rich plasma (Figure 3).

Hemler *et al*[6] (1976) purified cyclooxygenase that forms prostaglandins. Moncada *et al*[7] and Vane *et al*[8] (1976) isolated the enzyme prostaglandin synthetase in endothelial cells from arteries that transformed cyclic endoperoxides to prostacyclin, that strongly inhibit platelet aggregation (Figure 2, 1976 concept of Michiels and Van Vliet). AA is metabolized in platelets and endothelial cells by cyclooxygenase to unstable cycloendoperixides PG2 and PGH2, which in turn is broken down to the stable prostaglandins PGE2, PGF2a and PGD2 (Figure 2)[7,8]. The cyclic endoperoxides in EC are metabolized by prostacylin synthetase into unstable PGI2 (a strong platelet aggregation inhibitor) and its stable inactive endproduct. The cyclic endoperoxides in platelets are metabolized by thromboxane synthetase into the unstable thromboxane A2 (a strong platelet aggregation agonist) and its stable inactive thromboxane B2 (Figure 2). Several reports between 1975 and 1980 confirmed prostacycline formation in endothelial cells (ECs) of the vessel wall. The formation from platelet membrane phospholipids of aracidonic acid (AA) is the substrate for cyclooxygenase to synthesize prostaglandin endoperoxides in endothelial cells and platelets (Smith *et al*[5] 1976, Moncada and Vane in 1979, Figure 2)[9-19]. Thromboxane A2 produced by platelets has vasoconstrictive and platelet aggregation stimulating properties, whereas prostacyline produced in endothelial cells (EC) causes vasoconstriction and strongly inhibits platelet aggregation (Figure 2). Half life times of prostacycline is two to three minutes. Half life time of thromboxane A2 is a few hours and broken down to its endproduct thromboxane B2, which is secreted by the kidney. Prostacycline is broken down into prostaglandin 6-keto-PGF-1-alpha (Figure 2). Prostacyline inhibit platelet aggregation through stimulation of adenyl cyclase and subsequent increase of cyclonic AMP concentration in platelets (Figure 2). Thromboxane A2 induce platelet aggregation through inhibition of adenyl cyclase and subsequent decrease of cyclic AMP in platelets (Figure 2). Prostacycline plays an important physiological role in the prevention of platelet adhesion and aggregation to the intact vessel wall[13-15]. Disturbance of the balance between thromboxane A2 from platelets and prostacycline from ECs plays an important role in the pathogenesis of arterial thrombosis by activation of

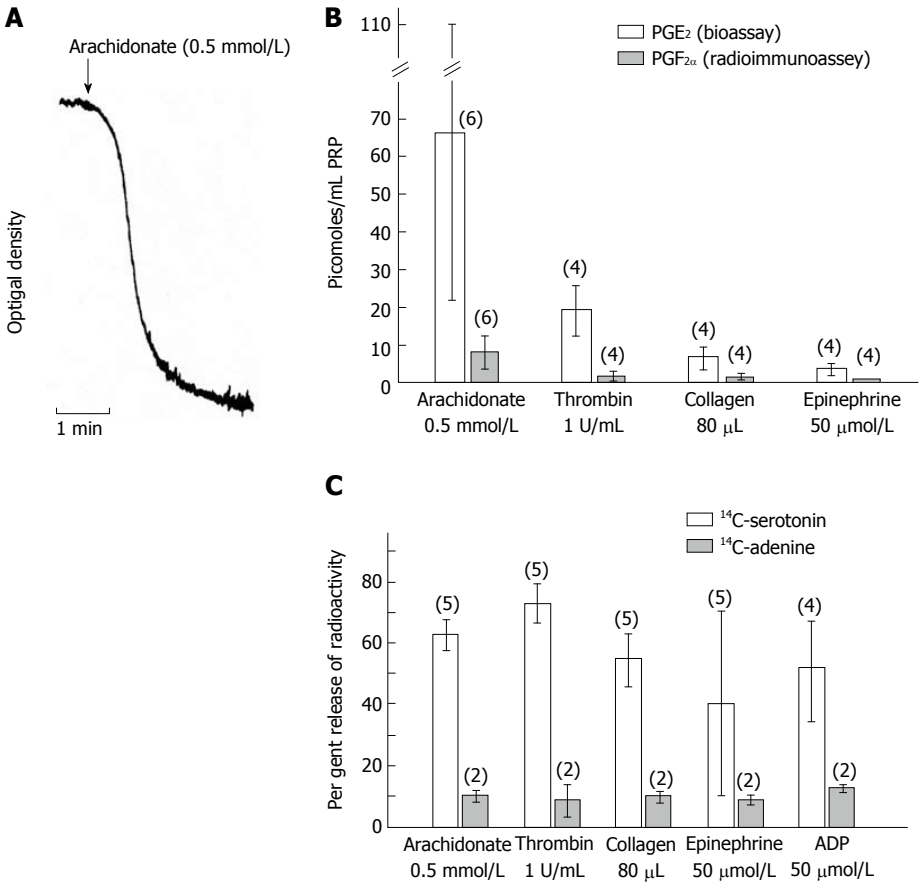

Figure 1 Arachidonic acid-induced human platelet aggregation and prostaglandin formation (Silver *et al*[3] 1973). Arachidonate acid (AA), 0.5 mmol/L induces a normal platelet aggregation curve (A). AA induces secretion of large amounts of prostaglandins after platelet aggregation, but little or no prostaglandins secretion occur after platelet aggregation induced by thrombin, collagen and epinephrin (B). AA, thrombin, collagen, epinephrine and ADP induce aggregation and secretion of rather equal amounts radioactivity from PRP preincubated with ^{14}C-serotonin or ^{14}C-adenine (C). These findings implicate that spontaneous *in vivo* shear induced aggregation of sticky JAK2 mutated platelet in the endarteriolar circulation is associated with high prostaglandin levels as the cause of the inflammatory pain and signs (redness, warmth and congestion) of erythromelalgia in JAK2-mutated thrombocythemia (Michiels *et al*[53] in 1985 and Michiels[80] in 2017).

platelet aggregation on damaged endothelial cells of the artheriosclerotic vessel walls.

Arachidonic (AA) stimulated platelets produce large amounts of prostaglandin endoperoxides PGE2, PGF2α, and thromboxane A2 (TxA2) and small amounts of prostaglandin D2 (Figure 1)[1,13]. Prostaglandine E2 is able to induce pain and inflammatory manifestations. Prostaglandin D2 has platelet aggregation inhibitory activity through stimulation of adenylcyclase[19]. In the absence of thromboxane A2 formation through irreversible inhibition of cyclo-oxygenase by aspirin, high concentrations of collagen, ADP and thrombin are still capable to induce platelet aggregation both *in vitro* and *in vivo* indicating that aspirin treated platelets retain their capability to adhere to subendothelium and aggregate in pathological situation like wound and arteriosclerotic vessel wall lesions. Secretion of the dense bodies contents ADP, ATP, calcium and serotonine during platelet activation subsequently propagate platelet aggregation, whereas serotonin also has vasoconstriction properties (Figure 1). During platelet aggregation alpha granules release platelet specific proteins like beta-thromboglobulin (beta-TG), platelet factor 4 (PF4), and platelet derived growth factor (PDGF), of which the latter stimulates proliferation of the smooth muscle cells in the media of arterioles and vessels[20,21].

ASSOCIATION OF ERYTHROMELALGIA AND THROMBOCYTHEMIA IN PV AND ET

The association of erythromelalgia and PV was known for a long time[22-26]. Oppenheimer recognized that the erythromelalgia in PV frequently progressed into acrocyanotic digital ischemia or gangrene diagnosed as thromboangiitis obliterans[23]. Dameshek and Henthel described a PV case with frequent episodes of erythromelagia complicated by gangrene of the third toe suggestive diagnosed as thrombo-angiitis oblitterans during longstanding follow-up of the PV: Hemoglobin 115%, erythrocytes 7 × 10^{12}/L (normal value less than 6 × 10^{12}/L), white blood cells 19 × 10^{9}/L, and platelets 2850 × 10^{9}/L[27]. Another PV case of Dameshek and Henthel[27] suffered from recurrent episodes of severe erythromelalgia since 5 years before PV could be diagnosed: Hemoglobin 116%,

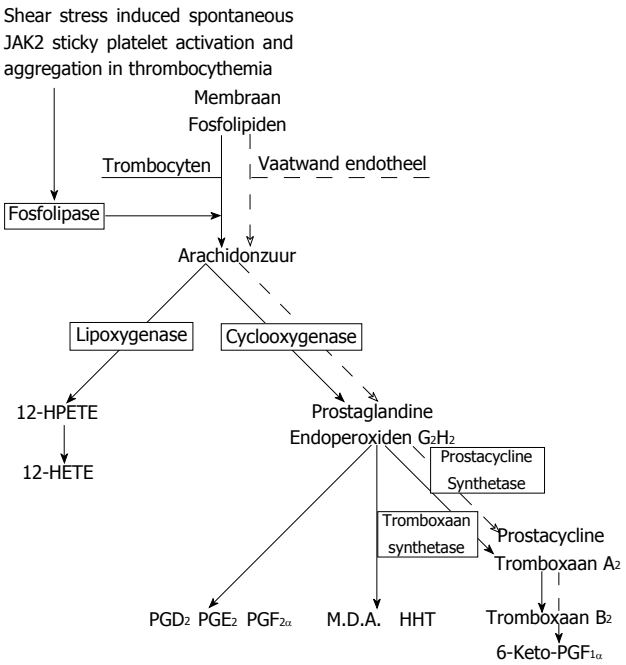

Figure 2 Dutch design by Michiels and Van Vliet on membrane phospholipid - arachidonic acid metabolism in platelets as compared to endothelial cells conceptualize in 1976, three years before the Moncada and Vane[13] publication in the *NEJM*. Arachidonic acid (AA) is metabolized by lipoxygenase into (12-HPETE) and 12 HETE, and by platelet cyclooxygenase into prostagandin endoperoxides G2 and H2, which consist of PGE2, PG2alpha, PGD2, malondialdehyde (M.D.A.) and HHT. Prostaglandin endoperoxides in platelets are metabolized by thromboxane synthetase into thromboxane A2. Thromboxane A2 is potent inducer of platelet aggregation and smooth muscl cell contraction. AA induced prostaglandin endoperoxides in endothelial cells are metabolized by prostacyclin synthethase into prostacycline. Endothelial cell (EC) derived prostacyclin causes vasodilatation and prevents platelet aggregation and platelet derived thromboxane causes vasoconstriction and platelet aggregation. Prostacyclin is continuously produced by endothelial cells, which have a nucleus to synthesize cyclo-oxygenase and prostacyclin. The biological half life times of prostacycline and thromboxne A2 are short and broken down to the inactived metabolites 6-keto-PGF-1-alpha and thromboxane B2, which are sereceted by the kidneys into the urine (Figure 15). Ticlopedine and clopidogrel inhibit ADP induced platelet aggregation without affecting platelet cyclo-oxygenase (Figure 13). Upon platelet activation of constitutively activated JAK2-platelets by shear stress starts the membrane phospholipids → phopspholipase A2 → arachidonic acid (AA) → cyclooxygenase biochemical pathway induced prostaglandin endoperoxides G2 and H2 production by platelets are the cause of the inflammatory signs erythromelalgia (Figure 4) featured by fibromuscular intimal proliferation and occlusive platelet thrombi (Figures 9 and 10). Release of platelet derived growth factor accounts for the fibromuscular intimal proliferation (Figures 6, 9 and 10) followed by von Willebrand (VWF) rich occlusive platelet thrombi (Figure 15). As platelets do not have a nucleus, irreversible inhibition of platelet cyco-oxygenase (COX-1) persist for the rest of platelet life time in the circulation and cures erythromelagia and migraine-like cerebrovascular ischemic manifestations[37,52,53].

erythrocytes 7.6 × 10^{12}/L white blood cells 14 × 10^{9}/L and platelets 1350 × 10^{9}/L.

The spectrum of erythromelalgia complicated by painfull acrocyanosis and digital gangrene has been descibed as the first manifestation of ET[28-32]. Erythromelalgia patients in the study of Smith and Allen discovered that one dose of aspirin (350 to 500 mg) immediately relieved erythromelalgic pain within one hour and holded on for three days, which is much longer than the analgesic effect of acetylsalicylic acid (Figure 3)[33]. This lasting effect of aspirin for three days due to irreversible platelet cycolooxygenase inhibition appeared pathognomonic for erythromelalgia and became the clue for the diagnosis of myeloproliferative thrombocythemia indicating a causal relation between erythromelalgia and clonal thrombocythemia in ET and PV patients (Figures 3 and 4)[26,28,29,34-37]. Aspirin responsive erythromelalgia is the presenting symptom of ET at platelet counts above 400 × 10^{9}/L, but has never been observed in reactive thrombocytosis. PV when accompanied by erythromelalgia had increased platelet count indicative for associated thrombocythemia[37]. The complete relief (cure) of burning pain and red congestion by one dose aspirin (350 to 500 mg) for a few days is diagnostic for thrombocythemia in ET and PV patients[34,37]. Michiels and Van Vliet used since 1976 malondialdehyde (MDA) production in platelet rich plasma after incubation of platetel rich plasma with NEM according to Smith *et al*[4] as an objective measure for the inhibtion of cyclo-oxygenase and prostaglandin endoperoxide formation in thrombocythemia platelets by which we discovered that the longlasting pain relief of erythromelalgia by aspirin (500 mg) was of similar duration as that one dose aspirin (500 mg) irreversible inhibited platelet cycooxygenase for a few days (Figure 3)[37]. Reversible inhibtion of platelet COX-1 activity by indomethacin 25 mg TID is an alternative to relief erythromelalgia (Figure 3)[37]. In contrast, sodium salicylate has no effect on platelet cyclo-oxygenase activity and did not affect erythromelalgia. Sodium salicylate but also ticlopedine (platelet ADP-receptor inhibitor) and other platelet inhibiting agents like dipyridamol did not inhibit platelet cycolo-oxygenase activity and were absolutely not effective in the treatment of erythromelalgia[37]. Michiels and Van Vliet (1978) concluded that erythromelalgia is caused by ongoing platelet cyclooxygenase-mediated inflammatory and microvascular ischemic thrombotic processes restricted to myeloproliferative ET thrombocythemia in patients with ET and PV. This was the start of prospective clinical, laboratory, histopathology and platelet kinetic studies initiated by Michiels to further explore the pathophysiology of Erythromelalgia in Thrombocythemia[37] at the Hematology Department of the Academic Hospital Dijkzigt, Erasmus University Rotterdam.

ROTTERDAM CLINICAL AND PATHOLOGIC FOR ET AND PV

Dameshek[38] (1950), Kurnick *et al*[39] (1972) and Michiels[37] (1980) showed that trilinear bone marrow hypercellularity of megakaryo/erythro/granulopoiesis combined with increased erythrocytes above 6 × 10^{12}/L is a pathognomonic diagnostic for PV (Table 1) and clearly differentiates between PV from primary or secondary erythrocytosis obviating the need to measure red cell mass[37]. Bone marrow histopathology is the

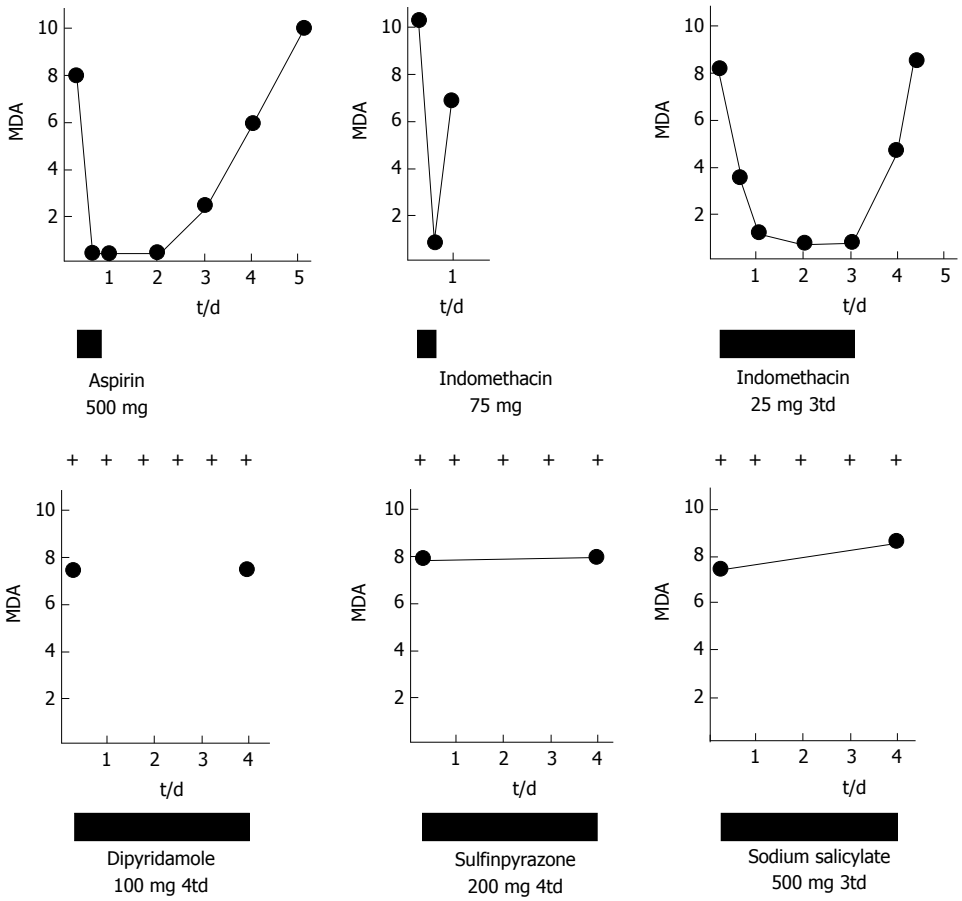

Figure 3 The effect of platelet aggregation inhibiting drugs on stimulated platelet aggregation, erythromelalgia and malondialdehyde concentration in N-ethylmaleimide stimulated platelet rich plasma. The effect of aspirin (acetylsalicylic acid), indomethacin, dipyridamol, sulfinpyrazon and sodium salicylate on erythromelalgia and MDA production by arachidonic acid stimulated platelets in platelet-rich plasma of symptomatic thrombocythemia patients with ET or PV complicated by erythromelalgia. MDA: Malondialdehyde.

most accurate diagnostic clue to ET and early and overt stages of PV by the demonstration of increase of clustered large and mature megakaryocytes (Table 1). Symptoms of erythromelalgia and atypical migraine-like atypical transient ischemic attacks (MIAs) in thrombocythemia patients already occurred at platelet counts above 400 × 10^9/L in ET and PV patients (Table 1). Bone marrow histology shows an increase of clustered, mature, large megakaryocytes with normal or slightly increased cellularity in ET and increased cellularity due to increase of erythropoiesis in early PV (Figure 5)[37]. Confirmative criteria for the diagnosis of ET and PV were normal erythrocyte sedimentation rate (ESR) and elevated score for leukocyte alkaline phosphatase (LAP) in the absence of infection. Bone marrow histology has the power to differentiate myeloproliferative ET from reactive thrombocytosis, from thrombocythemia in Philadelphia-chromosome positive (Ph+) ET and from 5q-minus syndrome with thrombocytosis[37]. The megakaryocytes in Ph+ ET and in the chronic phase of Ph+ CML the megakaryocytes are smaller than normal with round nuclei showing little lobulation (Figure 5)[40,41]. The megakaryocytes in the 5q-minus syndrome are small with dysplastic nuclei.

The minimum platelet count of 1000 × 10^9/L was required by the Polycythemia Vera study Group (PVSG, 1975) for the diagnosis of ET[42]. In 1980 Michiels[37] defined ET as a novel early stage MPD at platelet count between 400 and 1000 × 10^9/L overlooked by the PVSG (Table 1). Wasserman[43] (1972) and Berlin[44] (1975) proposed a new set of major (A) and minor (B) criteria for PV patients to be included in the randomized clinical trial PVSG 01 study[44]. The clinical PVSG criteria did not use bone marrow pathology and are crude to be sure that patients included in the PVSG 01 study indeed suffered from PV and not from secondary erythrocytosis (Wasserman personal communication)[45]. Pearson and Whetherley-Mein showed in 1979 significant shortcomings of the 1975 PVSG criteria for PV in a prospective evalution of 30 PV patients by the demonstration that the PVSG criteria overlook the early erythrocythemic PV cases with normal leukocytes, platelets and spleen size[46].

SPECTRUM OF EYTHROMELALGIC THROMBOTIC THROMBOCYTHEMIA: ETT

The time lapse between the appearance of erythro-

Table 1 The 1980 Rotterdam Clinical and Pathological criteria for essential thrombocythemia and polycythemia vera

The 1980 RCP criteria for prefibrotic ET[37,52]	
Major criteria	
A1	Persistent platelet count in excess of 400 × 10^9/L
A2	Increase and clustering of enlarged megakaryocytes in bone marrow biopsy
A3	No or slight increase of reticulin fibers (RF 0 or RF 1)
Confirmative criteria	
B1	Presence of large platelets in a peripheral blood smear
B3	No or slight splenomegaly on ultrasound sonography (length diameter normal value < 12 cm)
B4	Increase of LAP-score and no signs of fever or inflammation
Exclusion criterion	
Ph+ chromosome and any other cytogenetic abnormality in blood or bone marrow nucleated cells	
The 1980 RCP criteria for prefibrotic PV to replace the crude 1975 PVSG criteria for PV	
Major	
A1	The combination of erythrocyte count of > 6 × 10^{12}/L and bone marrow hypercellularity due to EM or EMG hyperproliferation is pathognomonic diagnostic for PV (Dameshek and Henthel[27] 1940, Dameshek[38] 1950, Kurnike *et al*[39] 1972) obviating the need to measure raised red cell mass
A2	Increase in bone marrow biopsy of clustered, enlarged pleomorphic megakaryocytes with hyperlobulated nuclei and moderate to marked increase cellularity of megakaryopoiesis/erythropoiesis or typically trilinear mega-erythro-granulopoiesis (EMG). Such a typical PV bone marrow picture excludes all variant of primary and secondary erythrocytosis[37-39]
Minor	
B1	Thrombocythemia, persistant increase of platelet > 400 × 10^9/L
B2	Leukocytosis, leucocyte count > 10^9/L and low erythrocyte sedimentation rate
B3	Raised leukocyte alkaline phosphatase score > 100, absence of fever or infection
B4	Splenomegaly on ultrasound sonography
A1 + A2 establish PV and exclude erythrocytosis. One or more of B confirm PV	

RCP: Rotterdam Clinical and Pathological; ET: Essential thrombocythemia; PV: Polycythemia vera; EM: Erythrocythic megakaryocytic; EMG: Erythrocytic megakaryocytic granulocytic.

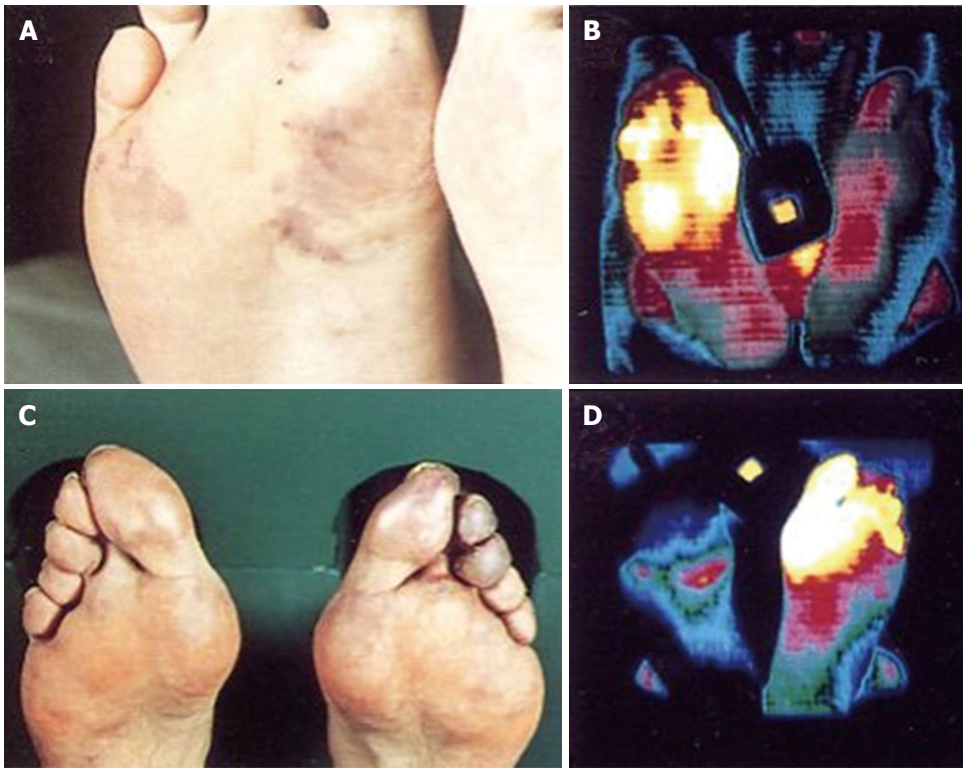

Figure 4 Isothermpgrams of two essential thrombocythemia patients with erythromelalgia in toes and fore foot sole. Typical mottled red blue congestion and thermographic visualization of erythromelalgia in the fore foot and toes. Skin surface temterature: blue 24 ℃-25 ℃; green 26 ℃-27 ℃; purple 28 ℃-29 ℃; red 30 ℃-31 ℃; yellow 32 ℃ and white 33 ℃. Complete correction of the upper leg thermograms after effective treatment with aspirin.

Table 2 Localization of erythromelalgia in feet/toes *vs* fingers and skin, and the presence of peripheral gangrene and history of acute coronary syndrome or migraine-like cerebral ischemic attacks, and time lap between first manifestations of erythromelalgia and diagnosis of thrombocythemia in essential thrombocythemia (n = 11) and polycythemia vera (n = 13)

Patient	Diagnosis	Feet toes	Fingers	Skin	PG	ACS	MIAs	Time lap (mo)
1	ET	Bilateral		Present	Yes	Yes	Yes	45
2	ET	Bilateral			Yes		Yes	154
3	ET							60
4	ET	Unilateral			Yes	Yes		12
5	ET	Unilateral	Unilateral	Present	Yes			4
6	ET					Yes	Yes	20
7	ET						Yes	60
8	ET							30
9	ET	Bilateral			Yes			20
10	ET	Bilateral	Bilateral	Present	Yes			30
11	ET	Bilateral		Present				30
12	PV	Unilateral						24
13	PV	Unilataral						3
14	PV	Bilataral						0
15	PV		Unilateral	Present	Yes	Yes		36
16	PV	Bilateral					Yes	48
17	PV	Unilateral						1
18	PV	Bilateral					Yes	18
19	PV		Bilateral					2
20	PV	Bilataral			Yes			24
21	PV	Unilateral						4
22	PV	Unilateral		Present				3
23	PV	Unilateral	Unilateral					24
24	PV		Unilateral					6

PG: Peripheral gangrene; ACS: Acute coronary syndrome; ET: Essential thrombocythemia; PV: Polycythemia vera; MIAs: Migraine-like cerebral ischemic attacks.

melalgic symptoms and diagnosis of thrombocythemia in my first cohort of 24 patients with erythromelalgic thrombotic thrombocythemia (ETT: 11 ET and 13 PV) ranged from a few months in eight, and from 1 to 5 years in 15 cases due to the lack of knowledge of a causal relation between erythromalalgia and thrombocythemia[37,47]. The lowest platelet count in ET at which erythromelalgia occurred was around 400 × 10^9/L[47]. Twenty four ETT (11 ET and 13 PV) patients, presented with erythromelalgia complicated by microvascular disturbances including peripheral acrocyanosis or gangrene (thromboangiitis oblitterans) in 8, acute coronary syndrome in 4 and transient neurologic ischemic attacks in 6 (Table 2). Erythromelalgia was localized in toes and foot soles in 17, in fingers in 8 and the skin of lower or upper legs in 6 (Table 2)[37]. Localization of erythromelalgia in the skin in 6 trombocythemia patients (4 ET and 2 PV) was misdiagnosed as superficlal thrombophlebitis (Table 2 and Figure 6). Thermographic measuring of the skin surface temperature using a Bofors Mark Ⅱ camera showed that the burning pain and red congestion of erythromelalgia started to occur when the skin surface temperature exceded the critical level of 31℃, and ameliorated to disabling suffer at increasing skin temperature above 31 ℃ (Figures 4 and 6)[37]. This is in accordance with observation of Brown[48] (1932) and Smith and Allen[33].

PLATELET KINETIC STUDIES IN THROMBOCYTHEMIA COMPLICATED BY ERYTHROMELALGIA

Platelet kinetic investigations according to Branehög[49,50] was used to document the involvement of platelets in the etiology of erythromelalgia in thrombocythemia. Platelet kinetic studies were performed in 4 control persons, 6 asymptomatic thrombocytosis (3 with reactive thrombocytosis: RT and 3 chronic myeloid leukemia: CML), 6 asymptomatic thrombocythemia patients, and 8 thrombocythemia patients complicated by erythromelalgia (Figure 7 and Table 3)[37]. Measuring of half life times (T1/2, mean survival (MS) and maximal life times (MaxLS) of ^{51}Cr labeled autologous platelets in the circulation appeared to be a trustful objective method to demonstrate platelet consumption in ongoing thrombotic processes (Figure 7)[37]. MS and MaxLS are equal in T (reactive thrombocytosis) and in E- indicating that the Cr platelet disappearance curves are linear or near to linear indicating a normal platelet survival with a T1/2 of around 4 d (Figure 7). In E+ MS is significantly shorter than MaxLS indicating a shortened Cr platelet survival with a T1/2 of around 2 d with curvilinear platelet disappearance curves (Figure 7).

Aspirin treatment of 7 symptomatic thrombocythemia resulted in the disappearance of erythromelalgia, significant increase of peripheral blood platelet counts,

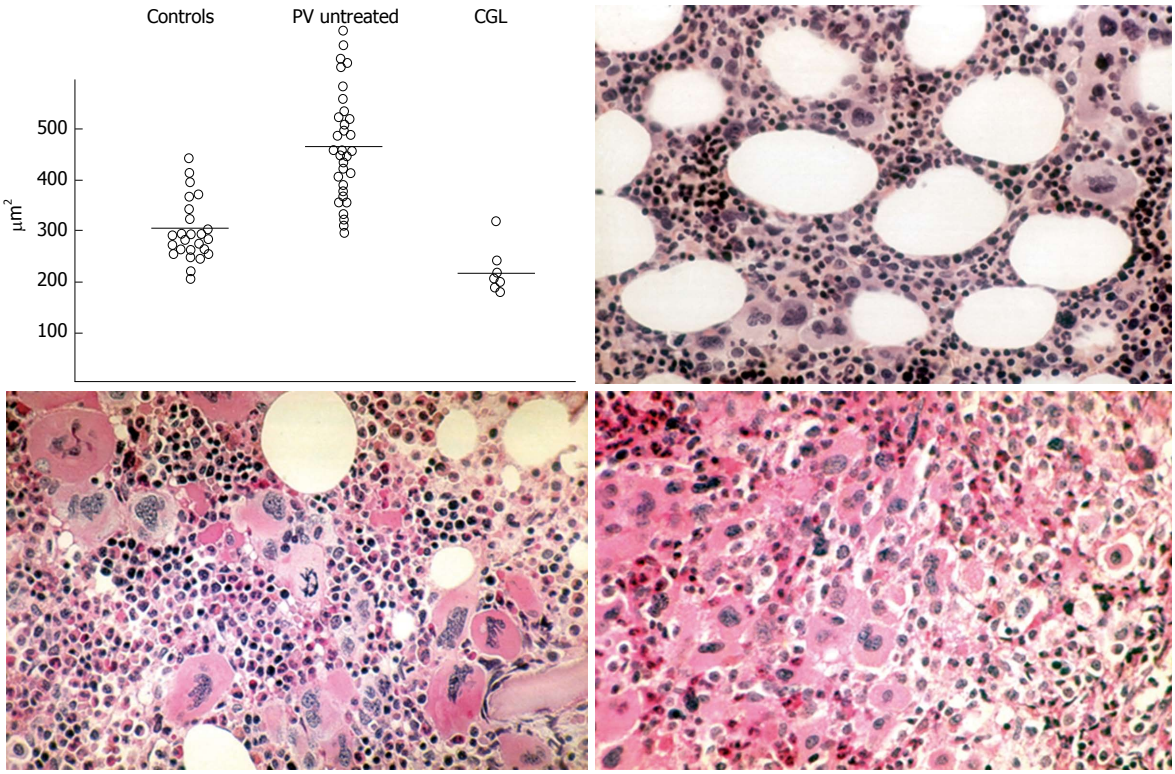

Figure 5 Planimetry of megakaryocyte sizes (μm^2) from bone marrow smears in controls, polycythemia vera and chronic granulocytic leukemia upper left: Normal size megakaryocytes in controls; large megakaryocytes in untreated polycythemia vera and small sized megakaryocytes in chronic granulocytic leukemia (Frantzen *et al*[40]). Demonstration by Michiels (1981) of a spectrum of clustered large megakaryocytes with hyperlobulated nuclei and a normocellular bone marrow in essential thrombocythemia (ET) *vs* increased bone marrow cellularity duet o increased erythropoiesis in ET and polycythemia vera (PV) *vs* increased trilinear eythrocythemic, megakaryocythemia and granulocythemic (EMG) proliferation in classical PV according to Dameshek[38] (1950) and Kurnicke *et al*[39].

correction of platelet survival times and platelet disappearance curves to normal (Figure 8)[37]. These data on platelet consumption and its correction by aspirin in symptomatic thrombocythemia patients demonstrate that erythromelalgic thrombotic complications of thrombocythemia including transient cerebral and ocular ischemic attacks are caused by spontaneous activation of hypersensitive platelets at high shear in the arteriolar endartrial circulation as as first documented by Michiels and Ten Kate in skin biopsies (Figures 9 and 10)[37,51,52]. The platelet-mediated erythromelalgic microvascular thrombotic complications are cured by aspirin and platelet reduction to normal (< 400 × 10^9/L), but not by coumadin and not by the ADP inhibitor ticlopedin[37,52]. Erythromelalgic thrombotic complications in thrombocythemia associated with Ph+ ET or CML is rare[37]. Despite the high platelet counts, patients with reactive thrombocytosis and thrombocytosis in Ph+ CML patients do not present with erythromelalgic miscrovascular ischemic events. In Ph+ thrombocythemia, the platelets are small, indolent and non-reactive, whereas the platelets in thrombocythemia of ET and PV patients are large and hypersensitive with clinical evidence of platelet-mediated erythromelalgic thrombotic manifestations[37,41].

HISTOPATHOLOGY OF ERYTHROMELALGIA IN THROMBOCYTHEMIA

As there were no reports in the 1970s on the histopathological substrate of aspirin responsive erythromelalgia, Michiels and Ten Kate performed in 1984 skin punch biopsies for histopathological investigations in ET patients from recently relapsed red congested erythromelalgia in the fore foot sole within one week after discontinuation (Figure 9)[37,51,52]. The arterioles in the deep reticular dermis show strong proliferation and degenerative vessel wall changes and the venules, capillaries and nerves are not involved (Figure 9). The zone of proliferated cells in the intima is two to three layers thick and distinct from the smooth muscle cells of the media. Immunofluorence studies using antibodies aganst FVⅢ and smooth muscle cells revealed that the intimal proliferation was caused by proliferation by smooth muscle cells covered by one layer of endothelial cells (Figure 9). The endotheilal cells (EC) are swollen and have large nuclei indicative for activated ECs (Figure 9). The venules, capillairies and nerves were not involved[37,51]. The membrana elastica interna (mei) at places of fibromuscular intimal proliferation is broken up and splitted by the proliferating smooth muscle cells (Figure 9)[37,51]. Histopathology of skin punch biopsies from relaped acrocyanotic erythromelalgia three weeks after discontinuation of aspirin are featured by fresh thrombotic occlusion on top of fibromuscular intimal proliferation in arterioles, whereas the venules, capillaries and nerves were not involved (Figure 10)[37,51,52]. The histopthology of longstanding untreated erythromelalgia complicated by digital gangrene mainly

Table 3 Results of ^{51}Cr autologous platelet survival studies in 4 controls (Group Ⅰ), in 3 cases of thrombocytosis in chronic myeloid leukemia and 3 cases of reactive thrombocytosis (Group Ⅱ), in 6 cases of asymptomatic thrombocythemia in essential thrombocythemia, myelofibrosis and polycythemia vera (Group Ⅲ), and in 8 cases of thrombocythemia in essential thrombocythemia, myelofibrosis and polycythemia vera complicated by erythromelalgia (Group Ⅳ)

Patient group	Diagnosis	Platelet, × 10/L	E	T1/2 (d)	Mean life span	Maximal life span
Ⅰ	210		No	3.6	5.4	9.9
	181		No	4.2	9.0	9.1
	193		No	3.9	7.1	7.8
	138		No	3.7	6.0	8.8
Mean	180			3.9	6.9	8.9
Ⅱ	722	CML	No	4.0	8.6	8.2
	1487	CML	No	3.9	7.6	7.3
	2244	CML	No	4.0	7.4	7.7
	1015	RT	No	4.0	6.7	8.7
	736	RT	No	4.0	6.6	7.8
	866	RT	No	4.9	9.7	9.2
Mean	1178			4.1	7.9	8.2
Ⅲ	1722	ET	No	3.4	5.9	6.8
	1167	ET	No	3.0	4.6	7.3
	511	MF	No	3.1	4.5	8.8
	935	PV	No	3.8	6.2	9.0
	506	PV	No	3.5	5.8	8.8
	614	PV	No	3.3	5.7	7.5
Mean	918			3.3	5.4	8.0
Ⅳ	666	ET	Yes	2.1	2.9	6.4
	637	ET	Yes	2.6	4.0	6.8
	1018	ET	Yes	2.7	4.2	7.2
	539	MF	Yes	1.8	2.6	6.1
	489	PV	Yes	2.7	4.0	7.9
	1028	PV	Yes	2.5	1.7	7.3
	1036	PV	Yes	2.0	3.4	5.6
	1180	PV	Yes	3.1	6.0	5.8
Mean	824			2.4	3.6	6.6

CML: Chronic myeloid leukemia; RT: Reactive thrombocytosis; ET: Essential thrombocythemia; PV: Polycythemia vera; MF: Myelofibrosis.

show complety occluded arterioles by fibrotic organized thrombi (Figure 10). If overlooked and not treated with aspirin endstage erythromelalgia result in painfull acrocyanotic cold toes and fore foot showing onion-like structures of occluded arterioles due to vascular and perivascular fibrosis (Figure 10)[37,51,52].

ERYTHROMELALGIA CURED BY ASPIRIN AND CORRECTION OF PLATELET NUMBER TO NORMAL

Twenty three patients with erythromelalgia (Figure 11) were treated with aspirin between 1974 and 1985)[37,52]. The cure of erythromelalgia by aspirin could be documented in 15 thrombocythemia patients. Some erythromelalgic thrombocythemia patients had already themselves discovered the favorable effect of aspirin on erythromelalgia. Remission of thrombocythemia by busulfan was defined by reduction of platelet count to below 350 × 10^9/L, and relapse by increase of

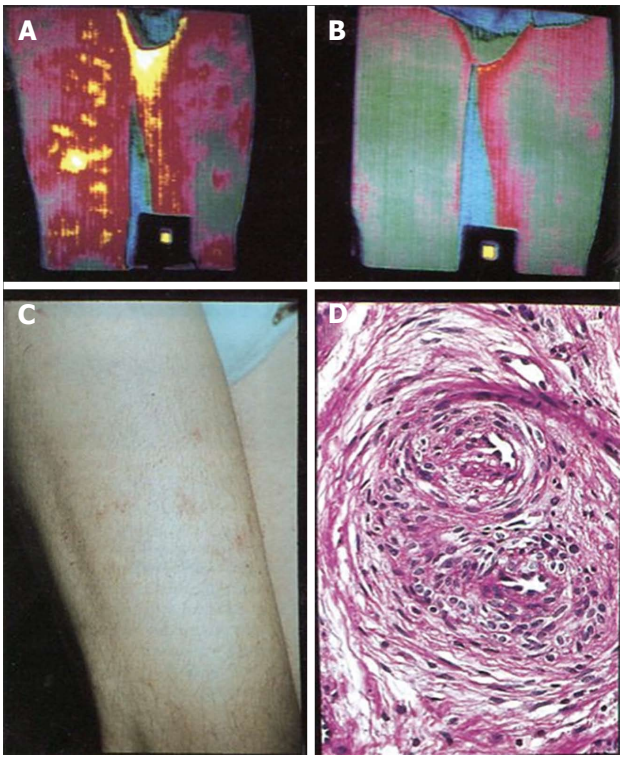

Figure 6 Isothermpgrams of upper legs showing "superficial thrombophlebitis" (A) in the right upper leg, which completely disappeared after treatment with aspin once daily (B) and superficial thrombophlebitis manifested as red painful indurated hot spots, erythromelalgia of the skin (C) in the upper leg caused by fibromuscular intimal proliferation (endarteritis oblitterans) as documented by histotolgy from skin punch biopsies (D) from the red spots.

platelet count above 400 × 10^9/L. The erythromelagia disappeared completely after reduction of platelet count to less than 400 × 10^9/L and did not re-appear after discontinuation of aspirin at platelet count below 400 × 10^9/L[37,52]. Aspirin was discontinued in busulfan induced thrombocythemia with normal platelet count in 13 ET and 11 PV patients patients (Figure 11)[37,52]. Erythromelalgia recurred in 8 of 12 patients (9 ET and 2 PV) already at platelet counts between 400 to 550 × 10^9/L (Figure 11). Remission duration of thrombocythemia (platelet 400 to 500 × 10^9/L) by one course busulfan lasted from 2 to more than 9 years (long busulfan remittors), which was associated with the disappearances of erythromelalgia and with no reappearance of erythromelalgia after discontinuation of aspirin in ET patients 1 to 6 and 9 and 10 (Figure 11)[52]. Phlebotomy in PV did not improve erythromelalgia in PV. Patients 16, 19, 20 and 26 with PV received long-term aspirin therapy, which gave complete symptomatic relief of erythromelalgia and cure circulatory disturbances for the duration of aspirin administration at increased platelet counts (Figure 11). Busulphan induced normal platelet count was reached in all PV patients, but the remission duration of erythromelalgic thrombocythemia was much shorter (short busulfan remittors) as compared to ET[52]. The final follow-up of the effect of one course busulfan in 20

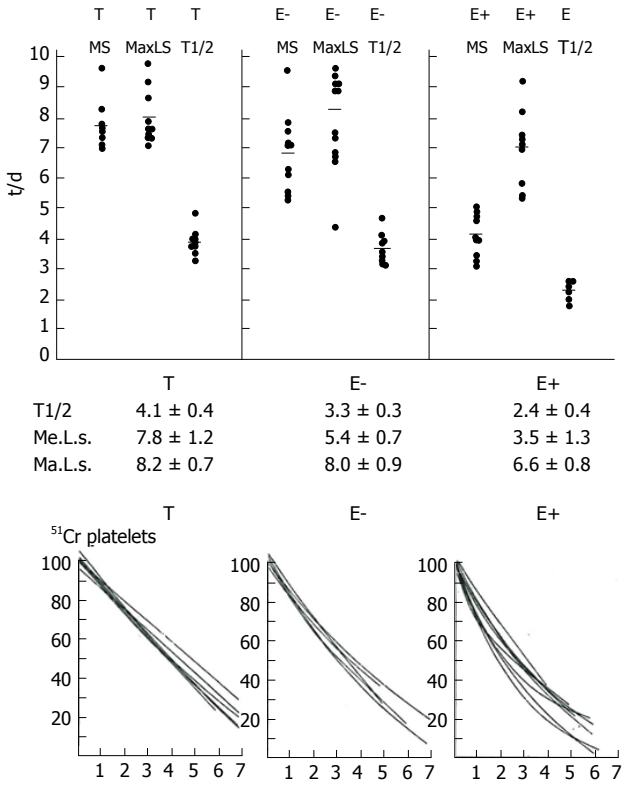

Figure 7 Platelet survival times and platelet disappearance curves according to Branehög *et al*[50] in 10 thrombocythemia patients complicated by erythromelalgi (E+), 10 asymptomatic thrombocythemia patients (E-), and 11 asymptomatic patients with thrombocytosis (T). Curvilinear platelet survival curves in E+ indicates a consumptive diappearance of thrombocythemic platelets from the circulation: Slight curvilinear platelet survival curves in E- suggest slight, but insignificant random platelet consumption; and linear platelet survival curves in group T with reactive thrombocytosis indicate a non-random, age-related disappearance of platelets from the circulation. MS = mean survival. T1/2; MaLS = maximal life span according to Branehög *et al*[50].

symptomatic ET in the period between 1974 to 1986 has been reported in 1999 in great detail[47].

PATHOPHYSIOLOGY OF ASPIRIN RESPONSIVE PLATELET MEDIATED ERYTHROMELALGIA

Spontaneous activation and aggregation at high shear in arterioles of JAK2 constitutively activated (sticky) platelets induce high levels of arachidonic acid (AA) release from platelet membrane phopholipds with the subsequent transition of AA cyclooxygenase in to large amount of prostaglandin endoperoxides followed by the generation of thromboxane A2 (Figure 1) appear to be of critical importance for the inflammatory signs, fibromuscular intimal proliferation and platelet thrombi in JAK2^{V617F} mutated thrombocythemia (Figure 12). In this process secondary activation of platelets by ADP (P2Y12), thrombin or collagen receptor mediated aggregation does not play any role, therby explaining the ineffectiveness of ticlopedin and clopidogrel in the treatment of erythromelalgia

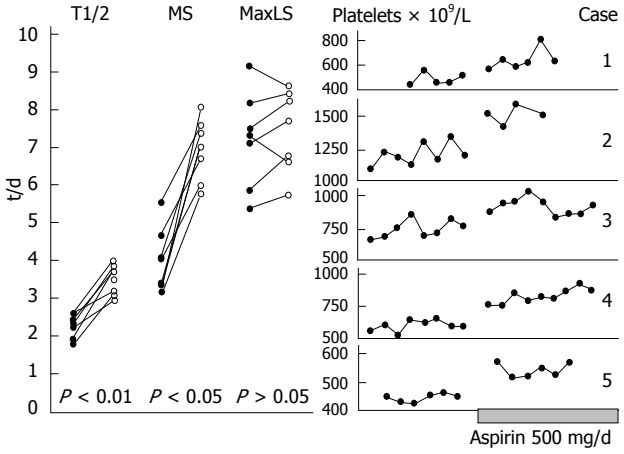

Figure 8 Platelet survival curves in seven E+ thrombocythemia patients before and after aspirin 500 mg/day, and peripheral blood platelet counts before and after maintenance aspirin treatment 250 mg/d. MS: Mean survival; T1/2; MaLS: Maximal life span according to Branehög *et al*[50].

(Figure 13). This novel insight has very important clinical implications in our current understanding that spontaneous activation of hypersensitive JAK2^{V617F} mutated thrombocythemic platelets at high shear in arterioles causes erythromelalgia due to the release of large amounts of prostaglandin endoperoxides and thromboxane A2, that can explain both the pronounced inflammatory, fibromuscular intimal proliferation and thrombosis in arterioles. The cure of erythromelalgia by aspirin is due to complete inhibition of prostaglandin endoperoxide (PGE2) and thromboxane A2 through irreversible inhibition of platelet cyclooxygenase (Figures 1, 3, 12 and 13). Aspirin indeed became a wonder drug that cured platelet mediated erythromelalgia in myeloproliferative JAK2^{V617F} mutated thrombocythemia in ET and PV patients by irreversible inhibition of platelet cyclooxygenase[5,37,51,52] (Figures 3, 12 and 13). The novel key observation in this report anno 2017 is that spontaneous activation and aggregation of hypersensitive JAK2^{V617F}-mutated sticky platelets is associated with the generation of large amounts of AA induced cyclic endoperoxides including PGE2 and thromboxane A2 as compared to ADP (P2Y12) induced aggregation by ticlopedin and clopidogrel Figures 3, 12, 13). This lucid insight can fully explain the occurrence of the inflammatory manifestations of erythromelalgia caused by shear stress induced activation of hypersensitive platelets in thrombocythemia as the first stage of red congested erythromelalgia (Figures 3, 12 and 14) followed by fibromuscular intimal proliferation in skin areas of erythromelalgia (Figure 9). If not treated with aspirin, occlusion by von Willebrand factor (VWF) platelet rich thrombi occur at places of vessel wall damage of fibromuscular intimal proliferation (Figure 15)[51-53]. Coumadin and the platelet ADP (P2Y12) inhibitors ticlopedin and clopidogrel are ineffective. Treatment with a loading dose 350 to 500 mg followed by 100 mg once daily cures erythromelalgia, its acrocyanotic complications

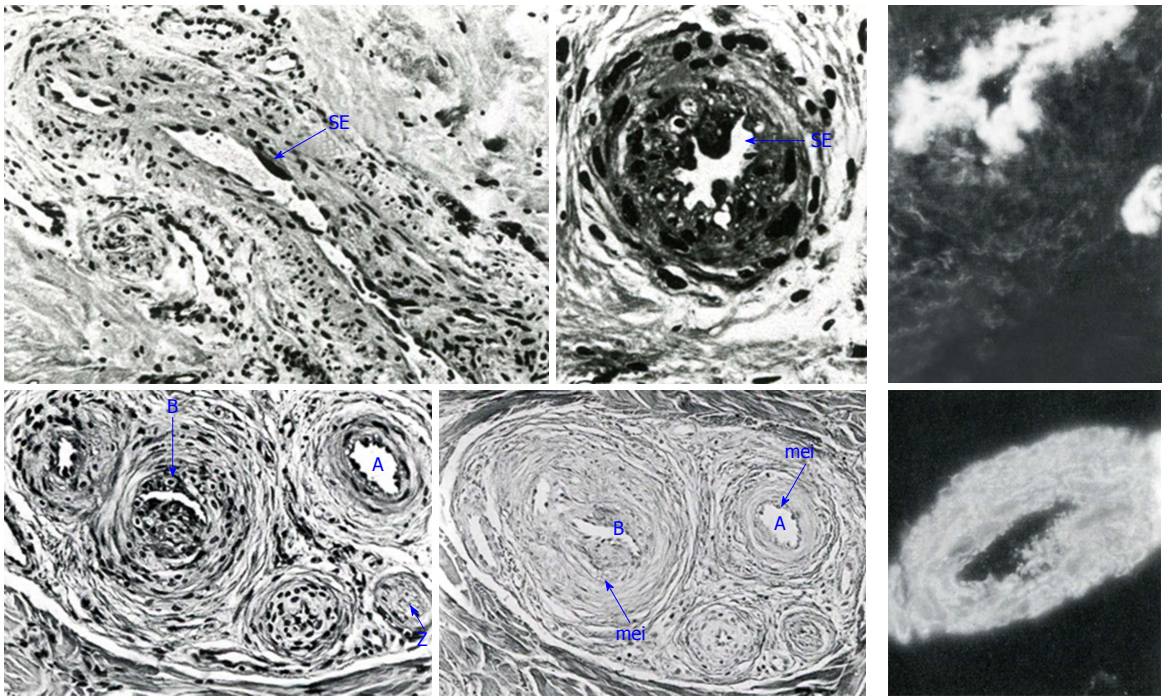

Figure 9 Arterioles with swelling of endothelial cells, proliferation of cells of the inner layer below the media and normal venules, capillaries and nerves (upper and left), and elastica von Gieson stain showing a normal membrana elestica interna (mei) in a normal arteriole (A). Source Michiels 1981. The membrana elstica interna (mei) is splitted up and fragmented between the proliferating cells in arteriole B with intimal proliferation in in skin areas of very typical red congested erythromelalgia within one week after discontinuation of aspirin in two cases with essential thrombocythemia. Source Michiels 1981. Immunofluorescence of proliferating cells in the intima of affected arterioles shows on layer endothelial cells with antiserum against factor VIII and multilayer proliferation of smooth muscle cells with antiserum against smooth muscle cells indcative for fibromusclar intimal proliferation of affected arterioles in erythromelalgic thrombocythemia (upper). Partial and complete occlusion by a fresh thrombus in acrocyanotic erthomelalgia three weeks after discontinuation of aspirin (lower). Source Michiels 1981.

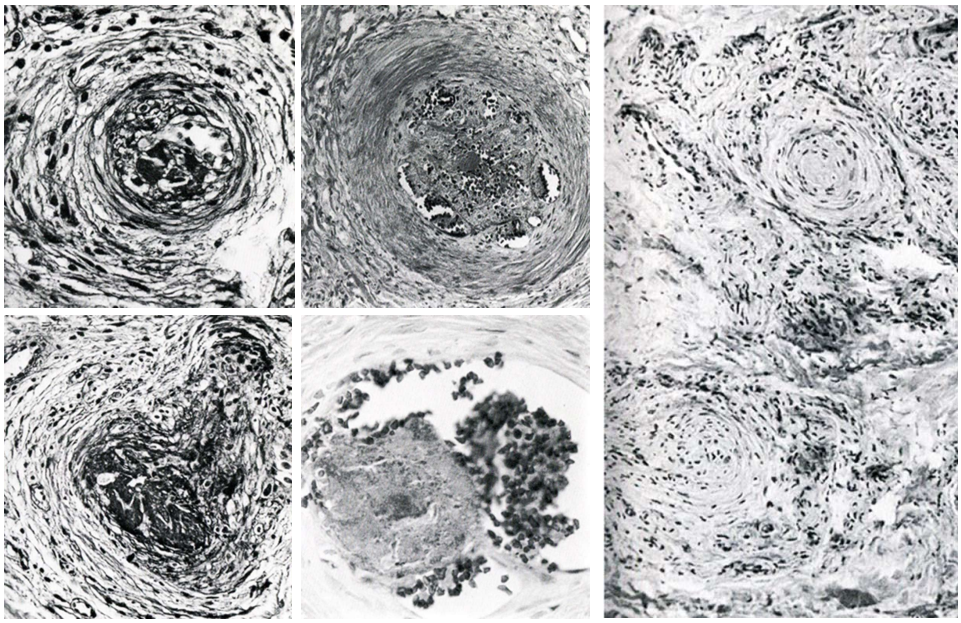

Figure 10 Thrombotic occlusion of arterioles on top of fibrouscular intimal proliferation. Thromboangiitis oblitterans (left panel) and recanalisation of arterioles showing vessel wall fibosis of arterioles in two cases of erythromelalgia complicated by acrocyanosis and digital gangrene (middle panel). Source Michiels 1981. Oniony structure by vascular and perivascular fibrosis of occluded fibromusclar intimal proliferation in acrocyanotic digital ischemia of untreated endstage erythromelalgia that had transformed into aspirin resistent Raynaud phenomenon (right). Source Michiels 1981.

as well as the migraine-like atypical TIAs (MIAs) and acute coronary syndromes (ACS) through irreversible inhibition of platelet cyclo-oxygenase (Figures 13-15). The cure of erythromelalgia by aspirin OD could be attributed to maintained irreversible inhition of platelet cyclo-oxygenase keeping the prostacycline cyclo-

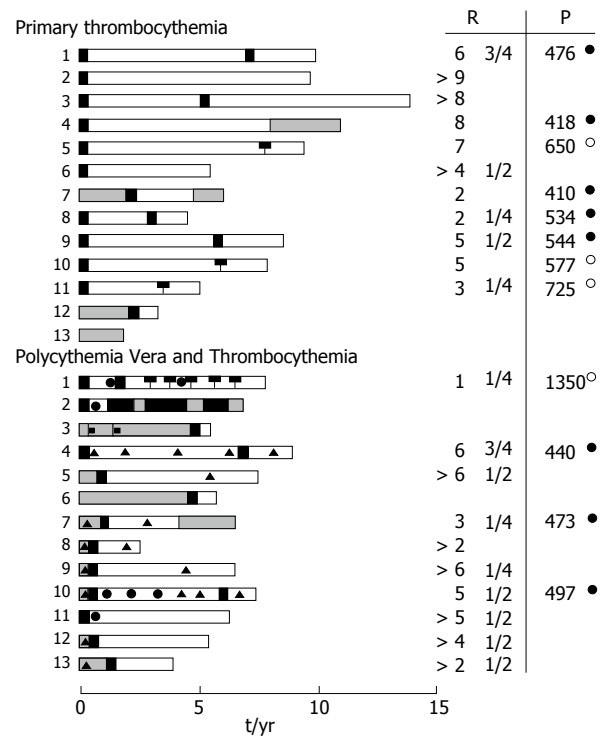

Figure 11 Results of treatment in 13 patients with primary or essential thrombocythemia and 13 patients with polycythemia vera and thrombocythemia. Source Michiels *et al*[53], 1985. Busulfan induced remission of thrombocythemia (platelet counts < 350 × 10^9/L) lasted 2 to 9 years (long busulfan remitters) in essential thrombocythemia (ET) patients, which became asymptomatic with no recurrence of erythromelalgia after discontinuation of aspirin during thrombocythemia remission periods of 2 to 9 years (R). Erythromelalgia recurred in eight [5 ET, 3 polycythemia vera (PV)] of twelve (8ET, 4PV) at platelet counts between 400 to 550 × 10^9/L (P) after thrombocythemia remission periods of 2 to 8 years. Busulfan induced remissions of thrombocythemia in the majority of PV patients lasted several months to a few years (short buslfan remittors) indicating the need to treat with repeated courses of busulfan.

oxygenase in endothelial cells intact (Figures 2 and 15)[37,52,53]. The platelet inhibiting drugs sulfinpyrazone, dipryridamole and ticlopedine do not inhibit platelet cyco-oxygenase activity and had no effect on erythromlalgia (Figures 3 and 15)[37,53]. Spontaneous activation, aggregation, secretion of JAK2 constituively activated, hypersensitive sticky platelets became the key cause in the etiopathogenesis of erythromelalgia, MIAs and ACS in JAK2-mutated thrombocythemia (Figures 14 and 15). PDGF in this process accounts for the fibromuscular intimal proliferation[20,21]. Vaso-active substances, prostaglandins endoperoxides and other factors released during $JAK2^{V617F}$ mutated platelet aggregation account for the inflammatory symptoms (Figure 15)[52,53]. Platelet kinetic studies demonstrated that in the presence of erythromelalgia platelet consumption is increased as the final proof of platelet cyclo-oxygenase mediated etiology of erythromelalgic inflammatory and arteriolar (end-arterial) microvascular thrombosis in $JAK2^{V617F}$-mutated thrombocythemia in ET and PV patients (Figures 7 and 8)[37]. Biopsies from erythromelalgic areas in five ET patients show arteriolar leasions of fibromuscular intimal proliferation without involvement of venules, capillaries and nerves (Figure 9)[37,51]. If left untreated erythromelalgia leads to ischemic symptoms of acrocyanosis and peripheral gangrene due to thromboangiitic occlusions of arterioles on top of platelet cyclooxygenase mediated fibromuscular intimal proliferation (Figure 10)[37].

CLINICAL MANIFESTATIONS OF PV: THERAPEUTIC IMPLICATIONS

The presenting clinical manifestations in PV patients include microvascular events, ranging from erythromelalgic ischemia of a toe or finger, amputation of one or more gangrenous digits (thrombo-angiitis obliterans), attacks of transient blindness (amaurosis fugax), MIAs, facial weakness or aphasia, superficial thrombophlebitis and major thrombosis including stroke, coronary artery disease, deep vein thrombosis, splanchnic vein thrombosis and pulmonary embolism[54-59]. The intrinsic blood changes in PV as a trilinear MPN (Table 1)[37-39] are increased platelets, erythrocytes, hematocrit, activated leukocytes and blood cellular viscosity, which are responsible for this altered distribution of minor and major vascular complications in PV as compared to the high incidence of microvascular and low incidence of major thrombotic manifestations in the rotterdam clinical and pathologic (RCP) defined ET of the Dutch Collaborative Low-dose Aspirin in ET (Dutch CLAT) studies[37,52,60-70]. Low-dose aspirin in ET and combined aspirin and phlebotomy in PV are highly effective in the reduction of erythromelalgia, and microvascular ischemic disturbances in ET and PV, but partially reduce major thrombosis in PV, and do not influence the natural history of the JAK2 mutated trilinear myeloproliferative neoplasms (MPNs) in terms of leukocytosis, erythrocytosos, splenomegaly and myelofibrosis. On top of the erythromelalgic thrombotic microvascular disease of thrombocythemia (ETT) the high incidence of major thrombotic events in PV was related to high blood hematocrits due to increased erythrocyte counts above 6 × 10^{12}/L (Table 1[37-39] and Figure 12[54-60]). In PV phlebotomy reduces the incidence of major arterial and venous thrombosis but does not improve the aspirin responsive erythromelalgia, acrocyanotic digital complications, and migraine-like atypical transient cerebral and ocular attacks (MIAs) (Figure 15 and 16). The lowest incidence of major thrombosis has been found in PV treated to achieve adequate control hematocrit to around 0.40[58,59], but

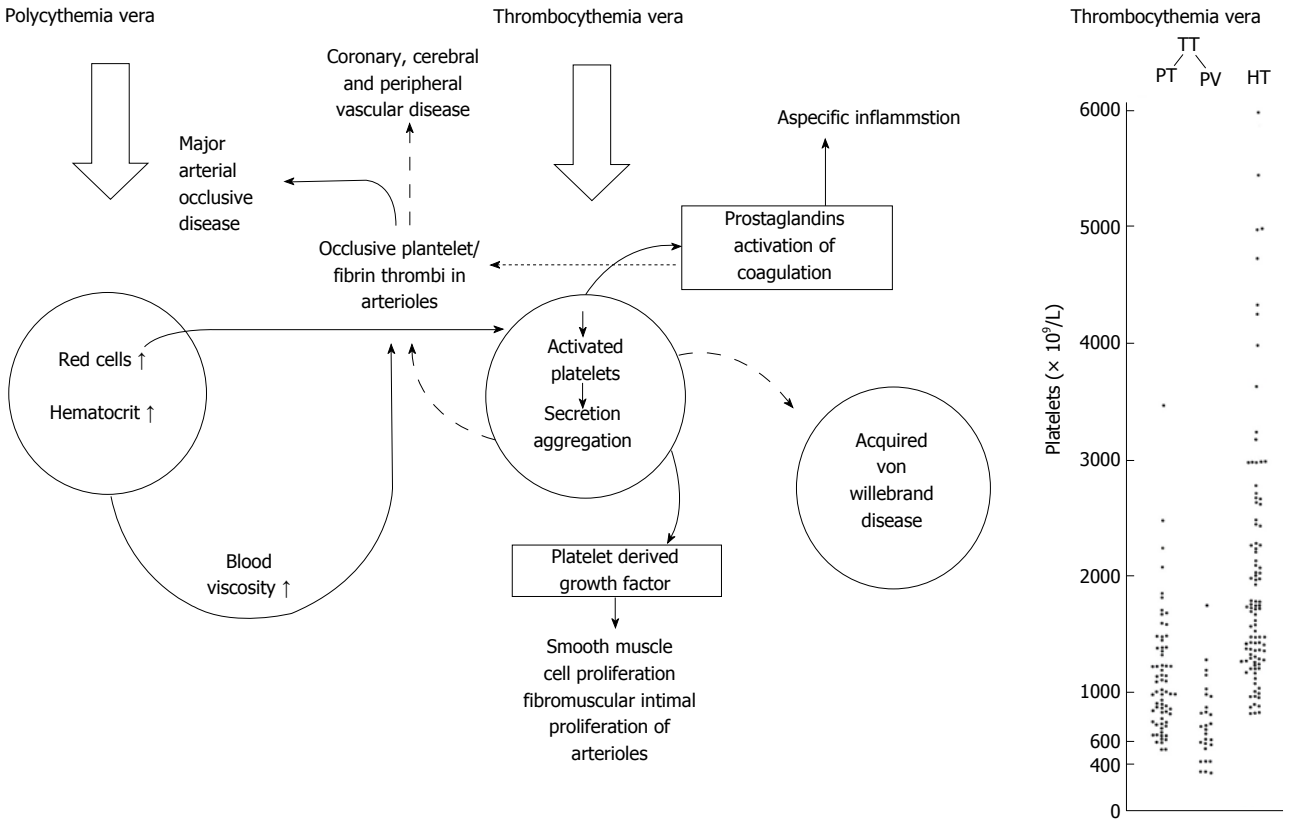

Figure 12 Pathophysiology of erythromelalgia as multicellular processes caused by platelet mediated microvascular erythromelalgic arteriolar inflammation and thrombosis in myeloproliferative thrombocythemia vera and major arterial thrombotic disease in polycythemia vera. Shear induced production of prostaglandin endoperoxides from activated platelets in arterioles account for red warm congested swelling. Platelet derived growth fator (PDGF) released during platelet secretion can readily explain the fibromuscular intimal proliferation of aretierioles first descibed by Michiels in 1981 and published by Michiels *et al*[52,53] in 1984 and 1985. Right: Platelet counts in 99 case histories of erythromelalgia thrombotic thrombocythemia (ETT) subdivided in ET (n = 69) and PV (n = 30) and in 100 case histories with hemorrhagic thrombocythemia (HT), Source Michiels 1981.

the microvascular erythromelalgic occlusive syndrome of thromocythemia at platelet counts above 400 × 10^9/L persists in PV in remission by phlebotomy[58,59]. Weitherley-Mein and Michiels discussed their common experiences on microvascular disturbance, major thrombosis and bleeding in myeloproliferative ET and PV[53-59] and strongly recommended since 1985 the use low dose aspirin for the treatment and prevention of erythromelalgic cerebral, ocular and coronary ischemic attacks in ET and PV patients in the United Kingdom and The Netherlands[59-61]. Cure of erythromelalgia, microvascular ischemic disturbances preceding PV or in the early stages of PV patients in complete remission by phlebotomy are obtained with aspirin 40 to 50 mg OD on top of keeping the hematocrit around 0.40 in males and females at platelet between 400 to 1000 × 10^9/L)[53,55,58,69,70]. It became evident that the JAK2^{V617F} mutated platelets in trilinear MPN are large and hypersensitive (sticky) in patients carrying the JAK2^{V617F} in ET and PV. Platelet in MPL^{515} mutated ET[71] are also constitutively activated and hyperreactive (sticky). This novel insight can easily explain the high risk (about 40% to 60%) of platelet-mediated erythromelalgic microvascular ischemic attacks in JAK2^{V617F} ET and PV and in MPL and CALR (calreticulin) mutated ET patients without features of PV patients[71]. If not treated with aspirin as was the case in the Vannucchi study[71], the incidence of major thrombosis at diagnosis and during follow-up in JAK2^{V617F} mutated ET and PV was high, but less frequent in JAK2 negative ET and PV patients[71].

In the Dutch ET/PV studies two third of PV patients were on aspirin/phlebotomy alone and only one third needed hydroxyurea and 16% used IFN, whereas two third of PV patients treated according to the WHO recommendations were on maintained hydroxyurea treatment[72-80]. In the Netherlands low dose pegylated interferon (IFN) became the first line treatment option in symptomatic PV with leukocytosis and mild splenomegaly to postpone the use of the leukemogenic agent hydroxyurea during long-term or even life long follow-up[78,79]. In the ECLAP (European Collaboration on Low-dose Aspirin in PV) study[72-74] treatment modalities at time of randomization into aspirin *vs* placebo were: Hydroxyurea in 44%, busulphan in 1%, pipobroman in 5.4%, IFN in 4.2% and phlebotomy alone 28%, or as adjuvant in 72%. There were no differences of vascular risk factors (like hypertension, diabetes, hyperlipidemia, previous thrombosis, *etc.*) in the aspirin and the placebo group. Mean values in randomized treated PV patients were 0.45 for hematocrit and 330 × 10^9/L for platelet count. In this setting treatment with aspirin (100 mg OD) *vs* placebo

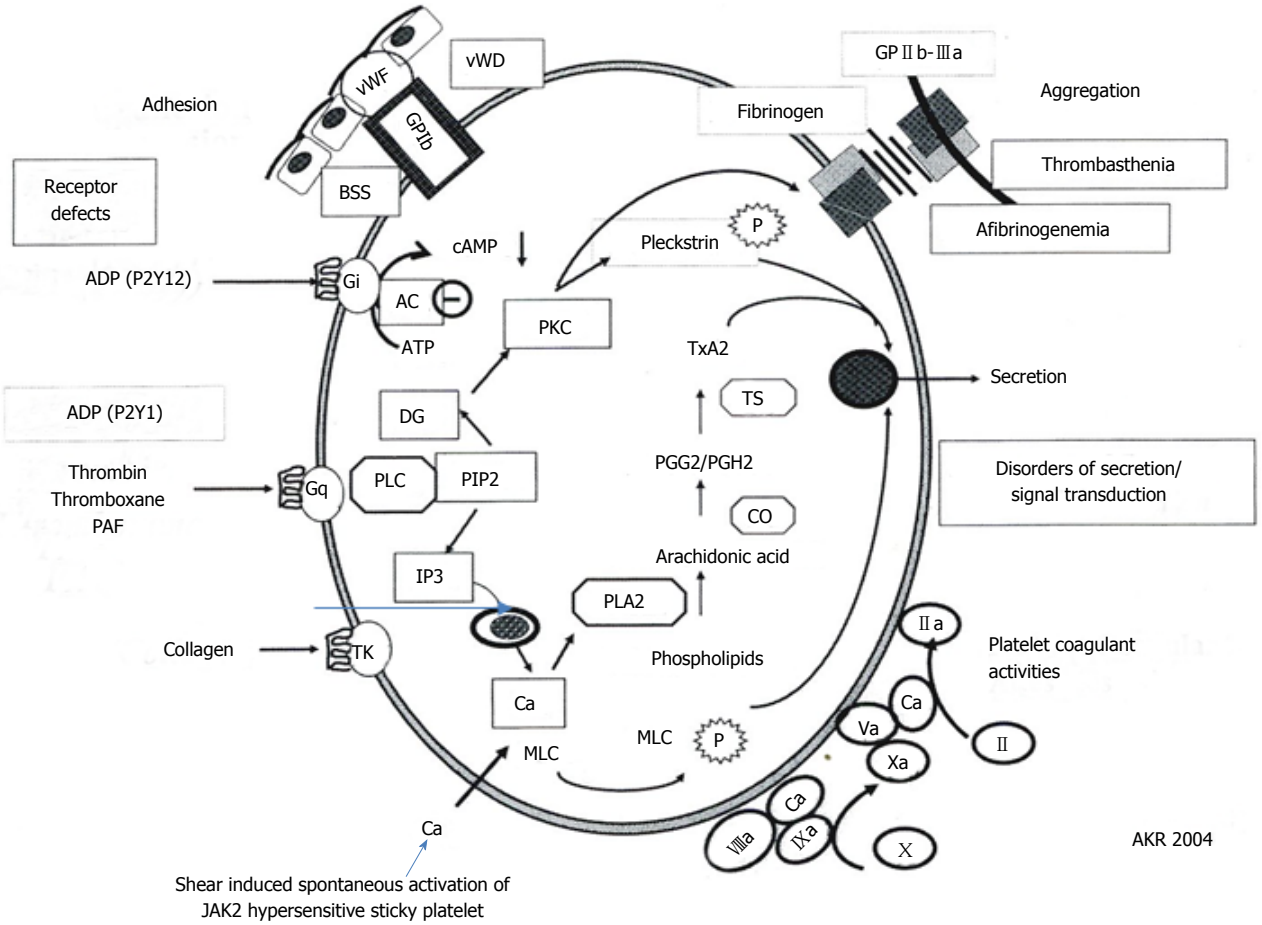

Figure 13 A schematic representation of platelet adhesion through von Willebrand factor and glycoprotein Ib disturbances in Bernard Soulier syndrome, von Willebrand disease and aggregation disturbances in Glanzmann's thrombasthenia, and receptor defects of ADP (P2PY12, P2Y1), thrombin, thromboxane, platelet activating factor (PAF and collagen induced intracellular metabolites). Source: Rao *et al*. Inherited defects in platelet signaling. *Sem Thromb Hemostas* 2004; 30: 525-535. Shear induced platelet activation of JAK2 hypersensitive (sticky) platelets (blue arrow) in arterioles starts with liberation of arachidonic acid from platelet membrane phopholipids by phopholipase A2 (PLA2) and metabolization of arachidonic acid by cyclooxygenase (CO) into prostaglandin endoperoxides PGG2/PGH2 and subsequent thromboxane A2 (TxA2) generation by thromboxane synthetase (TS). Spontaneous activation and aggregation of JAK2 sticky platelets (arrow) occur in the arteriolar peripheral and cerebral endarterial circulation with the production of large amount of prostaglandin endoperoxides as the cause of aspirin responsive inflammatory component of erythromelalgia followed by fibromuscular intimal proliferation and occlusion platelet thrombi in arterioles (Figures 9 and 10). Aspirin cures erythromelalgia in JAK2 thrombocythemia by irreversible inhibition of platelet cycolooxygenase (CO)[37]. In this process the activation of platelets by ADP (P2Y12), thrombin and collagen receptors are not involved in the etiology of erythromelalgia and cerebral vascular disturbances[52,53,79,80]. vWF: Von Willebrand factor; BSS: Bernard Soulier syndrome; vWD: Von Willebrand disease; Ca: Calcium; MLC: Myosin light chain; IP3: Inositoltriphosphate; PLC: Phospholipase C; PIP2: Phosphatidylinositol biphosphate; DG: Diacylglycerl; AC: Adenylcyclase; PKC: Protein kinase C.

in the ECLAP study significantly reduced the overall risk of a combined end point of microvascular and major vascular complications, including cardiac death, non-fatal myocardial infarction and stroke and major venous thrombosis from 15.5% to 6.7% during 2.7 years follow-up[73]. Absolute risk reduction was 8.4% and the number needed to treat to prevent one thrombotic event is 12[71,72]. Subsequently low dose aspirin was added for prevention of vascular events in PV. Treatment of PV anno 2017 include low dose aspirin, phlebotomy, low dose IFN, hydroxyurea in combination with phlebotomy and or ruxolitinib to reduce the dose and duration of the leukemogenic agent hydroxyurea (Figure 17)[53,78,79]. Recent insights indicate the IFN reduces both platelet and leukocyte count to normal obviating the need of aspirin and hydroxyurea life long in JAK2^{V617F}-mutated prodromal PV, classical PV and hypercellular ET with PV features (masked PV) or without PV faetures in CALR thrombocythemia[78-80] (personal experiences, manuscript in preparation).

MOLECULAR ETIOLOGY OF STICKY PLATELETS IN JAK2-THROMBOCYTHEMIA OF ET AND PV PATIENTS

With the advent of the JAK2^{V617F} mutation as the cause of trilinear myeloproliferative neoplasms (MPNs) ET and prodromal, classical and advanced PV, it became clear that JAK2^{V617F} mutated megakaryocytes are constitutively activated and do produce JAK2^{V617F} positive hypersensitive "sticky platelets" which spontaneously activate at high shear in the peripheral, ocular, cerebral and coronary endarteriolar circulation as the cause of platelet mediated arteriolar inflammation (platelet thrombophilia) in JAK2-mutated thrombocythemia

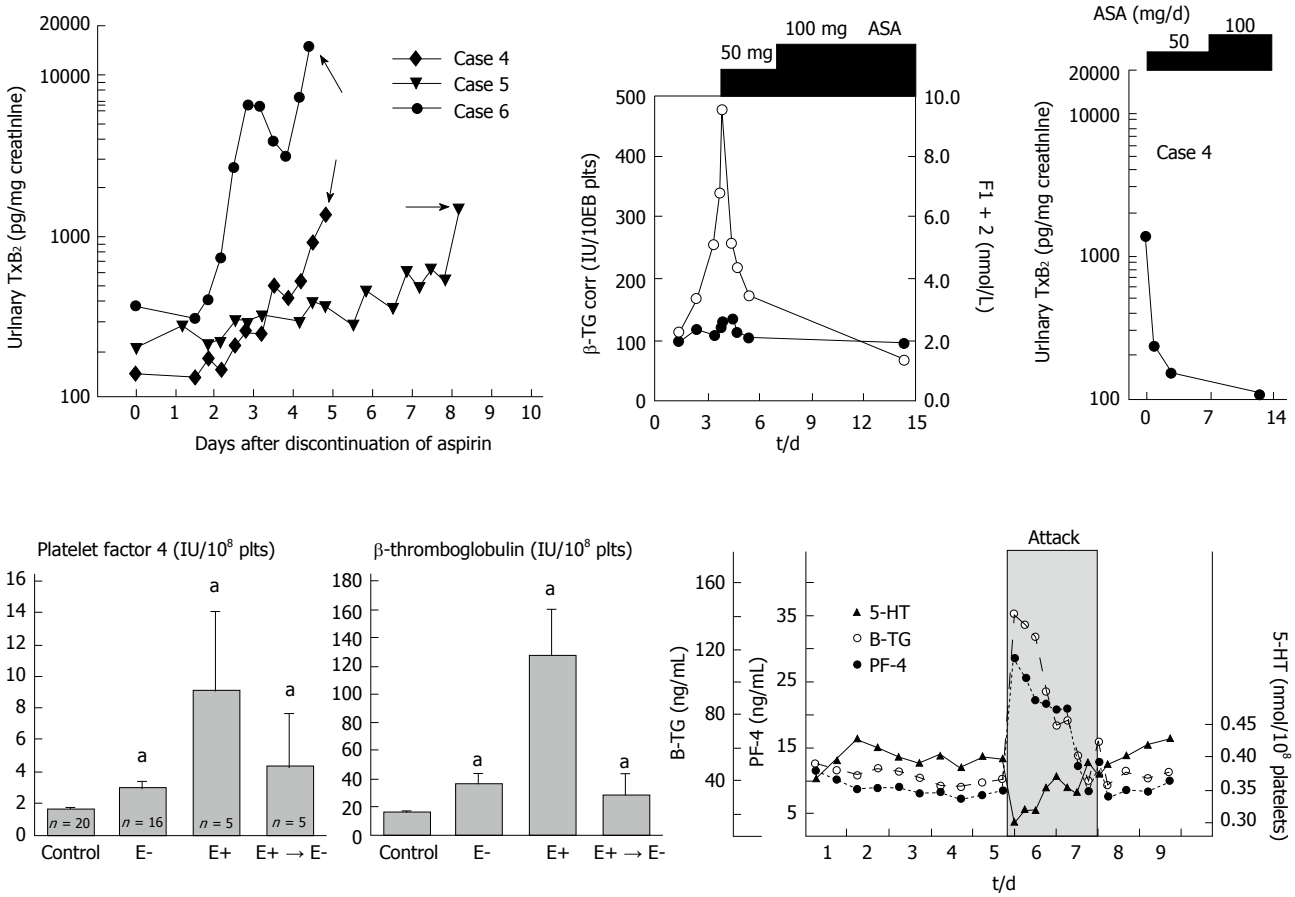

Figure 14 Vaso-active substances, prostaglandins endoperoxides and other factors released during JAK2 mutated platelet aggregation account for the inflammatory symptoms in JAK2-thrombocythemia of ET and PV patients. Upper part: Simultaneous study of clinical signs and symptoms of erythromelalgia, platelet activation markers and increased urinary thromboxane B2 (TxB2) excretion (right) in three ET patients during attacks of erythromelalgia after discontinuation of aspirin. This was associated with large amounts of urinary thromboxane B2 (TxB2) excretion (right) and high levels of beta-thromboglobulin (middle) at time of aspirin responsive erythromelalgic symptoms in JAK2 thrombocythemia. Erythromelalgia was successfully treated with a platelet-specific aspirin regimen of 50 mg per day, which was associated with correction of beta-TG to normal (right) and correction of TxB2 urinary excretion to normal (right). Treatment with 100 mg aspirin per day did even further decrease platelet activation markers beta-TG and TxB2 urinary excretion reaching completely normal levels[80]. Lower part: The effects of intervention with aspirin on platelet factor 4 (PF4) and bete-thromboglobulin (beta-TG) in 20 controls, 16 cases of thrombocythemia without erythromelalgia (E-), in 5 cases of thrombocythemia complicated by erythromelalgia (E+) and no aspirin, and in 5 cases after curative treatment of erythromelalgia in thrombocythemia patients (E+ → E-left and middle)[66,67]. Decreased platelet 5-HT and increased beta-TG and PF-4 during a documented migraine attack (grey zone) demonstrating that in such patients migraine is a platelet disorder with documented *in vivo* platelet activation during the attack[53,62,64,67]. [a]$P < 0.05$ *vs* control.

(Figure 15)[75]. The platelet mediated platelet thrombophilia in JAK2-mutated thrombocythemia of ET and PV patients was incurable and became curable by two subsequent discoveries in the history of Nature Medicine. First, Hoffmann[75] (1897) synthesized acetyl salicylic acid (aspirin, Bayer[R]), which appeared to inhibit platelet aggregation due to irreversible platelet cycolooxygenase[1-5]. Second, aspirin cures erythromelalgia and migraine-like cerebral microvascular disturbances through platelet cyclo-oxygenase inhibition[37,51,52] in JAK2-mutated thrombocythemia[76-79], could be labeled as a novel disease of platelet prostaglandin metabolism caused by JAK2 induced consitutively activated sticky platelets[80]. Heterozygous JAK2^{V617F} mutation with low JAK2 allele burden do present with the clinical picture of ET patients at high risk for aspirin-responsive erythromelalgic microvascular circulation disturbances[76] and low risk for major arterial and venous thrombosis[76-80]. Low dose aspirin in ET and aspirin/phlebotomy in PV aiming at hemotocrits of about 0.40 significantly improve the quality of life, prolongs life expectancy by the prevention of microvasvular and major thrombosis, but does not influence the natural history and progression of JAK2^{V617F} trilinear MPN and CALR and MPL thrombocythemia into myelofibrosis (Table 4)[52,76,79]. Progression of heterozygous JAK2^{V617F} mutated ET (Step 1) into combined heterozygous and homozygous JAK2^{V617F} mutated early PV and homozygous (Step 2) mutated advanced stages of PV is due to mitotic recombination of the mutated chromosome 9p[80]. This molecular event profoundly changed the clinical biological and pathological phenotype of trilinear MPN (Table 4 and Figure 17)[76-80]. About one third of JAK2^{V617F} mutated MPNs with prodromal PV or with advanced masked PV is associated with significant splenomegaly, leukocytosis and major thrombosis, who are candidates for pegylated interferon as the first treatment option to

Table 4 2017 Clinical, Laboratory, Molecular and Pathobiological classification and staging of JAK2V617F trilinear Myeloproliferative Neoplasms: Therapeutic Implications

PV: CLMP stage	0	1	2	3	4	5	6
Clinical Diagnosis	Prodromal PV	Erythrocythemia	Early PV	Classical PV	Masked advanced PV	Inapparent PV: IPV → Spent phase	Post-PV MF
LAP-score, CD11B	↑	↑	↑	↑	↑/↑↑	↑	Variable
EEC	+	+	+	+	+	+	+
Red CELL MASS	N	N	↑	↑/↑↑	↑/↑↑	↑ N or ↓	Variable
Erythrocytes × 10^{12}/L	< 5.8	> 5.8	> 5.8	> 5.8	N	N	↓
Leukocytes × 10^{9}/L	< 12	< 12	< or > 12	< or-> 15	> 15	N or ↑	> 20
Platelets × 10^{9}/L	> 400	400	< or > 400	> 400	+1000	N or ↑	Variable
Bone marrow histology	EM	EM	EM	EMG	EMG	MG-MF	MF
BM cellularity (%)	50-80	50-80	60-100	80-100	80-100	60-100	↓
Grading RF	RF 0-1	RF 0-1	RF 0-1	RF 0/1	RCF2/3	RCF 2/3	RCF 3/4
Grading MF[57]	MF 0	MF 0	MF 0	MF 0	MF 1 2	MF 1 2	MF 2/3
Spleen size:							
On echogram	< 12-15	< 13	12-15	12-16	18- > 20	16 > 20	> 20 cm
Below MCL	0-3	NP	0-3	4-6	> 6	> 6	> 8 cm
$JAK2^{V617F}$ load	Low +(++)	low +(++)	Moderate < 50% +	Mod/High + / ++	High > 50% ++	High → 50% ++	High → 50% ++
Granulocytes %							
Risk stratification → Therapeutic implications according to guidelines	Low	Low	Low Moderate	Inter-mediate	High early MF	JAK2 inhibitor	Post-PV MF

Source Michiels *et al*[68,70,80] 2006, 2017. ↑: Increased; ↓: Decreased; N: Normal; +: Present or heterozygous; ++: Homozygous; RCT: Randomized clinical trial; ET: Essential thrombocythemia; PV: Polycythemia vera.

postpone or eliminate the use of hydroxyurea (Table 4 and Figure 17)[71,76-80]. The gradual progression of JAK/Stat signalling kinase activity at the molecular level of $JAK2^{V617F}$ mutated heterozygous into combined heterozygous and homozygous and homozygous stages of overt and advanced stages PV[80] is associated with the acquisition of major thrombosis and constitutional symptoms in PV due to increased JAK2 mutated load, increase of activated leukocytes, and hematocrit (Table 4) on top of platelet mediated microvascular microvascular thrombotic syndrome of associated thrombocythemia[76-80].

Vannucchi *et al*[71] (2007) assessed the incidence of major thrombosis in a large retrospective study of 962 JAK2-MNP patients with thrombocythemia (MNP-T) subdivided in 323 PV and 639 ET patients heterozygous or homozygous for the $JAK2^{V617F}$ mutation. Aspirin responsive platelet thrombophilia or microvascular symptoms due to microvessel disorder including migraine-like headache, acral paresthesia, erythromelalgia, transient neurological and visual disturbances (Sticky Platelet Syndome) were excluded by definition in this retrospective analysis[71]. One hundred seventy-six patients (18.3%) had a major thrombotic event at diagnosis with a similar frequency in PV (19.2%) and ET (17.8%). During long-term follow-up, major thrombosis (usually not on aspirin) occurred in 122 patients (12.7%), corresponding to 14.9% in PV and 11.6% in ET patients. It should be emphasized that erythromelalgia and thrombocythemia may precede PV for several to about 10 years before latent MPN patients meet the PVSG or WHO criteria of PV[23,37,52,68,76]. Up to date, the causal association of early functional vasomotor disturbances of erythromelalgia with thrombocythemia and MIAs as the presenting symptom of early stage JAK2 mutated ET and PVs overlooked by internists, hematologists and neurologists[52,76,78] and therefore not treated with aspirin untill major thrombotic events of transient cerebral ischemic attacks (TIAs), major stroke, myocardial infarction had develeped[71].

The molecular pathology of $JAK2^{V617F}$ mutated MPN can explain the gradual progression of JAK/Stat kinase activity at the molecular level of heterozygous into homozygous $JAK2^{V617F}$ mutated MPN, which is associated with the sequential occurrence of ET, PV and MF phenotypes[76,80,81]. This evolution of MPN disease burden in ET, PV and MF patients has significant therapeutic implications to adapt the treatment opions from the use of low dose aspirin, aspirin/phlebotomy, pegylated IFN, hydroxyurea and ruxolitinib during long-term follow-up. Thromboxane A2 inhibition by dazoxyben[82], and platelet inhibition by dipirydamol[37,52], ticlopedin[37,52], clopidogrel[83] and coumarin[37,52] are well documented to have no effect on ongoing arteriolar platelet-mediated inflammatory and thrombotic processes in JAK2-thrombocythemia of ET and PV patients[53,60,77,80]. The association of erythromelalgia and MIAs has also been observed in congenital ET due to germline gain of function mutations in the TPO, JAK2 and MPL gene[79,80,81]. These germline gain of function mutation constitutively activate bone marrow megakaryopoiesis *via* the MPL receptor (TPO-receptor) signalling mechanism and increased production of hypersensitive sticky platelets as the cause of autosomal dominant aspirin responsive sticky platelet syndrome[70,81]. At platelet counts from below to above 1000 × 10^{9}/L erythromelalgic thrombotic thrombocythemia (ETT) is complicated by

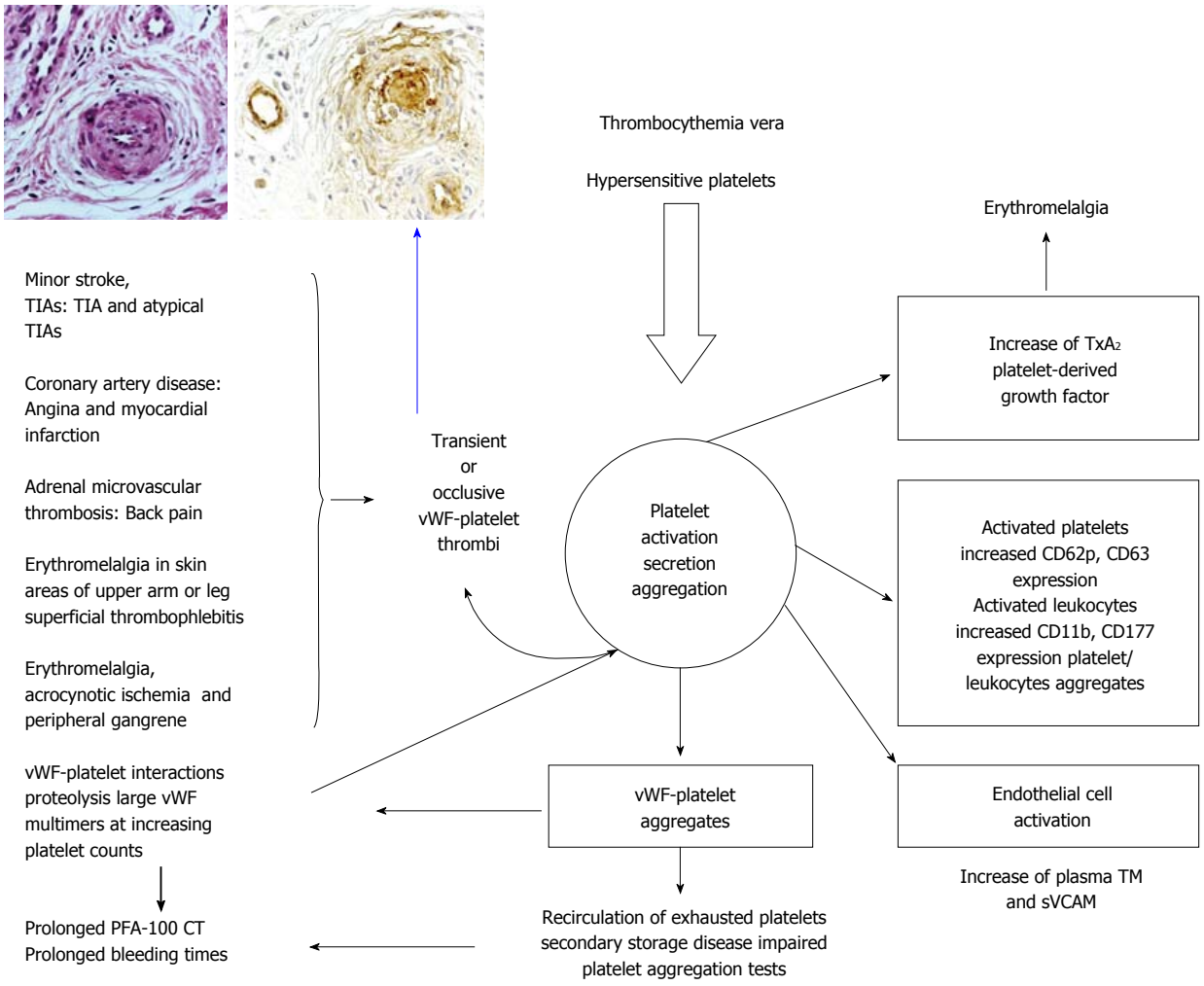

Figure 15 Shear induced platelet activation of constitutively JAK2^{V617F} hypersensitive sticky platelet with increased CD62p and CD 63 expression) in thrombocythemra vera of JAK2^{V617F}-positive ET and PV patients is the cause of a broad spectrum of platelet-mediated arteriolar inflammatory and thrombotic manifestations of erythromelalgia, digital ischemic complications, superficial thrombophlebitis, MIAs, TIAs, adrenal microvascular thrombosis and TIAs or even stroke and acute coronary syndrome in particular when thrombocythemia[79,80] is associated with increased activated leucocytes and erythrocyte count of polycythemia vera (increased cellular blood viscosity (Figure 12). In this process of *in vivo* platelet activation and secretion, reversible VWF-platelet aggregates activate endothelial cells to secrete thrombomoduline (TM) and sVCAM[79,80], whereas secreted PDGM accounts for the fibromuscular intimal proliferation (inset left) followed by occlusion of arterioles by VWF-rich platelet thrombi (inset right). After reversible aggration the platelets recirculate as exhausted platelets with secondary storage pool deficiency and impaired platelet functional defects. The platelet - Von Willebrand factor (VWF) interactions leads to proteolysis of large vWF multimers at increasing platelet counts from below to above 1000 × 10^9/L (Figure 13 right and Figure 16 peak 1 and 4).

spontaneous hemorrhagic thrombocythemia (HT) or paradoxical ETT/HT due to acquired von Willebrand disease type 2A (Figure 15)[53,77,78], which is reversible by reduction of platelet counts from above to below or 1000 × 10^9/L or to normal preferentially with interferon (Pegasys^R) or anagrelide in JAK2, MPL and CALR thrombocythemias[37,53,61,69,79,80]. CALR and MPL mutated thrombocythemias usually present with high platelet count around or above 1000 × 10^9/L complicated by ETT/HT or HT. Since CALR and MPL thrombocythemias have no features of PV the incidence of major arterial and venous thrombosis is low[80,81].

ROLE OF DUAL ANTIPLATELET THERAPY IN ACUTE CORONARY SYNDROMES

Erythromelalgia is successfully cured by a platelet-specific aspirin maintenance regimen of 50 to 100 mg OD, which is associated with correction of TxB2 urinary excretion to normal leaving the prostacycline synthesis in endothelial cells intact (Figure 14, Van Genderen *et al*[62-64]). Inhibition of platelet ADP (P2Y12) receptor by clopidogrel[83] leaving cyclooxygenase activity intact (Figure 13) does not prevent shear induced spontaneous activation of JAK2 induced hypersensitive sticky platelets in the endarterial circulation in ET and PV patients in the absence of vascular pathology or arterioclorotic disease.

ASPIRIN AND ADP (P2Y12) RECEPTOR INHIBITORS IN ACUTE CORONARY SYNDROME

Low dose aspirin 75 mg OD *vs* placebo in 796 patients

1978
Three year history of burning painful red or blue toes and forefoot sole of the right foot (erythromelalgia)
Spontaneous hernorrhages of large ecchymoses on the chest and subcutaneous hematomas
Erythromelalgia, which characteristically disappeared by treatment with low dose aspirin
During aspirin treatment the platelet counts increased from 1.101 × 10^9/L to 1.700 × 10^9/L
After cytoreduction of platelet counts to normal by busulphan the symptoma did not recur

May 1992. Third relapse of thrombocythemia
Platelets 861 × 10^9/L. Asprin responsive erythromelalgia big toe/forefoot
September 1992
Severe ischemic attacks of unsteadiness
Monoparesis of the right arm and dysarthriaparadoxycally occurred
Subsequent treatment with aspirin was associated with:
No recurrence of cerebral ischemic attacks
Increase of platelet count to about 1500 × 10^9/L
Persistence of bleeding symptoms

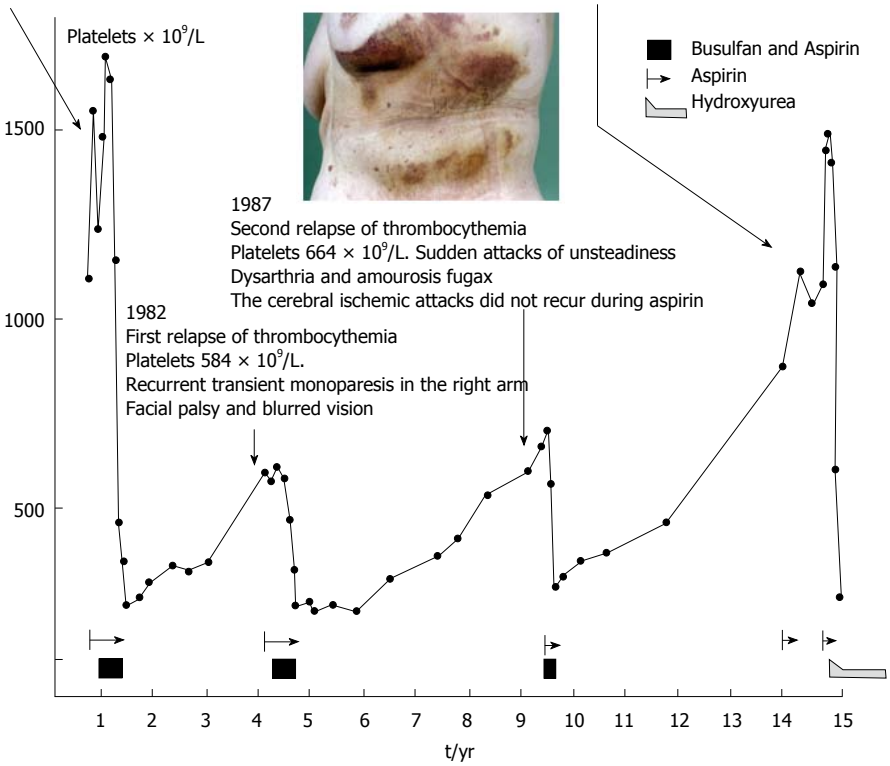

Figure 16 Longterm observations in a 72-year-old woman with a three years history of burning painful red or blue toes and forefoot sole of the right foot (erythromelalgia) presented in December 1978 with spontaneous hemorrhages of large ecchymoses on the chest and subcutaneous hematotomas. Paradoxical occurrences of platelet mediated thrombosis and bleeding in this case with thrombocythemia (ET with features of PV in the bone marrow: Prodromal PV) 1978 peak 1 in the figure. The first episode of erythromelalgia for 3 years followed by simultaneous occurrence of thrombotic thrombocythemia and hemorrhagic thrombocythemia at platelet counts around 1100 × 10^9/L in 1982. Aspirin was effective at peak 1 for the relief of erythromelalgia, which was associated with a further increase of platelet count to about 1500 × 10^9/L. During periods of thrombocythemia at peak 1, 2, 3 and 4 busulphan induced complete remissions of thrombocythemia resulted in normal platelet counts below 400 × 10^9/L, which was associated with no recurrence of erythromelalgia when not on aspirin. At peak 2 and 3 in the figure, recurrence of a second and third episode of thrombotic thrombocythemia occurred at platelet counts between 600 and 800 × 10^9/L. In 1992 at peak 4 the patient suffered from an episode of thrombotic and hemorrhagic thrombocythemia at platelet counts of 1040 × 10^9/L. Again aspirin relieved the erythromelalgia, which was associated with a further increase of platelet count to to around 1500 × 10^9/L but the hemorrhagic manifestation persisted which associated with acquired von Willebrand factor deficiency type 2A (acquired von Willebrand syndrome: AVWS and disappeared after correction of platelet count to normal) (Figure 15).

with unstable angina or non-Q myocardial infarction in the presence of arterioclerotic vascular pathology was effective to reduce the probability of death or myocardial infarction during one year follow-up (Figure 18)[84]. After revascularization or stent implantation in the percutaneous cutaneous intervention (PCI) setting the endothelial cell layer of the treated coronary artery has been removed with the consequence of platelet adhesion and aggregation as the cause of reocclusion if left untreated. We hypothesize that treatment with ADP (P2Y12) receptor blocker allow platelets to adhere to the subendothelium by VWF-GPIb and collagen receptor mediated adhesion to the subendothelium, while ADP (P2Y12) receptor inhibtion does inhibit the propagation of platelet aggregation thereby preventing reocclusion of the damaged coronary artery after PCI (Figure 13). Treatment of ACS patients with successful stent implantation (PCI) in 517 patients randomized for Marcoumar (INR 3.5-4.5) + aspirin 100 mg BID *vs* Ticlopedine 250 mg BID + aspirin 100 mg BID showed superiority of dual antiplatelet therapy of platelet cycolooxygenase and ADP (P2Y12) receptor inhibtion as compared to aspirin and vitamin K antogonist (Figure 18)[85]. The conclusion is that oral anticoagulation does not play a role in the reocclusion of coronary artery after PCI as compared to dual antiplatelet therapy and ticlopedin is superior to aspirin in the PCI setting of stent implantation. This could be confirmed in the

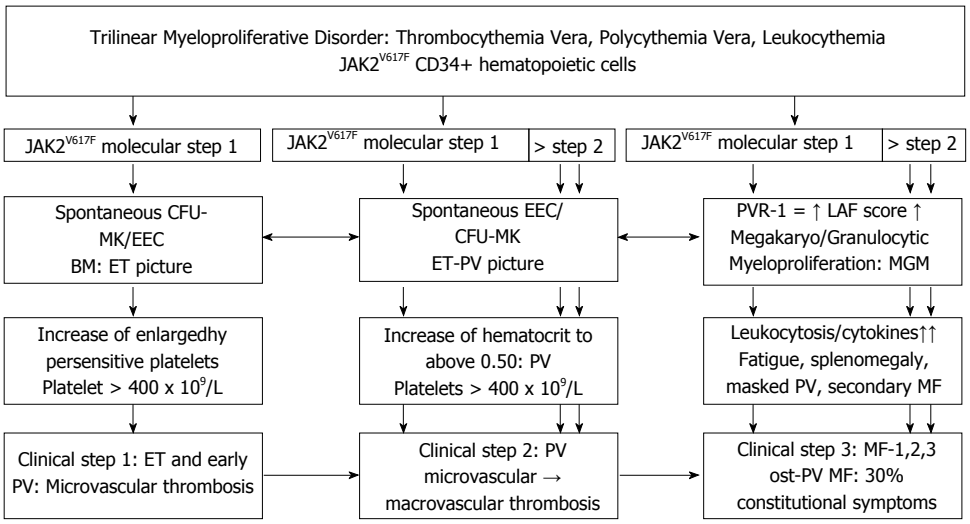

Figure 17 The molecular etiology of JAK2^{V617F} heterozygous mutated essential thrombocythemia (essential thrombocythemia picture Step 1) and evolution into combined herozygous/homozygous or homozygous JAK2^{V617F} mutated sequential stages of prodromal polycythemia vera classical PV (ET/PV pictures) and post-polycythemia vera myeloid metaplasia of bone marrow and spleen complicated by chronic idiopathic or secondary myelofibrosis.

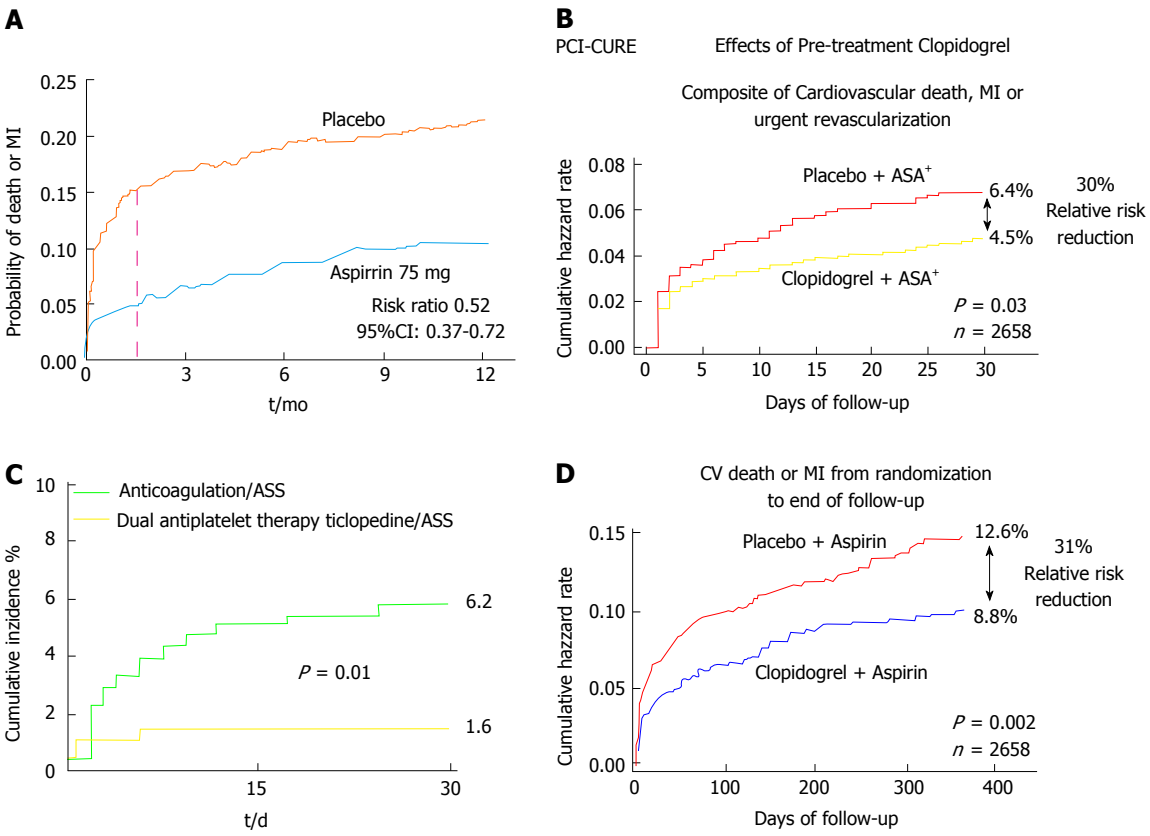

Figure 18 Low dose aspirin 75 mg OD *vs* placebo in 796 patients with unstable angina or non-Q myocardial infarction in the presence of arteriosclerotic vascular pathology was effective to reduce the probability of death or myocardial infarction during one year follow-up. A: Aspirin 75 mg OD *vs* placebo in 796 patients with unstable angina or non-Q myocardial infraction (MI) reduced the probability of death or MI from about 20% to 10% during 1-year follow-up[82]; B: Aspirin/marcoumar *vs* aspirin/ticlopedin after percutaneous cutaneous intervention (PCI) reduced the combined events of cardiac death, MI, bypass or recurrent PCI from 6.2 to 1.6% after 1-mo follow-up[83]; C: Aspirin/placebo *vs* aspirin/clopidogrel in 2625 treated PCI patients reduced the composite of cardiovascular death, MI, or urgent revascularization from 6.5% to 4.5% in the PCI-CURE study[84]; D: The extended substudy of the PCI-CURE reduced the combined cardiovascular death and MI reduced from 12.6% to 8.8% after 1-year follow-up[84].

Clopidogrel in Unstable Angina to Prevent recurrent Events Trial (PCI-CURE) in non-ST-elevation acute coronary syndrome patients. The PCI-CURE study examined whether the addition of clopidogrel to aspirin (dual aspirin-clopidogrel) *vs* aspirin alone in the PCI setting improves the outcome in terms of cardiovascular death, myocardial infarction (MI) or urgent revasularization (Figure 19)[86]. Clopidogrel was

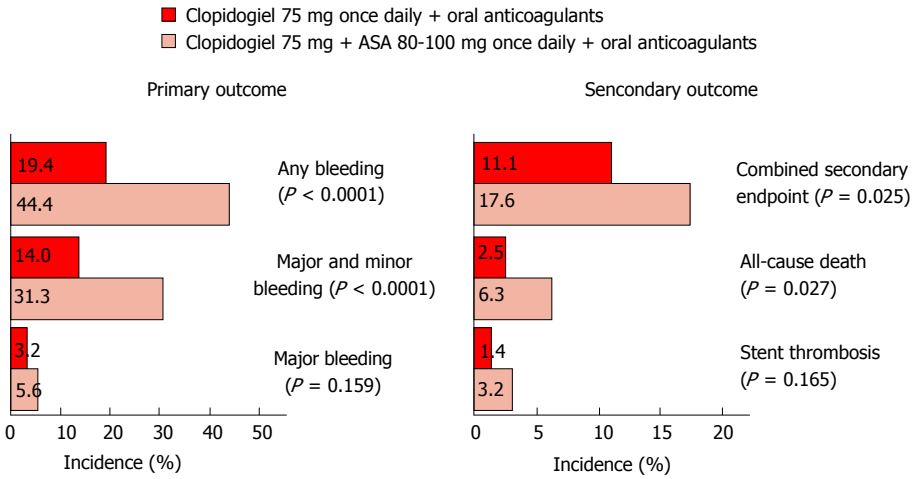

Figure 19 Dual clopidogrel and coumarin compared to triple clopidogrel coumarin and aspirin in 573 patients undergoing PCI receiving oral anticoagulation for another reason in the WOEST study was equal in terms of death, reoclusion and revascularization but superior for dual clopidogrel/coumarin in terms of any bleeding (44.4% *vs* 19.4%), major and minor bleeding (31.3% *vs* 14%) and major bleeding (5.6% *vs* 3.2%)[85].

given orally in an immediate dose of 300 mg followed by a maintenance dose of 75 mg once daily. There was a 30% relative risk reduction (RRR) from 6.5% to 4.5% in 2658 treated PCI patients during the first month follow-up and a 31% RRR from 12.6% to 8.8% after one year follow-up in a subgroup of PCI Cure patients (Figure 18)[86]. The likely explanation is that ADP inhibition by clopidogrel on top of aspirin does allow platelet adhesion to subendothelium of the coronary artery after PCI, but prevent subsequent platelet mediated reocclusion of the coronary artery after PCI by the double aspirin and clopidogrel inactivated platelets. As shown in Figure 13 the ADP induced pathway of platelet inactivation is not needed in aspirin responsive erythromelalgia and microvascular ischemic disturbances in JAK2-muated thrombocythemia. In the PCI setting, the ADP (P2Y12) pathway inhibition of platelet by clopidogrel is of critical importance in the reduction of the reocclusion rate after PCI, while the aspirin sensitive AA pathway plays a minor role (Figure 13). The critical question is whether aspirin is needed on top of clopidogrel in the PCI setting to prevent reocclusion. The WOEST evaluated the safety and efficacy of dual clopidogrel/oral anticoagulation (Clop/OAT) therapy (n = 284) for one year after PCI compared with triple clopidogrel/aspirin/coumarin (Clop/Asp/OAT) in patients undergoing PCI receiving oral anticoagulation for another reason atrial fibrillation in particular (n = 289)[87]. The primary efficacy endpoint was a combined end point of minor, moderate or major bleeding complication during the initial hospitalization and one year follow-up. The secondary efficacy endpoint was a combined event of death, myocardial infiarction, stroke, systemic embolization and target vessel revascularization during one year follow-up (Figure 19). After one year follow-up, any bleeding had occurred in 54 patients (19.4%) in the dual Clop/OAT patients as compared to 126 (44.4%) in the triple Clop/Asp/OAT patients[87]. The incidence of major and minor bleeds was significantly higher in the triple Clop/Asp/OAT group; 31.2% as compared tot he dual Clop/OAT group, 14.0%[87]. Secondary outcome events occurred in 31 patients (11.1%) in the double Clop/OAT group and in 50 patients (17.6%) in the triple Clop/Asp/Oat group. At one year, 7 patients (2.5%) in the double Clop/OAT and 18 patients (6.3%) in the triple Clop/Asp/Oat had died from any cause (Figure 19)[87]. In patients taking oral anticoagulation and undergoing PCI, dual Clop/OAT is superior to triple Clop/Asp/OAT treatment in terms of bleeding complications and there was no evidence of increased thrombotic risk after PCI without the use of aspirin.

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MINIREVIEWS

Oxidative alterations in sickle cell disease: Possible involvement in disease pathogenesis

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Abstract

Sickle cell disease (SCD) is the first molecular disease in the literature. Although the structural alteration and dysfunction of the sickle hemoglobin (HbS) are well understood, the many factors modifying the clinical signs and symptoms of the disease are under investigation. Besides having an abnormal electrophoretic mobility and solubility, HbS is unstable. The autooxidation rate of the abnormal HbS has been reported to be almost two times of the normal. There are two more components of the oxidative damage in SCD: Free radical induced oxidative damage during vaso-occlusion induced ischemia-reperfusion injury and decreased antioxidant capacity in the erythrocyte and in the circulation. We will discuss the effects of oxidative alterations in the erythrocyte and in the plasma of SCD patients in this review.

Key words: Oxidative stress; Sickle cell disease; Iron; Protein oxidation; Carbonyl group; Sulfhydryl group; Low-density lipoprotein; High-density lipoprotein

Core tip: Oxidative alterations in the plasma and erythrocyte of sickle cell disease may indicate disease progression and phenotype. Detected oxidative modifications may be used as disease markers. Novel drugs targetting oxidative damage of plasma and cellular components may be important as promising therapeutic options.

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INTRODUCTION

Sickle cell disease (SCD) is an autosomal recessive disease which was first reported by an American

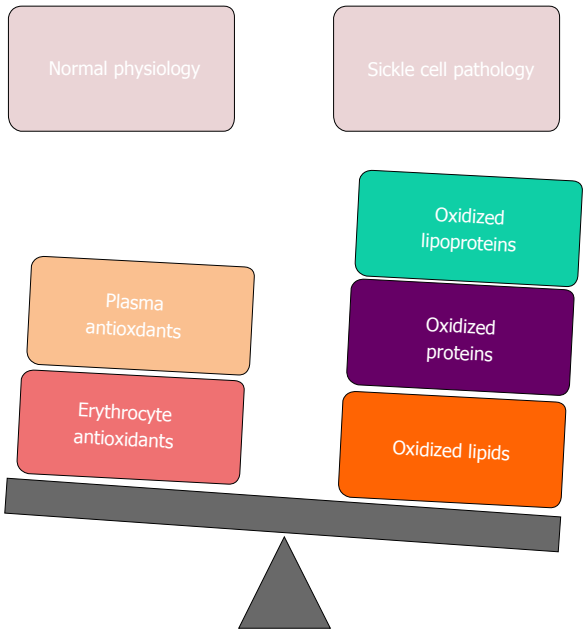

Figure 1 The balance between antioxidants and oxidants in sickle cell disease is altered towards the increase of oxidative stress and production of oxidized lipids, proteins and lipoproteins.

physician, James Herrick in 1904[1]. It was noted for being the first molecular disease after demonstration of the point mutation in beta globin gene in 1949[2]. Acidic glutamate residue at the sixth position was exchanged with a hydrophobic valine in the beta subunit of sickle hemoglobin (HbS). Solubility of abnormal HbS decreases in deoxygenation, dehydration and acidosis resulting with formation of long and solid polymers in the erythrocyte where it interacts with the cytoskeleton forcing the cell to get an almost sickled shape. Although the erythrocyte has a high capacity to move through the narrowest capillaries, the sickle erythrocyte loses its elasticity and tends to slow down and accumulate resulting with vaso-occlusion.

SCD is characterized by anemia, vaso-occlusion and chronic inflammation. Ischemia, and necrosis develop after vaso-occlusion concomitantly resulting with organ dysfunction[3]. Acute vaso-occlusive crisis is the most common clinical presentation that results with hospitalization. Pulmonary hypertension, leg ulcers, priapism and stroke may develop as a complication of vaso-occlusive crisis. On the other hand frequent transfusions result with iron deposition in the tissues and organs of the patients with resultant organ dysfunction[4]. Iron induces generation of free radicals that produce oxidative stress and damages cell membrane, organelles and DNA[5].

Although the structural alteration and dysfunction of the HbS are well understood, the many factors modifying the phenotype of the patients and clinical presentation are under investigation. Understanding the spectrum of biochemical alterations produced by this genetic disease, novel therapeutic approaches can be developed to increase the life quality of the patients.

OXIDATIVE PROCESSES IN THE NORMAL ERYTHROCYTE

The erythrocytes have always been subjected to oxidative stress because they already transport oxygen in the circulation[6]. While there is a continuous flow of oxygen in the erythrocyte, it additionally contains iron (Fe^{2+}) bound to heme in the cytoplasm surrounded by a membrane containing unsaturated fatty acids[7]. However, under normal conditions Fe^{2+} is isolated in the pocket of heme group and the antioxidant enzymes work to prevent or limit the damage of the oxidant stress[8].

When deoxyhemoglobin binds oxygen, an electron from Fe^{2+} of hemoglobin is transferred to oxygen forming oxyhemoglobin also called superoxyhemoglobin[9]. Normally this is reversible, however occasionally O_2 leaves hemoglobin in the form of superoxide and l ferric hemoglobin named methemoglobin (MetHb) is formed. Normal erythrocytes have some amount of MetHb and superoxide formation. As a result hydroxyl radical is formed by dysmutation catalyzed by H_2O_2 and Fe^{2+}. Therefore there is always some amount of oxidative stress in the erythrocyte[10].

However, there is an excessive increase of oxidative stress in the sickle erythrocyte and plasma medium that the balance between antioxidants and oxidants is altered towards an increased production of oxidized lipids, proteins and lipoproteins (Figure 1).

OXIDATIVE PROCESSES IN THE SICKLE ERYTHROCYTE

A point mutation in beta globin gene results with an unstable HbS protein that has an abnormal electrophoretic mobility and solubility[11,12]. Therefore, MetHb formation and decomposition and heme release have tremendously increased[13]. It was first shown by Hebbel *et al*[9] that autooxidation of HbS was increased compared to normal hemoglobin, HbA. The autooxidation rate of HbS has been reported to be almost two times (1.7-1.9) of the normal with an increased formation of superoxide[9-14].

Excessive amount of lipid peroxidation has been observed in sickle erythrocytes[15] where the membrane damage due to peroxidation was demonstrated by increased permeability to potassium ion[16], altered membrane asymmetry[17], decreased erythrocyte deformability[18], dehydration[19,20] and hemolysis[21].

Iron and copper are particular elements that trigger Hb oxidation[22-24]. There are contradictory findings about the concentration of Fe^{2+} and Cu^{2+} in the sickle erythrocyte. Increased[24-26], similar or decreased amounts were reported in the sickle erythrocyte compared to normal[27,28]. Furthermore, there is an iron

deposit on the membrane of the sickle erythrocyte that is different from normal. Heme bound iron[11] and unbound Fe^{2+} ion[29] were shown on the membrane. This is a factor that further increases the oxidative stress on the membrane. In addition, Hb auto-oxidation and radical formation thereby increased as mentioned above.

There are two more components of the oxidative damage in SCD: Free radical induced oxidative damage during vaso-occlusion induced ischemia-reperfusion injury and decreased antioxidant capacity in the erythrocyte and in the circulation[30]. Increased oxidative stress in the sickle erythrocyte disrupts the reducing power and defense mechanisms of the cell, thus facilitates further damage by other oxidative agents. Free heme in the sickle erythrocyte inhibits some enzymes that protect the cell from oxidation; there is a decreased activity of hexose mono phosphate pathway as well as decreased glutathione[30]. Although this metabolic deterioration has not been understood in the sickle erythrocytes, it should have a strong implication on the disease pathogenesis.

Membrane proteins of sickle erythrocytes were reported to have oxidative alterations[20-31]. Irregularities in the membranous distribution of band 3 and glycophorin, show that the membrane structure of the sickle erythrocyte is disrupted[20]. It has been observed that, accumulation of aggregates of hemichrome at the cytoplasmic region of Band 3 results in the merging of Band 3 molecules which in turn increases sickle cell fusion to endothelium and recognition by macrophages through increased immunoglobin G and complement activation at Band 3 merging sites[32]. Spectrin, which is a membrane skeleton protein, cannot properly bind to the sickle membrane as a result of the anomalies in the membrane proteins of the sickle cell. There is direct evidence that membrane proteins such as ankyrin, spectrin, Band 3 and Band 4 may have oxidative damage[31].

It has been shown that, membrane lipids of sickle cells also suffer oxidative damage[15]. Excessive lipid peroxidation accompanied by loss of membrane fluidity in biological membranes result in decreased membrane potential and increased permeability of H^+ and other ions, followed with cell rupture and loss of cell contents and organelles.

ENDOTHELIAL DYSFUNCTION IN SCD AND OXIDATIVE ALTERATIONS IN THE PLASMA PROTEINS

Chronic intravascular hemolysis of SCD results with excessive production of heme that depletes endothelial nitric oxide[33]. Additionally vaso-occlusive crisis end up with ischemia-reperfusion injury where enzymes like xanthine oxidase, NADPH oxidase, nitric oxide synthase were activated inducing excessive production of free radicals[34,35]. Asymmetric dimethyl arginine, a nitric oxide inhibitor was found to be increased in SCD[36]. All these factors contribute to endothelial dysfunction and further aggravate oxidative stress resulting with a depletion of plasma antioxidants in SCD[37].

Plasma protein oxidation is monitored by measurement of protein carbonyl levels[38]. Increased protein carbonyl levels were reported in various diseases and regarded as a factor that might contribute to the disease pathology[39-41]. Carbonyl-modified plasma proteins were demonstrated to trigger endothelial dysfunction[42] which is regarded to be important in the pathogenesis of SCD. We reported increased protein oxidation by carbonyl modification in SCD patients' plasma where carbonyl levels were correlated to plasma iron and hemolysate zinc levels[43]. Sulfhydrl groups measured in the plasma are mostly from proteins[44]. In fact protein sulfhydryl groups are important antioxidants that can break the oxidation chain. Albumin is the major plasma protein and was been shown to be a strong antioxidant[12]. We found decreased sulfhydryl content in the plasma of SCD patients[43]. All these posttranslational modifications occurred as a result of oxidative stress and needs further investigation to understand their effect on protein function and turnover.

Albumin is the major plasma protein that has antioxidant capacity due to its sulfhydryl groups[45]. Therefore it is a major target for oxidative injury. It was previously reported that free ^{34}cysteine residue of albumin was the target for oxidizing agents[46,47]. A study using proteomics approach reported oxidative posttranslational modification of plasma albumin in the form of malondialdehyde adducts in SCD patients with pulmonary hypertension[48]. Our group showed that electrophoretic mobility of albumin from SCD patients was different than that of albumin from healthy controls[49]. The inflammatory and oxidative medium in SCD possibly targets albumin and induces structural modification. Methemalbumin formation was also reported in SCD patients[50]. This may be an antioxidant defense mechanism where plasma albumin binds oxidized heme and may by this way alleviate toxic effects of free heme on other low abundance proteins.

LIPID PEROXIDATION IN SICKLE ERYTHROCYTES

Malonyldialdehyde is a non-enzymatic oxidative by product of lipid peroxidation. Its main sources are oxidation of polyunsaturated fatty acids and cyclic endoperoxides released during eicosanoid synthesis[51]. Peroxidation of membrane lipids results in loss of membrane architecture that is essential for the deformability of the erythrocyte in passing through capillaries[52]. An erythrocyte with such membrane defects has a shorter life span and becomes a target for the reticuloendothelial system.

We previously reported MDA levels in the plasma and in the erythrocyte of SCD patients were higher than healthy controls[53]. Interestingly these patients had significantly lower blood cholesterol levels and there was a negative correlation between MDA and cholesterol in these patients.

Oxysterols are cholesterol oxidation products having metabolic roles as well[54]. 7-ketocholesterol is an oxysterol that is mostly formed due to increased oxidative stress[55]. There are two studies investigating cholesterol oxidation in the sickle erythrocytes. One study found sickle erythrocyte membranes contained higher 7-ketocholesterol levels than normal erythrocyte membranes[56]. In the other study, increased 7-ketocholesterol in sickle erythrocyte membrane was suggested to alter membrane dynamics and packaging capacity, therefore contributing to membrane pathology in SCD[57]. We found increased 7-ketocholesterol levels in SCD patients who also had hypocholesterolemia[58]. We suggested this cholesterol oxidation product, 7-ketocholesterol may modulate cholesterol biosynthesis at cytoplasmic or nuclear level.

LIPOPROTEIN OXIDATION

Low-density lipoprotein (LDL) oxidation is a complex procedure in which both the proteins and lipids of the LDL are oxidized, resulting in extensive damage to its structure[59,60]. Macrophages, through increased proteoglycan binding, recognize and scavenge this cytotoxic remnant of native LDL forming foam cells[61,62]. The oxidation of LDL particles draws attention primarily because of their effect on atherosclerosis and coronary syndromes[63]. However, LDL leakage across endothelium and its subsequent oxidation by radicals can result in macrophage activation in all vascular structures. Furthermore, it is known that without oxidation, LDL particles do not result in the accumulation of cholesterol esters in blood vessels[64,65]; we can infer that if LDL is being oxidized, the result will be damage in vascular structure.

For example, oxidation of apolipoprotein B-100 component of LDL resulted in conformational change and increased endothelial uptake of LDL[66]. Being reported previously in patients with thalassemia[67], increased oxidation of LDL in patients with SCD patients might result with its increased clearance from plasma. This may be an explanation for decreased LDL as well as cholesterol levels in patients with SCA[68]. Possibly chronic hemolysis and increased erythropoietic activity are more important in the consumption of plasma pool of cholesterol and the development of hypocholesterolemia in patients with SCD[69]. However, the possible link between LDL oxidation and hypocholesterolemia should be investigated in further studies.

High-density lipoprotein (HDL) is known as the apolipoprotein that carries cholesterol back into the liver[70]; although HDL function is not as simple as this sentence suggests, its primary ability to accept cholesterol from LDL and macrophage foam cells is why HDL is considered protective against atherosclerosis[71,72]. Oxidized HDL on the other hand, loses its ability to remove cholesterol[73]. Contrary studies exist, it has been shown that specific forms of oxidized HDL (tyrosylated HDL) may in fact increase cholesterol uptake and decrease atherosclerotic plaque formation[74]. However, the specific nature of these oxidations and the lack of data about the *in vivo* formation of oxidized HDL raise questions on the reliability of this data for in vivo consideration.

Another important role of HDL is its anti-inflammatory function[75]. Oxidized HDL loses this function almost entirely and may even act pro-inflammatory during the acute phase response[75,76]. Furthermore, HDL levels are also decreased by ongoing inflammation[77,78]. This data suggests that the ongoing inflammatory state, increased acute phase reactants, and the constant oxidative stress that SCD patients undergo can result in a vicious cycle that is a major contributor to HDL dysfunction in SCD[79].

HDLs have additional functions; lipopolysaccharide binding, endothelial cell movement and function modulation, platelet-activating factor inhibition, anticoagulant activity inhibition, anti-oxidant enzyme effects, prostacyclin binding, stimulation of NO release; these are either direct effects through their plasma lipid transport role or effects through enzymes that travel alongside the apolipoprotein[78,80,81]. Paroxonase is one of these enzymes and was shown to have a decreased activity in SCD and researchers suggested that pediatric patients with SCD who had chronic oxidative stress might have a higher incidence of vaso-occlusive crisis[82]. However, SCD patients who had hydroxyurea had normal paroxonase activity. HDL has important antioxidant capacity and HDL mimetic peptides keep a potential to be a therapeutic agent in vascular inflammation[83]. 4F, an HDL mimetic, was shown to be beneficial against endothelial dysfunction in a mouse model of SCD[84].

CONCLUSION

SCD is regarded as a high oxidative stress situation, because of HbS. It is not unexpected that iron of heme can trigger many oxidative events that may damage erythrocyte and plasma macromolecules. Besides iron, vaso-occlusion induced ischemia-reperfusion injury and chronic inflammation also trigger oxidative damage at the cellular and at the circulation. There are many oxidative markers being studied in SCD. The clinical correlations of molecular alteration of proteins and lipids are important and they may modify disease presentation. New options of therapy in SCD will possibly involve antioxidants-either being synthetic or being biomimetic as adjuvant.

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