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WJH covers topics concerning experimental, clinical, oncological and transplant hematology, transfusion science, hemostasis and thrombosis, evidence-based medicine, epidemiology and nursing. Priority publication will be given to articles concerning diagnosis and treatment of hematological diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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FRONTIER

Allogeneic stem cell transplantation in chronic myeloid leukemia patients: Single center experience

Nur Soyer, Ayse Uysal, Murat Tombuloglu, Fahri Sahin, Guray Saydam, Filiz Vural

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Abstract

Chronic myeloid leukemia (CML) is a myeloproliferative disease which leads the unregulated growth of myeloid cells in the bone marrow. It is characterized by the presence of Philadelphia chromosome. Reciprocal translocation of the *ABL* gene from chromosome 9 to 22 t (9; 22) (q34; q11.2) generate a fusion gene (*BCR-ABL*). BCR-ABL protein had constitutive tyrosine kinase activity that is a primary cause of chronic phase of CML. Tyrosine kinase inhibitors (TKIs) are now considered standard therapy for patients with CML. Even though, successful treatment with the TKIs, allogeneic stem cell transplantation (ASCT) is still an important option for the treatment of CML, especially for patients who are resistant or intolerant to at least one second generation TKI or for patients with blastic phase. Today, we know that there is no evidence for increased transplant-related toxicity and negative impact of survival with pre-transplant TKIs. However, there are some controversies about timing of ASCT, the optimal conditioning regimens and donor source. Another important issue is that BCR-ABL signaling is not necessary for survival of CML stem cell and TKIs were not effective on these cells. So, ASCT may play a role to eliminate CML stem cells. In this article, we review the diagnosis, management and treatment of CML. Later, we present our center's outcomes of ASCT for patients with CML and then, we discuss the place of ASCT in CML treatment in the TKIs era.

Key words: Chronic myeloid leukemia; Allogeneic stem cell transplantation; Tyrosine kinase inhibitors; Graft *vs* host disease; Survival

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Core tip: Tyrosine kinase inhibitors (TKIs) have changed the fatal outcomes of chronic myeloid leukemia (CML). Many studies showed that TKIs provided rapid response, few serious adverse event and impressive survival outcomes. Although, allogeneic stem cell transplantation (ASCT) is only curative treatment option for CML, since 1999, the numbers of ASCT have dropped. Currently, ASCT is offering for patients who are resistant or intolerant

to at least one second generation TKI or for patients with blastic phase. Here, we present our center's outcomes of ASCT for patients with CML and then, we discuss the place of ASCT in CML treatment in the TKIs era.

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INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal myeloproliferative neoplasm that characterized by the presence of Philadelphia chromosome[1]. The incidence of CML is 1-2 cases per 100000. Reciprocal translocation of the *ABL* gene from chromosome 9 to 22 t (9; 22)(q34;q11.2) generate a fusion gene (*BCR-ABL*). BCR-ABL oncoprotein had constitutive tyrosine kinase activity that is a primary cause of chronic phase of CML[2].

Approximately 50% of patients are asymptomatic that they diagnosed incidentally after their routine laboratory tests. If they are symptomatic, symptoms are left upper quadrant pain or early satiety, fatigue, night sweats, symptoms of anemia, and bleeding due to platelet dysfunction. Splenomegaly is the main physical finding, in slightly > 50% of patients[3].

Characteristic feature of complete blood cell count is leukocytosis with basophilia and with immature granulocytes (metamyelocytes, myelocytes and promyelocytes and few myeloblasts). Thrombocytosis is frequent but severe anemia is rare[4]. Bone marrow aspirates and biopsy with conventional cytogenetics is taken from untreated patients at diagnosis. Cytogenetics must be performed by chromosome banding analysis (CBA). Fluorescence *in situ* hybridization (FISH) for t (9; 22) (q34;q11.2) and quantitative reverse transcriptase PCR (qRT-PCR) for BCR-ABL can be performed on peripheral blood[3].

The disease is classified into chronic phase (CP, most patients at presentation), accelerated phase (AP), and blast phase (BP)[4]. Clinical and hematologic criteria for the definition of AP according to World Health Organization (WHO) is the presence of one or more of the following: Persisting or increasing splenomegaly and/or white blood cells (> 10 × 10^9/L) unresponsive to therapy, 10%-19% blast cells and/or > 20% basophils in peripheral blood or bone marrow, platelet counts > 1000 × 10^9/L uncontrolled by therapy or < 100 × 10^9/L unrelated to therapy or clonal chromosome abnormalities in Ph^+ cells. Clinical and hematologic criteria for the definition of BP according to WHO is the presence of one or more of the following: Blast cells ⩾ 20% and/or extramedullary involvement excluding liver and spleen, including lymph nodes, skin, CNS, bone, and lung[4]. European LeukemiaNet (ELN) criteria for the definition of AP and BP slightly differ from WHO criteria. According to ELN, the definition of AP is the presence of one or more of the following: 15%-29% blast cells and/or > 20% basophils in peripheral blood or bone marrow, platelet counts < 100 × 10^9/L unrelated to therapy or clonal chromosome abnormalities in Ph^+ cells. The definition of BP is blast cells ⩾ 30% in peripheral blood or bone marrow and/or clonal chromosome abnormalities in Ph^+ cells[5].

The differential diagnosis of CML includes Ph^- negative chronic myeloproliferative neoplasms, leukemoid reactions, Ph-negative CML or chronic myelomonocytic leukemia.

At the diagnosis, there are several prognostic scoring systems to assess the risk of poor outcome: The Sokal score, Hasford score and the European Treatment and Outcome Study score (Table 1)[6-8]. Additionally, the stage of disease and response to tyrosine kinase inhibitor (TKI) are important factors for prognosis.

According to ELN response criteria, the complete hematologic response (CHR) is defined as white blood cell < 10 × 10^9/L, no immature granulocytes, basophils < 5%, platelet count < 450 × 10^9/L, and non-palpable spleen. The complete cytogenetic response (CCyR) is defined as no Ph (+) metaphases by CBA or < 1% BCR-ABL1-positive nuclei of at least 200 examined nuclei by FISH of peripheral blood. The partial, minor, minimal and no CyR is defined as 1%-35% Ph^+ metaphases, 36%-65% Ph^+ metaphases, 66%-95% Ph^+ metaphases and > 95% Ph^+ metaphases by CBA, respectively. Molecular response is assessed with the international scale (IS) as the ratio of BCR-ABL1 transcripts to ABL1 transcripts. Major molecular response (MMR) is defined as < 0.1% BCR-ABL1 expression. Deep molecular responses are defined as $MR^{4.0}$ (detectable disease with, 0.01% BCR-ABL1 IS or undetectable disease in cDNA with > 10000 ABL1 transcripts) and $MR^{4.5}$ (detectable disease with, 0.0032% BCR-ABL1 IS or undetectable disease in cDNA with > 32.000 ABL1 transcripts in the same volume of cDNA used to test for BCR-ABL1). Molecularly undetectable leukemia is defined as undetectable BCR-ABL with assay sensitivity ⩾ 4.5 or 5.0 logs[9].

It is recommended that either a molecular or cytogenetic test or both can be used for monitoring of CML. It's depends on local conditions of center. Routine blood counts with differentials are recommended every 1-2 wk until complete hematological response. Then, every three months, it should be evaluated to assess any side effects of TKIs. Every three months, molecular monitoring with qRT-PCR is recommended until major molecular response. Then, it can be performed every 3-6 mo. CBA of marrow cell metaphases was used for cytogenetic analysis at 3, 6 and 12 mo until CCyR. Then, it can be performed every twelve months. FISH on blood cells can be used for monitoring when the CCyR has been achieved. If patients fail to achieve therapeutic targets, progress to accelerate or blastic phase or show dysplastic changes, bone marrow biopsy and cytogenetic tests are recommended. Mutational analysis should be performed in case of progression or treatment failure[9].

Table 1 Calculation of relative risk

	Sokal score	Hasford score	EUTOS
Calculation	0.0116 × (age - 43.4) + 0.0345 × (spleen - 7.51) + 0.188 × [(platelet count/700)^2 - 0.563] + 0.0887 × (blast cells - 2.10)	0.666 when age ≥ 50 + (0.042 × spleen) + 1.0956 when platelet > 1500 × 10^9/L + (0.0584 × blast cells) + 0.20399 when basophils > 3% + (0.0413 × eosinophils) + 100	Spleen × 4 + basophils × 7
Risk definition	Exponential of the total Low risk: < 0.8 Intermediate risk: 0.8-1.2 High risk: > 1.2	Total × 1000 Low risk: ≤ 780 Intermediate risk: 781-1480 High risk: > 1480	Total Low risk: ≤ 87 High risk: > 87

Age is given in years. Spleen is given in centimeters below the costal margin (maximum distance). Blast cells, eosinophils, and basophils are given in percent of peripheral blood differential. All values must be collected before any treatment. EUTOS: European Treatment and Outcome Study.

TREATMENT OF CML

Imatinib, the first TKI, improved the 10-year survival rate from 10%-20% to 80%-90%[10]. Since its approval, two other TKIs, nilotinib and dasatinib, were approved first for second line then also for first line treatment for CML[11,12]. TKIs are now considered standard therapy for patients with chronic myelogenous leukemia.

First line treatment of CP-CML

Currently, imatinib (400 mg once daily), nilotinib (300 mg twice daily), and dasatinib (100 mg once daily) are recommended in first line therapy of CP-CML[9].

The main study of imatinib is International Randomized Study of Interferon and STI571 (IRIS). Patients with CML were randomized to receive imatinib 400 mg/d or INF-α plus low-dose subcutaneous cytarabine in this study. After a median follow-up of 19 mo, CCyR rate was 74% in imatinib arm and 9% in INF-α plus low-dose subcutaneous cytarabine ($P < 0.001$)[13]. In 8-year follow-up of the IRIS study, 53% of patients who treated with imatinib still had CCyR, although estimated event free survival (EFS) and overall survival (OS) rate were 81% and 93%, respectively[10].

The Dasatinib *vs* Imatinib Study in Treatment-Naive CML Patients (DASISION) and the Evaluating Nilotinib Efficacy and Safety in Clinical Trials-Newly Diagnosed Patients study (ENEST-nd) are randomized, prospective studies that showed superiority of dasatinib and nilotinib *vs* imatinib in newly diagnosed CML patients. In DASISION study, CCyR rate at 12 mo was 77% in dasatinib arm and 66% in imatinib arm ($P = 0.007$)[11,14]. In 3-year follow-up, responses were deeper and faster than imatinib arm. The 3-year OS and progression free survival (PFS) rates were similar both arms, but transformation to AP-CML and BP-CML was lesser than imatinib arm[15]. In ENEST-nd study, MMR rates at 12 mo were 44% in the arm of nilotinib 300 mg orally twice daily, 43% in the arm of nilotinib 400 mg orally twice daily, and 22% in the arm of imatinib ($P < 0.001$). The CCyR rates at 12 mo were significantly higher for nilotinib (80% for the 300-mg dose and 78% for the 400-mg dose) than for imatinib (65%) ($P < 0.001$)[12]. In 3-year follow-up, responses were deeper and faster than imatinib arm and transformation to AP-CML and BP-CML was lesser than imatinib arm[16].

Widespread using of TKIs is associated with drug resistance. One of the most common mechanisms of resistance involves point mutations in the kinase domain of BCR-ABL. The optimal treatment for patients failing imatinib treatment is imatinib dose escalation, a second-generation TKI or allogeneic stem cell transplantation (ASCT)[1]. Recently, there are some experimental studies using combination of TKIs to overcome the drug resistance[17-19]. They reported that combination of TKIs could overcome and prevent resistance. Combined TKIs approach should be investigated in further clinical trials in the subset of patients with TKI resistance.

Patients should be followed up according to definition of ELN response criteria (Table 2). If patients do not achieve a CHR by 3 mo, switching to a second TKI should be considered. If patients had > 10% BCR-ABL1 transcript level at 3 mo, it is recommended that serial molecular monitoring should be performed for 3 mo. If patients had > 10% BCR-ABL1 transcript level at 6 mo, therapy should be changed. If patients do not achieve CCyR by 12 mo, it requires a change in therapy. At any time, therapy should be changed, if patients loss of CHR or CCyR or PCyR or confirmed loss of MMR or determined new mutations and/or CCA/Ph$^+$[9].

Second line treatment of CP-CML

For patients who had intolerance to first line TKI, anyone of the other TKIs approved first line therapy can be used. Patient's comorbidities and toxicity profile of TKIs are considered in the choice of therapy[9].

For patients who had failure of TKI in first line, other TKIs approved first line therapy that patient did not use, bosutinib or ponatinib were recommended. Bosutinib was studied in patients that were resistant to or intolerant of imatinib. The CHR and CCyR rates were 86% and 41%, respectively. The 2-year PFS and OS rates were 79% and 92%, respectively[20]. Ponatinib is the only TKI with activity in patients with the T315I mutation. In phase Ⅱ Ponatinib Ph ALL and CML Evaluation study, among 267 patients with chronic-phase CML, 56% had a major cytogenetic response, 46% had a complete cytogenetic response, and 34% had a major molecular response[21]. So, bosutinib (500 mg once daily) and ponatinib (45 mg once daily) have been approved for patients resistant to prior therapy.

Table 2 European LeukemiaNet response criteria to tyrosine kinase inhibitors at first line

	Optimal	Warning	Failure
Baseline	NA	High risk or CCA/Ph$^+$, major route	NA
3 mo	BCR-ABL1 ≤ 10% and/or Ph$^+$ ≤ 35%	BCR-ABL1 > 10% and/or Ph$^+$ 35%-95%	Non-CHR and/or Ph$^+$ > 95%
6 mo	BCR-ABL1 ≤ 1% and/or Ph$^+$ 0	BCR-ABL1 1%-10% and/or Ph$^+$ 1%-35%	BCR-ABL1 > 10% and/or Ph$^+$ > 35%
12 mo	BCR-ABL1 ≤ 0.1%	BCR-ABL1 > 0.1%-1%	BCR-ABL1 > 1% and/or Ph$^+$ > 0
Then, and any at time	BCR-ABL1 ≤ 0.1%	CCA/Ph$^-$ (-7 or 7q-)	Loss of CHR Loss of CCyR Confirmed loss of MMR mutations CCA/Ph$^+$

In 2 consecutive tests, of which one with a BCR-ABL1 transcripts level ≥ 1%. This table was originally published in Baccarani *et al*[5]. CCA/Ph$^+$: Clonal cytogenetic abnormalities in Ph-positive cells; CCA/Ph$^-$: Clonal cytogenetic abnormalities in Ph-negative cells; Ph: Philadelphia chromosome; CCyR: Complete cytogenetic response; MMR: Major molecular response; NA: Not applicable.

Treatment of AP-CML

The therapeutic approach in AP-CML differs according to whether the patient is TKI naive or has progressed from CP while taking a TKI. All recommendations are based on results of single-arm, retrospective and prospective studies. For TKI naive patients; it is recommended a TKI (imatinib 400 mg twice daily or dasatinib 70 mg twice daily or 140 mg once daily). Allogeneic donor search should be done. ASCT is recommended for the AP patients who do not achieve an optimal response with TKI[9]. Response rate was reported higher with second generation TKIs than imatinib[22].

For patients who progressed from CP to AP-CML while taking a TKI; it is recommended anyone of the TKIs that were not used before progression. Allogeneic donor search and ASCT should be performed all patients[9].

Treatment of BP-CML

It is recommended combinations of induction chemotherapy and TKIs for patients with BP-CML. ASCT is recommended for all BP-CML patients who are eligible[9].

ASCT

ASCT is a highly effective treatment for CML. Since TKIs were used routinely in first line treatment and were safe and highly effective at controlling CP-CML, the numbers of allografts performed for CML have dramatically decreased[23]. Although outcomes of ASCT improved over years, HSCT is still limited to patients with an available donor and remains associated with significant morbidity and mortality[24]. However, ASCT remains an important therapeutic option for CML, especially for patients who are resistant or intolerant to at least one second generation TKI or for patients with blastic phase[9]. Another issue is keep in mind that BCR-ABL signaling is not necessary for survival of CML stem cell and TKIs were not effective on these cells[25,26]. ASCT is still had the potential for cure.

In this report, we present our single center experience of the outcomes of ASCT for patients with CML. Then, we will review our data with the literature of ASCT for CML.

CASE SERIES

Ten patients (3 female and 7 male) with CML were treated with ASCT in our center between October 2000 and December 2015. The median age at the transplantation was 50 (range 22-65) years. All patients were in chronic phase at diagnosis. Only one patient did not receive imatinib, this patient treated with interferon and hydroxyurea. Others received at least imatinib. One patient had primary imatinib resistance and 8 had lost their response. Four patient who had lost their response to imatinib received second-line TKIs. At the time of transplantation, 5 of all were in first CP, 3 were in 2nd CP and 2 was in AP. Time from diagnosis to ASCT was 61.5 (range 14-133) mo. Nine of all transplantation were matched sibling donor and one was an antigen mismatched (HLA A antigen) unrelated donor transplantation. Patient's characteristics are shown in Table 3.

Seven patients received myeloablative conditioning regimens (busulfan 3.2 mg/kg per day 4 d and cyclophosphamide 60 mg/kg per day 2 d) and 3 patients were received non-myeloablative regimens (fludarabine 30 mg/m^2 per day 5 d and busulfan 3.2 mg/kg per day 2 d; fludarabine 30 mg/m^2 per day 5 d, busulfan 3.2 mg/kg per day 2 d and, cyclophosphamide 350 mg/m^2 per day 3 d). Cyclosporine (2 mg/kg per day day -1, levels maintained at 200-300 μg/L until dose reduction) and methotrexate (15 mg/m^2 on day +1, 10 mg/m^2 on day +3, +6, +11 for myeloablative regimens and 10 mg/m^2 on day +1, +3 and +6 for non-myeloablative regimens) were used for graft *vs* host disease (GVHD) prophylaxis. In all patients, peripheral blood stem cell grafts were used.

All patients were engrafted. The median neutrophil and platelet engraftment times were 13 (10-25) d and 14.5 (10-30) d, respectively (Table 4). The median follow-up was 16.5 (3-117) mo. Only 3 patients are still alive without disease. The median follow-up of these patients were 87 (50-117) mo. Five patients died of complications after ASCT including acute GVHD (n = 3), and infection (n = 2). Two of all patients relapsed at 19 (molecular relapse) and 6 (hematological relapse) months from the date of ASCT.

Although our cohort is small, most of patients achieved molecular remission after transplantation. Only 2 patients died because of blastic crises and granulocytic sarcoma.

Table 3 Patients characteristics

Patient	Sex	Age at ASCT (yr)	Disease phase at ASCT	Time from diagnosis to ASCT (mo)	Indication for ASCT	Donor sex
1	M	22	1st AP	50	Resistance to imatinib and clonal evolution	M
2	F	51	2nd CP	14	Previous myeloid blastic phase	M
3	M	25	2nd CP	61	Previous lymphoid blastic phase	M
4	M	33	1st CP	37	Resistance to imatinib and dasatinib	M
5	F	54	1st AP	103	Resistance to imatinib, nilotinib and dasatinib 1st accelerated phase	F
6	M	65	1st CP	133	Resistance to imatinib and nilotinib	F
7	F	49	1st CP	62	Resistance to imatinib	M
8	M	53	2nd CP	63	Previous myeloid blastic phase	M
9	M	41	1st CP	51	Resistance to imatinib	M
10	M	61	1st CP	75	Resistance to imatinib, nilotinib and dasatinib	F

ASCT: Allogeneic stem cell transplantation; AP: Accelerated phase; CP: Chronic phase; M: Male; F: Female.

Others died because of acute GVHD and infection. However, only 3 patients achieved long term survival, ASCT has a place for treatment of CML.

DISCUSSION

As we mentioned above, ASCT is still important therapy for CML patients. In 1982, different groups were reported ASCTs with bone marrow graft from HLA-matched siblings[27-29]. Then, it was shown that CML patients who received T-cell depleted transplants with or without GVHD had higher probabilities of relapse than recipients of non-T-cell depleted allografts without GVHD. These data support graft-*vs*-leukemia (GVL) effect independent of GVHD[30]. Other reports showed that donor leukocyte infusions (DLI) for treatment of recurrent CML after ASCT could achieve stable remissions[31-33].

Outcomes of ASCT for CP-CML patients continued to improve with general improvements in transplant management and powerful GVL effect of DLI. In the post-TKIs era, there are some reports evaluating outcomes of ASCT and potential negative effect of TKIs[34-38].

According to European Society for Blood and Marrow Transplantation (EBMT) data, the 2-year OS, transplantation-related mortality (TRM) and relapse rate in patients transplanted between 2000 and 2003 were 61%, 30% and 22%, respectively[34]. Eighty-four patients with CML who underwent ASCT were evaluated in 3 groups according to the reason of ASCT: Group Ⅰ (early transplantation in low-risk patients, EBMT scores 0-1), group Ⅱ (imatinib failure in first CP), and group Ⅲ (advanced disease). At a median follow-up of 30 mo, the 3-year OS was 88% in group I, 94% in group Ⅱ and 59% in group Ⅲ. TRM was 8%[35]. In a cohort study, it was compared the outcomes of imatinib *vs* ASCT for AP-CML. In ASCT arm, median follow-up was 51 mo, and the 6-year OS, EFS, and PFS were 83.3%, 71.8% and 95.2%, respectively. In imatinib arm, median follow-up was 32 mo, and the 6 year OS, EFS and PFS were 51.4%, 39.2% and 48.3%, respectively. Patients treated with ASCT were significantly higher OS (P = 0.023), EFS (P = 0.008) and PFS (P = 0.000) than patients treated with imatinib[36]. Pfirrmann *et al*[37] compared two consecutive German studies Ⅲ (recruitment from 1995 to 2001) and ⅢA (recruitment from 1997 to 2004) on chronic myeloid leukemia. They reported that HLA matching, age of transplantation ⩽ 44 and time from diagnosis to ASCT ⩽ 1 year had a significant association with improved survival. They also reported that improvement of transplantation practice over years was associated with better survival. These findings suggested that the timing of ASCT is an important factor on survival outcomes.

According to Center for International Bone Marrow Transplantation Research (CIBMTR) data in TKI era, 3-year OS and disease free survival (DFS) rates were 36% and 27% in second CP, 43% and 37% in AP, and 14% and 10% in BP. Pre-transplant imatinib had no association with transplant outcomes, including acute and chronic GVHD[38]. In a study with CIBMTR data, they reported that pre-transplant imatinib therapy was associated with improved survival after transplantation and, they showed similar acute GVHD rates both using and not using imatinib before transplantation[39]. Fifty-one patients with CML underwent ASCT for advanced disease at diagnosis or for treatment failure with TKIs. At a median follow-up of 71.9 mo, the 8-year OS, EFS, relapse, and non-relapse mortality (NRM) were 68%, 46%, 41% and 23%, respectively[40]. Another study demonstrated that OS, DFS, relapse and NRM rates were similar between pre-transplant imatinib arm and no imatinib arm. On the other hand, mortality was higher in CP patients with suboptimal response than in CP patients with CCyR or major CyR on imatinib[41]. In a retrospective study, it was demonstrated that there was no evidence for increased transplant-related toxicity with pre-transplant dasatinib and nilotinib therapy[42]. In a small study, they showed that using dasatinib and nilotinib before ASCT did not increase transplant-related toxicity or GVHD[43]. According to these data, there is no evidence for increased transplant-related toxicity and negative impact of survival with pre-transplant TKI.

Goldman *et al*[44] reported that 15-year OS and relapse rates were 88% and 8% for sibling donor ASCT and 87% and 2% for unrelated donor ASCT, respectively. Recent randomized, prospective study evaluated differences between early allogeneic HSCT (group A) and best drug

Table 4 Transplantation outcomes

Patient	PNL engraftment (d)	PLT engraftment (d)	Acute GVHD	Chronic GVHD	Post-transplant disease status	Last status
1	12	18	No	No	Molecular relapse and granulocytic sarcoma	Died
2	15	16	No	No	Blastic crises	Died
3	18	30	No	No	Remission	Died
4	14	14	Yes	No	Remission	Died
5	25	21	Yes	Yes	Remission	Alive
6	19	15	Yes	No	Remission	Died
7	10	10	No	No	Remission	Alive
8	10	10	Yes	No	Remission	Died
9	11	14	Yes	No	Remission	Alive
10	12	13	Yes	No	Remission	Died

GVHD: Graft *vs* host disease; PNL: Neutrophil; PLT: Platelet.

treatment (group B) in patients eligible for both strategies. The 10-year OS was not different between group A (76%) and group B (69%). Patients of group A with low risk EBMT score (10-year OS 85%) had significantly higher survival (median $P < 0.001$) compared with patients with high-risk (10-year OS 41%) and non-high-risk Euro score in group B (median $P = 0.047$; 10-year OS 73%)[45]. The studies demonstrated that ASCT is still an option for selected CML patients.

Myeloablative vs non-myeloablative regimens

The curative effect of ASCT in CML is largely associated with the immune effect (GVL) mediated by alloreactive donor T cells. Reduced intensity conditioning (RIC) regimens provided reduced toxicity and rapid engraftment for elderly patients or those with comorbidities. In a small study was demonstrated that GVL effect may be insufficient and cytoreduction is required to provide cure with ASCT for CML[46]. The 5-year OS and DFS was 85% ± 8% with fludarabine, low-dose busulfan, and anti-T-lymphocyte globulin containing non-myeloablative (NMA) regimen[47]. In a study which was evaluated ASCT with RIC regimen for CML, after a median follow-up of 30 mo, 35.3% of patients were still alive[48]. Kebriaei *et al*[49] evaluated outcomes of 64 CML patients with advanced-phase disease who were treated with fludarabine-based RIC regimens. The 5-year OS and PFS were 33% and 20%, respectively. TRM was 33% at 100 d and 48% at 5 years after ASCT. In a study that were compared outcomes of MA conditioning regimen (56 patients) *vs* RIC regimen (28 patients), the 5- and 10-year leukemia-free survival and OS were similar. On the other hand, relapse rate was higher in patients receiving RIC regimen, whereas mortality rate was higher in patients receiving MA regimen[50]. In a large multicenter CIBMTR analysis compared RIC regimens with NMA regimens, relapse risk was lower and DFS was higher with RIC regimens than NMA regimens[51]. According to all these data, RIC regimens have had a place for elderly patients and patients who had comorbidities, but NMA regimens were inferior to RIC regimens.

In a study that was used data from the CIBMTR, they compared outcomes in patients who treated with ASCT following MA conditioning with cyclophosphamide (Cy) in combination with TBI, oral busulfan (Bu) or intravenous (IV) busulfan[52]. They concluded that Cy in combination with IV Bu was associated with less relapse than TBI or oral Bu. NRM and OS were similar.

GVHD prophylaxis

Another important issue is GVHD prophylaxis for ASCT. In CML, GVHD prophylaxis can influence the outcomes. T-cell depletion was associated with higher relapse rate, but DLIs were controlled the disease relapse in CML[30-33]. Zuckerman *et al*[53] evaluated 38 patients who treated with ASCT using partial T cell depletion (TCD) and preemptive DLI, without post-transplant GVHD prophylaxis. The 5-year LFS and OS were 78.95% and 84.2%, respectively. Acute GVHD rate was 18% in post-transplant patients and 24% in patients receiving DLI. They concluded that partial TCD and preemptive DLI was reduced the GVHD risk. In a small study, the CCyR was induced in 8 of 9 CML patients who treated with ASCT using an alemtuzumab-based RIC regimen. GVHD incidence was low but disease relapse was frequent[54].

Post-transplantation relapse

DLIs, TKIs, chemotherapy and second ASCT can be used for treatment of relapse CML after ASCT[55-59]. Olavarria *et al*[55] reported response to imatinib in 128 patients with CML relapsing after ASCT. The CCyR rate was 58% for patients in CP, 48% for AP and 22% for patients in BC. The CyR rates were 63% for CP or AP and 43% for BP in a small study that evaluated response to imatinib in 28 relapse CML patients after ASCT[58]. They concluded that imatinib is highly effective treatment for relapse CML after ASCT. A retrospective study was evaluated pre-DLI factors associated with prolonged survival in remission without secondary GVHD. They reported that approximately 50% of responding patients treated with DLI had GVL effect without secondary GVHD. Prolonged survival in remission without secondary GVHD was observed in patients who were given DLI beyond 1 year from ASCT for molecular and/or cytogenetic relapse[60].

Donor source

Transplantation from HLA-matched sibling donor (MSD) has been associated with the most favorable outcomes[61-64].

In a study from China, they compared the long-term outcomes of HLA-MSD with mismatched related donor (MRD) and unrelated donor (URD) ASCT for CML. They concluded that OS is similar between HLA allele-matched URD and MSD transplantation, but OS is lower in MRD and mismatched URD transplantation than MSD transplantation[64].

Although MSD transplantation has favorable outcomes, MSD is available for only one third of the patients. So, we can choice MRD, URD or haploidentical donor for ASCT. Previously, haploidentical donor transplantation has had inferior outcomes than MSD. These results are related to higher GVHD and TRM rates. Post-transplantation cyclophosphamide improved the outcomes of haploidentical transplantation. Ma *et al*[65] compared outcomes of 67 haploidentical ASCT and 23 MRD ASCT for patients with BP-CML or CP-CML from blast crisis. The 3-year OS and RFS rates were 60.0% and 51.1 for haploidentical transplantation and 55.3% and 47.8% for MRD transplantation, respectively. They concluded that haploidentical transplantation is an option for BP-CML with comparable survival to MRD transplantation.

Transplantation time

There are some reports that time from diagnosis to ASCT less than 12 mo is associated with better outcomes for patients advanced phase CML[36,37]. Xu *et al*[66] reported that for T315I mutation positive CML patients, haploidentical ASCT is highly curative treatment and immediate ASCT could result in promising survival for patients in CP/AP. There are different suggestions about patients in CP CML who failed second-line TKI. These patients could receive a third-line agent or be considered for SCT. Patients in BP should receive intensive chemotherapy with or without a TKI. If patients achieved second chronic phase, ASCT should be considered[9].

CONCLUSION

ASCT is still an important option for treatment of CML. There are some questions about timing of transplantation, optimal conditioning regimen and optimal GVHD prophylaxis. Some reports indicate that using TKI before ASCT is not associated with inferior ASCT outcomes. Non-myeloablative ASCT seems to be feasible for older and medically infirm patients. Relapse after ASCT can be manage with DLIs and TKIs.

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MINIREVIEWS

Current approach to disseminated intravascular coagulation related to sepsis - organ failure type

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Abstract

Disseminated intravascular coagulation (DIC) is a syndrome characterized by the systemic activation of blood clotting, which generates large amount of intravascular thrombin and fibrin. Various diseases may cause acceleration of the clotting cascade, inactivate the endogenous anticoagulants and modify fibrinolysis, having thus the formation of micro thrombi in the systemic circulation. The abnormalities in the hemostatic system in patients with DIC result from the sum of pathways that generate both hypercoagulability and augmented fibrinolysis. When the hypercoagulability state prevails, the main manifestation is organic failure. This subtype of DIC is often referred as "organ impairment" type, frequently seen in patients suffering from severe sepsis. To identify the underlying infection, early initiation of culture-based antimicrobial treatment, and to resolve any infection source promptly are keystone actions of DIC related to sepsis prevention and treatment. These should be combined with specific treatment related to each DIC subtype. In the context of septic shock, DIC is associated to increased severity, greater number and seriousness of organ failures, more frequent side-effects from treatment itself, and worse outcomes. Therefore, we ought to review the information available in the literature about approach and management of DIC in severe sepsis.

Key words: Septic shock; Disseminated intravascular coagulation; Coagulation impairment; Organ failure; Antithrombin; Sepsis

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Core tip: Disseminated intravascular coagulation (DIC) is a syndrome characterized by the systemic activation of blood clotting, which generates large amount of intravascular thrombin and fibrin. In the context of severe sepsis and septic shock, DIC is related to increased severity, greater number and seriousness of organ failures, more frequent side-effects from treatment itself, and worse outcomes. We ought to review the most important and updated information available in the literature about DIC in severe sepsis and septic shock.

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INTRODUCTION

Disseminated intravascular coagulation (DIC) is a syndrome characterized by the systemic activation of blood clotting that generates a large amount of intravascular thrombin and fibrin. This process results in small and medium vessel thrombosis and, eventually, organ failure and severe hemorrhage[1,2]. DIC could be the consequence of infections, hematologic malignancy, obstetric complications, trauma, aneurisms or hepatopathy. Each etiology signifies individual hazards related to the underlying disorder. Therefore, the diagnosis and treatment should be dictated by the disease[3,4].

In the context of septic shock, DIC is related to increased severity, number and seriousness of organ failures, more frequent side-effects from treatment itself, and worse outcomes, including death[5,6]. Therefore, we ought to review the most important and updated information available in the literature about DIC in severe sepsis and septic shock setting.

NORMAL HEMOSTASIS

Hemostasis is an organized process that aids to maintain vascular integrity. In the presence of endovascular damage, thrombin generation with simultaneous negative feedbacks and coordination of fibrinolysis occur, to avoid massive hemorrhage or excessive thrombosis. The first step in hemostasis is the formation of a platelet plug over the damaged zone[7]. On the surface of platelets, Integrins interact with each other and with endothelial cells surface through the von Willebrand factor and fibrinogen. Nevertheless, the formation of a platelet plug is not enough to achieve stable hemostasis, given that the contribution of a fibrin mesh to stabilize the structure of the clot is needed.

Clotting cascade

Physiologic clotting initiates with tissue factor (TF) and activated Factor Ⅶ (FⅦ) complexes that cleave Factor Ⅹ (FⅩ) into activated FX. This initial step has a short duration, due to quick inhibition of TF-aFⅦ complexes by the tissue factor inhibitor. The second pathway starts with Factor Ⅸ (FⅨ) that cracks into activated FⅨand joins activated FⅧ to transform FⅩ into activated FⅩ. Activated FⅩ forms a complex with activated Factor Ⅴ (FⅤ), with both phospholipids on platelet surface and calcium to turn prothrombin into thrombin[8]. Subsequently, thrombin turns fibrinogen into fibrin. At that time, activated Factor ⅩⅢ (FⅩⅢ) forms crossbred fibrin connections inside the clot, which serve as an additional support. Finally, fibrin clots are degraded by a protease called plasmin (Figure 1)[8].

DIC PATHOPHYSIOLOGY

Any alteration in hemostasis balance could generate hemorrhage or thrombosis[8]. In critically ill patients, this alteration is usually associated with sepsis, malignancy, and multiple trauma. These diseases usually accelerate the clotting cascade, inactivate endogenous anticoagulants, and modify fibrinolysis, resulting in micro thrombi formation in the systemic circulation[3].

The abnormalities in the hemostatic system in patients with DIC result from either hypercoagulability or hyperfibrinolysis[8] (Figure 2). When hypercoagulability prevails, the main clinical manifestation is organ failure. This type of DIC is referred as organ impairment type (both hypercoagulability and/or hypo-fibrinolysis exist)[9]. Organ impairment or organ failure DIC subtype is often seen in patients with severe sepsis. The activation of the coagulation cascade is an important part of the defense mechanisms to prevent infection dissemination. The increase in serum plasminogen activator inhibitor type 1 (PAI-1) caused by high levels of cytokines and lipopolysaccharides (LPS) in the blood of septic patients has been identified as one of the causes of hypofibrinolysis. Moreover, activated neutrophils in patients with sepsis liberate histones, neutrophil elastase and Catepsin G as a defense mechanism against pathogens[10]. Histones promote endothelial cell apoptosis, and platelet aggregation; meanwhile, neutrophil elastase inhibit Antithrombin (AT) and the Catepsin G decrease levels of the tissue factor pathway inhibitor (TFPI) promoting thrombus formation[10,11].

Cytokines

Endotoxin LPS are a component of the external membrane of gram negative bacteria, responsible of many of the cases of sepsis[12]. The entrance of endotoxin into systemic circulation causes the production of pro inflammatory cytokines. The consequent tissue damage is aggravated through free radicals generated by activated leucocytes. This causes an imbalance in normal hemostasis with the ulterior formation of thrombi in small and medium blood vessels that promote loss of vascular tone. All of this mechanisms contribute to the development of multiple organ failure[11-13].

Tumor necrosis factor: Tumor necrosis factor alpha (TNF-α) is synthesized in macrophages, and it is amongst the first cytokines to appear when endotoxin reaches blood circulation. It grasps it maximum concentration at 90 min from stimuli; then, it gradually disappears despite if the toxic stimulus remains. TNF-α has an important role initiating the inflammatory cytokine cascade and tissue damage. It has effects over monocytes, neutrophils, and vascular endothelium causing the production of other pro inflammatory interleukins (1b, 6 or 8). Furthermore, it stimulates the production of adhesion molecules such as

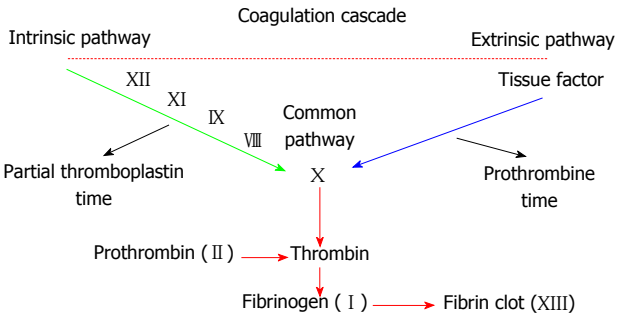

Figure 1 Schematic drawing of the coagulation cascade.

Intercellular Adhesion Molecule-1, vascular cell adhesion molecule-1 or E-Selectin.

Interleukin 1b: When the LPS enter the bloodstream, one can detect interleukin 1b (IL-1b) in plasma, and its presence serves as a severity marker. Patients with septic shock have high levels of IL-1b. It has been shown that the administration of this protein in primates induces a reduced fibrinolytic response equivalent to the one obtained with LPS or TNF-α. This suggests that IL-1b contributes to hypo-fibrinolysis mediated by PAI-1 in the presence of endo-toxemia[14,15].

IL-6: Endothelial cells synthesize IL-6 in presence of LPS. It also appears in the general circulation just after TNA-α shows up. IL-6 has a pathophysiologic role during sepsis as a clotting activator, and its concentration correlates with the disease severity[14,15].

Other cytokines: Other molecules participate in the inflammatory process in presence of the LPS: IL-12, IL-8, and interferon-γ. Nevertheless, their role in DIC is not yet well defined[15].

DIC DIAGNOSIS

At the bedside, is necessary to consider the clinical conditions that could alter the commonly used laboratory tests to diagnose DIC. Ergo, the diagnosis requires clinical expertise along biochemical workshop. The recurrently used test that might be affected include platelet count, prothrombin time (PT), fibrinogen, and fibrin degradation products (FDP), among others. Some clinical guidelines issued recommendations regarding this aspect[1,16,17]. In 2013 the International Society of Thrombosis and Hemostasis published recommendations for diagnosis and treatment of disseminated intravascular coagulation[18]. This guidance was based on a previous consensus by the British Committee for Standards in Hematology, the Japanese Society of Thrombosis and Hemostasis, and the Italian Society for Hemostasis and Thrombosis (Società Italiana per lo Studio dell'Emostasi e della Trombosi - SISET). They stated that in sepsis related DIC the major variation is either hyper-coagulation or hypo-fibrinolysis. As mentioned above, the main clinical manifestation

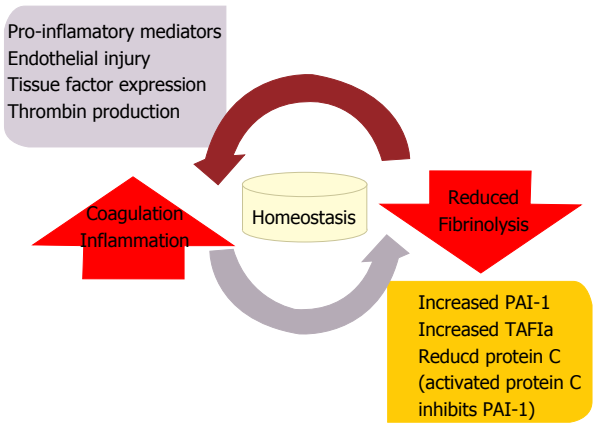

Figure 2 Mechanisms associated with hypercoagulability and/or hypofibrinolysis observed in sepsis related disseminated intravascular coagulation. PAI-1: Plasminogen activator inhibitor type 1; TAFIa: Thrombin activatable fibrinolysis inhibitor.

is organ failure, so several validated score systems to recognize DIC have been distributed using platelet count, prothrombin time, and anti-thrombin. The Japanese Association of Acute Medicine (JAAM) published a score system to detect sepsis related DIC, with a sensitivity and specificity of 100% and 65.0% respectively[5,19,20] (Table 1). Recently, Iba *et al*[21] proposed a modified version of the JAAM-DIC diagnostic criteria. They suggest to replace Systemic Inflammatory Response Syndrome (SIRS) by antithrombin activity, since SIRS is no longer used for the diagnosis of Sepsis. The new criteria could diagnose the same number of patients with comparable severity (mortality, 34.6% *vs* 34.8%). Also, mortality increased as the baseline antithrombin activity decreased (patients with a baseline antithrombin activity ⩾ 70% had a mortality of 26.5% *vs* 35.5% for those with an antithrombin activity < 70%). Despite this promising results, future studies to examine the worth of the modified scoring system in different populations are warranted[21].

Laboratory findings

A complete coagulation examination, including prothrombin time and platelet count is essential[4]. In some types of DIC (bleeding, massive hemorrhage, and asymptomatic) identifying the elevation of fibrin-associated biomarkers (D dimer, FDP, and soluble Fibrin) is useful to establish diagnosis[9]. Table 2 highlights the laboratory tests useful to diagnose DIC in a septic patient. It is important to consider that a coagulation disorder has around 35%-40% chance to be related to any other cause beside sepsis. A positive result does not guarantee the diagnosis. Delabranche *et al*[22] in 2016 published a multicenter, prospective observational study completed in 4 intensive care units in France. They used de JAAM score, sequential organ failure assessment score, and the acute physiology and chronic health evaluation Ⅱ to identify patients with DIC at early stage. They concluded that a combination of PT, endothelium-derived

Table 1 Score for disseminated intravascular coagulation diagnosis established by the Japanese Association of Acute Medicine

Parameter	Points
SIRS criteria	
3 or more	1
2-0	0
Platelet count (× 10^3/μL)	
< 80 or a reduction of > 50% in 24 h	3
80-120 or a reduction of > 30% in 24 h	1
> 120	0
Prothrombin time	
1.2 times over control or higher	1
< 1.2 times over control	0
Fibrin degradation products/fibrinogen (mg/L)	
25 or more	3
10 to 24	1
< 10	0
Diagnosis DIC: 4 or more points	

SIRS: Systemic inflammatory response syndrome; DIC: Disseminated intravascular coagulopathy.

Table 2 Laboratory findings in sepsis-related disseminated intravascular coagulation

Test	Alteration	Other causes
Platelet count	Reduction	Bone marrow abnormalities
Anti-thrombin/C protein	Reduction	Hepatic failure, capillary leakage syndrome
Prothrombin time	Extended	Hepatic failure, vitamin K deficiency
Soluble fibrin/thrombin	Increased	VTD, surgery
vWF-PP/PAI-1	Increased	Organic failure
aPTT	Bifasic wave	Infection
ADAMTS-13	Reduction	Hepatic failure, thrombotic microangiopathy
FDP/DD	Increased	VTD, surgery

VTD: Venous thromboembolic disease; vWF-PP: Von Willebrand factor pro-peptide; PAI-1: Type 1 plasminogen activator inhibitor; ADAMTS-13: A desintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; FDP: Fibrin degradation products; DD: D-dimer; aPTT: Activated partial thromboplastin time.

CD105$^+$-microparticles, and platelet count at admission could predict the absence of disseminated intravascular coagulation[22].

Liu *et al*[23] found four thrombin derived biomarkers that were triggered before PT, activated partial thromboplastin time (aPTT), or platelet count became altered. These markers include fibrinopeptide type A, soluble fibrin monomer complex, prothrombin fragment 1 + 2 (F1 + 2), and the thrombin-antithrombin complex. The F1 + 2 represents the total amount of fibrin produced, while the other three markers only show it partially. F1 + 2 is considered the most sensitive marker of thrombin production.

In the last few years the identification of endothelial damage markers and inflammatory cascade activators have made possible to find coincidences between the inflammation trigger mechanisms and coagulation. This extend the possibilities for future treatment targets[11].

Table 3 Treatment recommendations amongst different types of disseminated intravascular coagulation

Dysfunction	Recommended treatment
Pre-DIC	Treat cause and UFH 70 IU/kg per day or LWMH anti-Xa target: 0.8-1.2
Multiple organ failure	Treat cause and AT 30 IU/kg per day of 3 d
Hemorrhagic	Treat cause and Hemo-transfusion Anti-fibrinolytics Protease synthetic inhibitor
Massive hemorrhage	Treat cause and Hemo-transfusion Anti-fibrinolytics Protease synthetic inhibitor

DIC: Disseminated intravascular coagulopathy; UFH: Unfractionated heparin; LWMH: Low molecular weight heparin; Xa: Activated X factor; AT: Anti-thrombin.

DIC TREATMENT

To identify the underlying infection, early initiation of culture-based antimicrobial treatment, and to resolve any infection source promptly are keystone actions of DIC related to sepsis prevention and treatment. Table 3 lists key recommendations for the treatment of different types of CID.

The Surviving Sepsis Campaign guidelines[24], do not recommend treatment of any associated coagulopathy as for the lack of evidence to support it. Recently, Umemura *et al*[25] reported a meta-analysis of anticoagulation therapy in three different types of patients: (1) septic patients without coagulopathy; (2) patients with sepsis induced coagulopathy; and (3) patients with induced sepsis DIC. They identified that only septic induced DIC patients had a reduced mortality with no difference in the prevalence of hemorrhagic complications[25]. In septic patients, biomarkers of the homeostasis loss, such as histones (H3, H4), the TFPI, and the neutrophil extracellular traps are useful to determine whether to start treatment[26].

Antithrombin

AT has proven to be effective to revert sepsis induced DIC. As mentioned above, when germs disseminate throughout the organism, a diffuse coagulopathy that results in massive thrombi formation in small and medium blood vessels occur[13]. The KybertSept trial[27] was the first to evaluate the effectiveness of AT substitution in patients with severe sepsis and septic shock. The results showed an increase in the incidence of bleeding complications related to AT use. It is important to reflect that some of their patients used heparin as deep vein thrombosis prophylaxis. A sub-analysis of patients without heparin prophylaxis showed a reduction of adverse effects in AT group[27]. Later on, Gando *et al*[28] showed that in patients

with activated-AT levels of 50%-80%, the administration of AT at a dose of 30 UI/kg perday during 3 d improved platelet counts, and reduced the score punctuation for sepsis associated DIC without increasing bleeding events[28].

Heparin use

Antithrombin-Ⅲ (AT-Ⅲ) inactivates thrombin and other proteases, including FXa[29]. Heparin attaches to a AT-Ⅲ producing a conformational change that increases AT-Ⅲ activity. The unfractionated heparin (UFH) dose in Pre-DIC is 70 UI/kg per hour in continuous infusion for 5-7 d[23]. There are few randomized controlled trials evaluating the utility of heparin in DIC. Liu *et al*[23] shown that low molecular weight heparin was superior to UFH due to a higher inhibition of FXa[29]. The utility of other compounds like Fondaparinux and Danaparoid sodium is restricted to asymptomatic DIC for risk reduction of thrombotic events[9].

Blood components administration

Because of coagulation factors (specially fibrinogen) and platelet consumption, most clinical guidelines[1,16,17] recommend blood components administration only in hemorrhagic and massive hemorrhage DIC. The recommended platelet goal count has been established at 50 × 10^3/μL if active bleeding or 20 × 10^3/μL along high risk of hemorrhage. If PT or aPTT are 1.5 times over the standard, or fibrinogen is below 1.5 g/dL, fresh frozen plasma (15 mL/kg) is indicated. If volume restriction is intended, a concentrate of prothrombin complex, cryoprecipitates, or purify fibrinogen concentrates are preferred[1,16,17].

Human recombinant thrombomodulin

Thrombomodulin may reduce massive thrombotic events caused by the expression of extracellular histones observed in sepsis DIC[26]. In the double blind controlled study, Vincent *et al*[30] administered human recombinant thrombomodulin to patients with sepsis induced DIC that developed one or more organ failures and an international normalized ratio > 1.4. The dose of 0.06 mg/kg per day for 6 d along with conventional treatment reduced the severity of hematologic failure and reduced DIC incidence. Further trials are needed to safely recommend the therapy.

CONCLUSION

In critically ill patients, the early diagnosis of coagulopathy is essential to reduce morbidity and mortality. Identification of sepsis related DIC is difficult, especially when precise laboratory tests are not available. Clinicians should suspect the diagnosis in every severe sepsis or septic shock patient, and use whatever tools accessible to investigate it. It is important to treat promptly even subtle changes linked to coagulopathy, to diminish the extent of DIC.

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ORIGINAL ARTICLE

Basic Study

Intestinal heme absorption in hemochromatosis gene knock-out mice

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Abstract

AIM

To investigat the influence of hemochromatosis gene (Hfe) mutation on ^{59}Fe labelled duodenal heme absorption in mice.

METHODS

Heme absorption was measured in Hfe wild type and Hfe$^{(-/-)}$ mice by the duodenal tied loop and by oral gavage methods. The mRNA expression of heme oxygenase (*HO-1*), *Abcg2* and *Flvcr1* genes and levels were determined by quantitative polymerase chain reaction.

RESULTS

Heme absorption was significantly increased in homozygous Hfe$^{(-/-)}$ mice despite significant hepatic and splenic iron overload. While duodenal *HO-1* mRNA was highly expressed in the wild type and Hfe$^{(-/-)}$ heme-treated group following 24 h heme administration, *Flvcr1a* mRNA decreased. However, *Abcg2* mRNA expression levels in duodenum remained unchanged.

CONCLUSION

Heme absorption was enhanced in Hfe$^{(-/-)}$ mice from

both duodenal tied-loop segments and by oral gavage methods. *HO-1* mRNA levels were enhanced in mice duodenum after 24 h of heme feeding and may account for enhanced heme absorption in Hfe$^{(-/-)}$ mice. Implications for dietary recommendations on heme intake by Hfe subjects to modulate iron loading are important clinical considerations.

Key words: Hemochromatosis gene; Heme; Gavage; Iron; Absorption

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Core tip: These results indicate that loss of hemochromatosis gene (Hfe) protein results in increased dietary heme iron absorption that further contributes to the iron loading of the liver and other tissues of mice. Enhanced heme iron intake by homozygous Hfe subjects may contribute to body iron overload and early manifestation of phenotypic traits. This may have implications for dietary recommendations on heme intake by hemochromatosis subjects to avert tissue iron loading.

Laftah AH, Simpson RJ, Latunde-Dada GO. Intestinal heme absorption in hemochromatosis gene knock-out mice. *World J Hematol* 2017; 6(1): 17-23 Available from: URL: http://www.wjgnet.com/2218-6204/full/v6/i1/17.htm DOI: http://dx.doi.org/10.5315/wjh.v6.i1.17

INTRODUCTION

Dietary iron intake from both heme and non-heme sources is a key homeostatic step in iron metabolism, of which deficiency or enhanced absorption is associated with iron disorders in populations all over the world[1]. Heme from animal sources contributes about 10%-25% of total food iron and has a higher bioavailability (about 15%-38%) than non-heme iron[2] in humans. The absorption mode and molecular mechanism of both forms of iron are disparate. While non-heme iron is transported by a divalent metal transporter, a proton coupled symporter, heme is presumed to be transited into the enterocytes by endocytosis (passive pinocytosis or active receptor mediation), or proteins[3,4], that are yet to be fully characterised since HCP1 is a high affinity folate transporter[5]. Internalised heme is trafficked from the cytoplasm into endosomes[6] where it is catabolised by heme oxygenase (HO-1) to yield ferrous iron that converges with the labile non-heme iron pool for transit into circulation by ferroportin, the efflux regulatory protein[7]. On the other hand, basolateral efflux of intact heme has been shown in guinea pigs, and this may be *via* Flvcr1 or Abcg2[8].

Hereditary hemochromatosis (HH) constitutes heterogeneous mutations of genes in the hepcidin regulatory pathway. Homozygous C282Y mutation in Hemochromatosis gene (Hfe) is predominant in about 1:300 of Caucasian populations[9]. Coincidentally these populations are, in general, avid consumers of meat and animal products. HH patients are characterized by increased heme and non-heme iron absorption from the diet[10] coupled with excessive iron accumulation in parenchyma cells of the liver and the heart. This occurs because of low hepcidin expression due to loss of function of Hfe[11,12]. Hfe protein is vital for iron-sensing in the signal transduction cascade regulating hepcidin expression. Low serum hepcidin in Hfe patients permits sustained functional expression of ferroportin. Consequently, there is enhanced efflux of non-heme and heme iron by ferroportin into circulation[13,14]. There is, however, disparity in the phenotypic expression of HH which may be due to influences of other modifier genes, dietary factors or physiological iron requirements of the subjects[15]. Consequently, iron loading in HH subjects varies in severity[16,17]. Mouse strains have been shown to modulate phenotypic variability of Hfe severity[13].

Epidemiological studies generally agree that red meat consumption leads to higher iron stores in humans[18,19]. Moreover, dietary heme iron intake was found to be associated with high serum ferritin levels in HH subjects[18]. Of particular interest, however, is the question as to whether Hfe patients could benefit from dietary modifications of iron intake during treatment by phlebotomy.

Further work is needed to elucidate the effects of the loss of Hfe on the regulation of intestinal heme absorption[20]. Mouse knock-out models, however have contributed immensely to significant advances in understanding iron metabolism and disorders. The study, therefore, set out to investigate the effects of Hfe knock-out genotype on heme absorption in mice.

MATERIALS AND METHODS

Reagents

Chemicals and biochemicals were of Analar grade and were from either BDH-Merck Ltd (Poole, Dorset) or Sigma Chemical Company Ltd (Poole). ^{59}Fe (supplied as ferric chloride) was from PerkinElmer Life and Analytical Sciences (Wellesley, MA, United States, specific activity 185 GBq/g). ^{59}Fe-heme was prepared as described in[21]. To make ^{59}Fe-heme, a male Wistar rat was injected ip with 3.7 MBq ^{59}Fe citrate and housed in a metabolic cage for 1 wk. The animal was bled and the red cells washed three times in 10 volumes of saline and then lysed in 10 volumes of distilled water. Heme was then isolated from the haemoglobin by crystallization using the method of Labbe and Nishada[22].

Animals

Animal care and the regulation of scientific procedures met the criteria laid down by the United Kingdom "Animals (Scientific Procedures) Act 1986". Mice were housed in a light- and a temperature-controlled room with *ad libitum* access to standard pelleted diet and water unless stated. Hfe$^{(-/-)}$ breeders (C57/BL6 background strain; donated

by Srai K, Department of Biochemistry and Molecular Biology, Royal Free and University College Medical School, London, United Kingdom) were mated and subsequently genotyped by polymerase chain reaction (PCR). Wild-type and Hfe$^{(-/-)}$ homozygote breeders were established to produce age-matched male mice for experimental study. Mice at 3-5 wk of age were maintained on either iron-deficient (3 mg iron per kilogram) diet *ad libitum* during the treatment with either arginate (control) or heme:Arginate (200 mg/L heme and 3.3 mmol/L arginate) in drinking water for 24 h.

Iron absorption by tied loops in mice

In vivo Fe absorption was measured in tied-off duodenal segments as described previously[23]. In brief, the experiments were conducted in anaesthetised mice. A duodenal segment was tied at both ends followed by the injection of ^{59}Fe-heme arginate (100 μmol/L) into the tied-off segment. The segment was placed back into the abdominal cavity. After 10 min incubation, the duodenal segment was flushed with an ice-cold saline solution and weighed. Blood, liver and spleen were collected. Radioactivity in tissue samples and blood was measured using a gamma counter (1282 Compugamma; LKB Wallac, Turku, Finland), while carcasses were counted for radioactivity by a high-resolution bulk sample counter (J and P Engineering, Reading, United Kingdom). Radioactivity in the duodenum is referred to as mucosal retention while radioactivity in the carcass and other tissues is regarded as mucosal transfer (MT). TMU is the amount of total radioactive Fe absorbed from the gut lumen, and the percentage of MT (% MT) is the relative amount of Fe transfer into the body in comparison with total Fe uptake.

Heme iron absorption in mice after intragastric administration by gavage method

Food was withheld from the mice for 12 h prior to the oral dose, but they had free access to distilled drinking water during that period. Mice were then given 100 μL of physiological solution freshly prepared to contain heme:arginate labelled with 18 kBq ^{59}Fe ($FeCl_3$, in 0.1 M-hydrochloric acid, 1835 MBq/mg Fe; PerkinElmer) to provide target dosages of 4 mmol/kg body weight. This was gavaged as a single dose through the oesophagus and directly into the stomach of the animal through a 40 mm 13 gauge olive-tipped needle. No food was given to the animals after dosing and until tissue collection. The mice were then killed at approximately the same time (of 30 min) after the oral dose was administered. The abdomen was opened and after blood collection *via* a 1 mL syringe through a puncture into the heart, the whole gut was removed, externally rinsed, and divided into the stomach, duodenum, jejunum, ileum, caecum and colon. The lumen of each section was flushed gently with 3 mL of cold saline (9 g sodium chloride/L). Each section and the collected wash were counted for 1 min in a twin channel γ-counter (LKB, Wallac 1280, Helsinki, Finland). The carcass, minus gut, liver, spleen, kidney and blood, was counted in a high-resolution bulk sample counter for 2 min.

In the present study, it was found that 30 min were sufficient time to allow for passage of approximately 50% of the radiolabelled dose through the duodenum. Mucosal uptake of ^{59}Fe-heme measured only in the duodenum and jejunum were defined as the proportion of the initial dose of the label retained by the carcass plus duodenal and jejunal wall after dosing. Mucosal transfer at a given time was defined as the amount of ^{59}Fe in the carcass expressed as a percentage of the mean mucosal uptake[24].

PCR amplification procedures

Total RNA was extracted from tissue samples using Trizol reagent (Invitrogen, United Kingdom) according to manufacturer's instructions. Quantitative RT-PCR was carried out using an ABI Prism 7000 detection system in a two-step protocol with SYBR Green (ABI, Life Technologies, United Kingdom). The efficacy of the amplification was confirmed by a melting curve analysis and gel electrophoresis to confirm the presence of a single product. Quantitative measurement of each gene was derived from a standard curve constructed from known amounts of PCR product. The results were calculated by the ΔCt method that expresses the difference in threshold for the target gene relative to that of 18S RNA.

Statistical analysis

Data are presented as means with their standard deviations. The comparison of multiple groups for significant effects of two variables was determined by two-way ANOVA with a Bonferroni post hoc test. $P < 0.05$ was considered as significant. All statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla, CA, United States).

RESULTS

Heme absorption

Heme absorption, determined by the tied loop method was greater in Hfe$^{(-/-)}$ knockout mice than in control wild type mice (WT) (Figure 1). This was due to a significant increase in both the uptake and transfer phases of absorption. Moreover, a similar trend of absorption was observed when heme absorption was determined by oral gavage (Figure 2). Following the oral administration of ^{59}Fe-heme, intestinal uptake and transfer were elevated in Hfe$^{(-/-)}$ compared to WT after 30 min of heme administration.

Gene expression studies

To analyze the expression of genes involved in heme metabolism and transport, WT and Hfe$^{(-/-)}$ mice were administered arginate (control) or heme:arginate (200 mg/L heme and 3.3 mmol/L arginate) in drinking water for 24 h. Hfe$^{(-/-)}$ mice treated with heme and maintained on iron-deficient diets for 24 h showed an induction of *HO-1* expression (Figure 3A). The increase in *HO-1*

Table 1 Primer sequences of genes

Forward	Reverse
Mouse Flvcr1	
5′-CAGTTGATAGTCGGGTAGATCCAA-3′	5′-ACACCGGCTTCTTCAGAGTGA-3′
Mouse Abcg2	
5′-TCGCAGAAGGAGATGTGTTGAG-3	5CCAGAATAGCATTAAGGCCAGG-3′
Mouse HO-1	
5′-CAAGGAGGTACACATCCAAGCC-3′	5′-TACAAGGAAGCCATCACCAGCT-3′
Mouse 18S	
5′-GAATTCCCAGTAAGTGGCGGG-3′	5′-GGGCAGGGACTTAATCAACG-3′

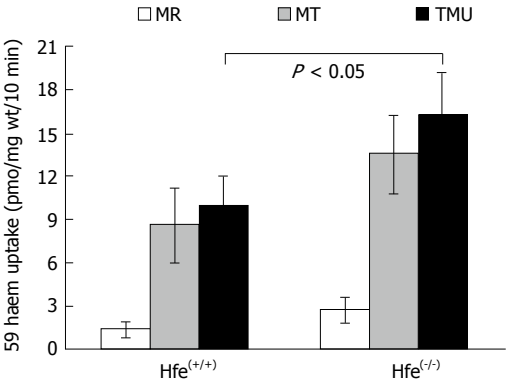

Figure 1 Tied loop mucosal uptake of ^{59}Fe-heme (100 μmol/L) in wild type and hemochromatosis gene$^{(-/-)}$ mice. Iron absorption was determined using tied-off duodenal segments. Data are means ± SD for 5 mice in each group ($P < 0.05$). MR: Mucosal retention; MT: Mucosal transfer; TMU: Total mucosal uptake of ^{59}Fe from *in vivo* tied-off duodenal segments; Hfe: Hemochromatosis gene.

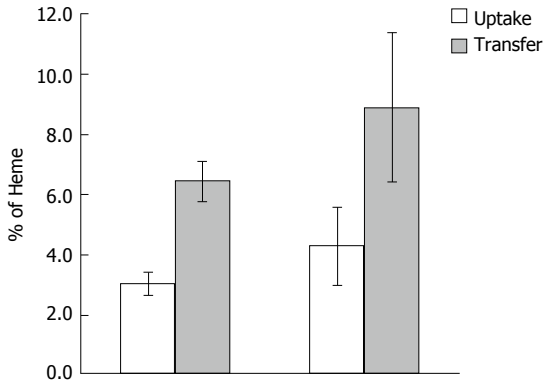

Figure 2 Heme absorption in wild type and hemochromatosis gene$^{(-/-)}$ mice by gavage method. Mice were orally gavaged with ^{59}Fe-heme (100 μmol/L) after an overnight fast. Mice were sacrificed 30 min after the oral dose was administered and tissues were collected as detailed in Methods. Values are mean ± SD, $n = 5$ per group.

expression in the duodenum of mice on the control iron-deficient diet was not significant (Figure 3A). *Flvcr1* mRNA level was lower in the duodenum of WT than Hfe$^{(-/-)}$ in mice fed the control iron-deficient diet. *Flvcr1* mRNA levels were significantly down regulated after 24 h heme feeding in drinking water (Figure 3B). *Abcg2* mRNA expression levels, however, were not significantly altered by heme feeding in drinking water (Figure 3C).

Serum and tissue iron levels

Serum and tissue iron status was determined in the mice after 24 h of heme feeding. Consistent with the literature, serum iron and transferrin saturation were significantly higher in Hfe$^{(-/-)}$ than WT (Table 1). Contrary to expectation, however, feeding heme to WT or Hfe$^{(-/-)}$ mice for 24 h showed no effect on serum iron and transferrin saturation (Table 2).

Endogenous non-heme iron levels in liver and spleen homogenates from Hfe$^{(-/-)}$ mice were significantly higher than WT (Figure 4; $P < 0.001$). Non-heme iron levels in liver homogenates were not significantly influenced by heme feeding. Although liver showed a trend towards being increased in Hfe$^{(-/-)}$ mice.

DISCUSSION

Heme as an exogenous source of iron is significant in nutrition because it is highly bioavailable for absorption by the gastrointestinal tract. In systemic metabolism, however, heme is derived endogenously from *de novo* biosynthesis for vital metabolic functions. Consequently, modulation of cytosolic, vesicular, membrane or plasma heme transport is regulated by a variety of extracellular and intracellular proteins[25,26]. While the luminal high affinity heme transport protein is not yet defined, heme absorption is enhanced in HH subjects and it is regulated by iron stores albeit by an order of magnitude less than non-heme iron absorption[10,27].

The current study demonstrates that heme feeding stimulates iron absorption in Hfe KO mice and provides evidence of increased iron storage in the spleen and hepatocytes of the mice. ^{59}Fe-Heme arginate absorption from the duodenal loop of the mice was significantly enhanced in Hfe$^{(-/-)}$ mice after 10 min of exposure (Figure 1). This trend was also confirmed in mice that were given ^{59}Fe-heme arginate by gavage and measuring absorption after 30 min (Figure 2). Hfe has been shown to have an impact on cellular iron trafficking and, indirectly, on intestinal iron absorption. The direct effects of Hfe on heme iron absorption are not clear. Alternatively, low levels of iron in the enterocyte of Hfe mice might induce heme absorption *via* increased HO-1 expression. It has been speculated that heme degradation by HO-1 might be the rate-liming step of heme absorption in the gut because the HO-1 activity was found to increase during Fe deficiency[28]. Increased heme iron absorption after

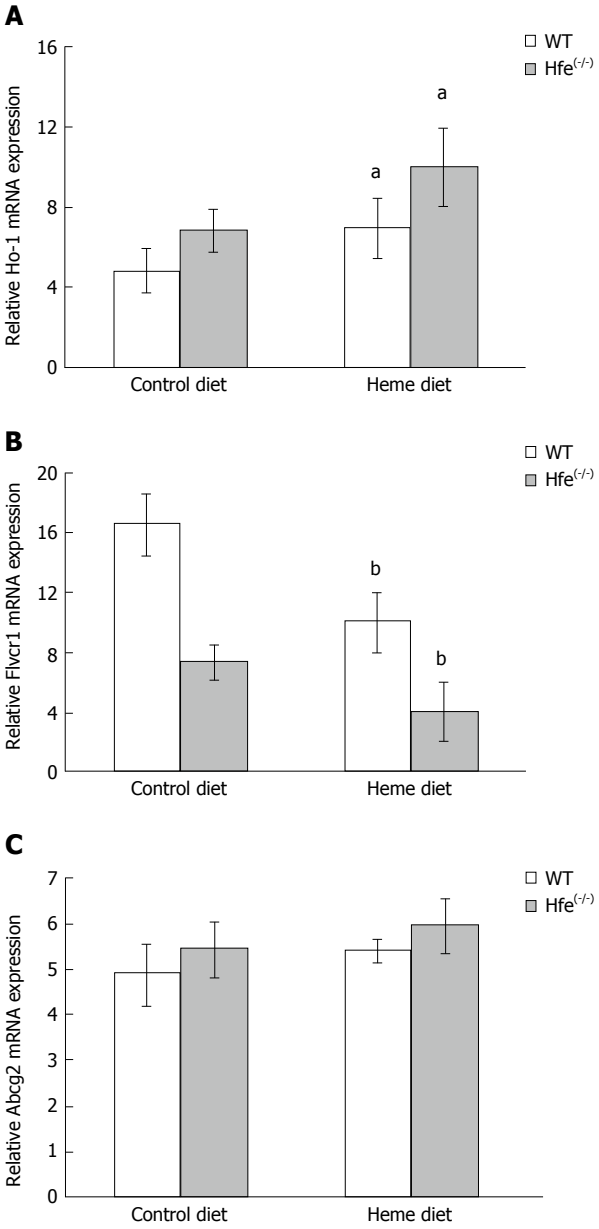

Figure 3 *HO-1*, *Flvcr* and *Abcg2* mRNA expression in wild type and hemochromatosis gene$^{(-/-)}$ mice fed control diet or heme for 24 h. Real-time polymerase chain reaction of mRNA of the genes from the duodenum of WT and Hfe$^{(-/-)}$ were determined and normalised β-actin (Actb) mRNA. Statistical analysis was performed by 2-way ANOVA with Bonferroni post-hoc test ($^{a}P < 0.05$ and $^{b}P < 0.001$). Hfe: Hemochromatosis gene; WT: Wild type.

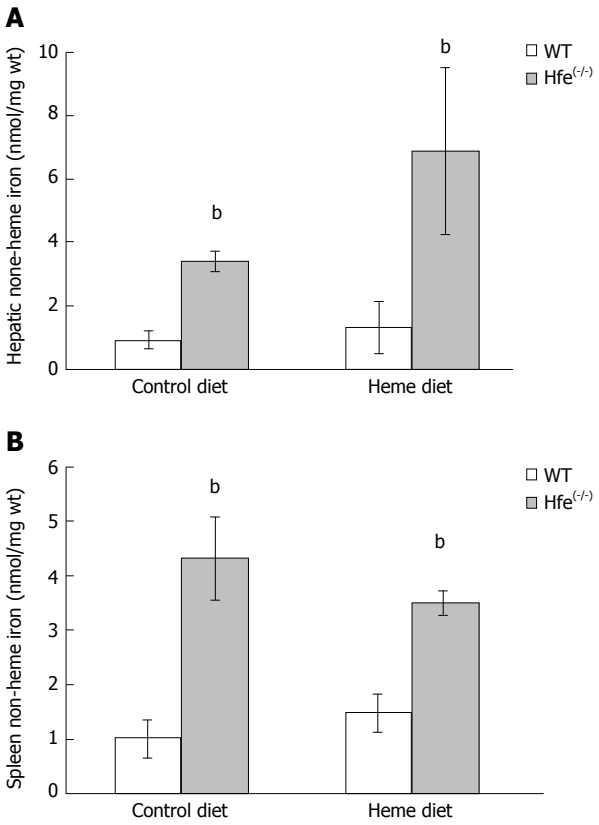

Figure 4 Tissue iron levels of mice. Effect of 24 h heme feeding on liver (A) or spleen (B) non-heme iron levels (nmol/mg) of WT and Hfe$^{(-/-)}$ mice. Results are means ± SD for 6-8 mice in each group ($^{b}P < 0.005$). Hfe: Hemochromatosis gene; WT: Wild type.

Table 2 Iron parameters of wild type and hemochromatosis gene$^{(-/-)}$ mice after 24 h of feeding heme

		Serum iron (μmol/L)	% Transferrin saturation
WT	Control	25.3 ± 4.4	32.1 ± 6.5
	Heme-24 h	26.8 ± 3.1	35.9 ± 7.9
Hfe$^{(-/-)}$	Control	49.8 ± 5.0	59.8 ± 6.1
	Heme-24 h	50.5 ± 10.6	61.0 ± 10.4

Hfe: Hemochromatosis gene; WT: Wild type.

24 h, shown in Figure 1, might be induced by enhanced expression of *HO-1* (Figure 3A). Augmented catabolism of heme by *HO-1* consequently may increase the inorganic iron pool that can be chaperoned into systemic circulation. It has also been speculated that a fraction of the heme in the enterocyte might be transferred intact into the circulation by the heme efflux proteins Flvcr or Abcg2. The expression of *Flvcr1* was down-regulated in Hfe mice (Figure 3B). Other modifiers such as TfR1 or TfR2 could interact with Hfe directly or, more likely, modify iron loading through independent mechanisms to increase or depress the effects of Hfe. This possibly might be due to hepatocyte regulation of hepcidin expression. A two-fold decrease was observed in hepcidin mRNA levels in the Hfe$^{(-/-)}$ mice used in the current study[29]. Reduced expression of hepcidin in Hfe$^{(-/-)}$ phenotype would lead to the maximal functional capability of FPN, hence the enhanced absorption of iron[30] in Hfe$^{(-/-)}$ mice. Hepcidin levels in the liver correlate negatively with serum ferritin which in humans is a biomarker of iron intake and iron status.

The increase in plasma iron and percentage transferrin saturation after feeding heme for 24 h might have contributed to increased liver and spleen non-heme iron levels in Hfe$^{(-/-)}$. The phenotype of HH patients of European descent attests to the higher iron absorption due to enhanced duodenal expression of transport proteins despite high iron stores[31]. Previous studies have attempted to use low iron intake and inhibitors of iron

absorption as dietary strategies to ameliorate the rate of tissue Fe deposition in Hfe patients[32].

While the feeding of high heme diet did not increase serum and hepatic iron levels of both $Hmox1^{fl/fl}$ and $Hmox1^{Vil\text{-}Cre}$ mice[33], Hfe knock-out mice demonstrated increased *HO-1* expression and enhanced heme absorption in the current study. Mouse strain differences have been shown to determine the severity of tissue iron deposition in Hfe knockout model of HH[13]. There might be species or strain differences in the absorption of heme iron, an earlier study however, showed that mice have the least heme absorption capacity, while canines are the highest (dog > guinea pig > rat > mouse)[34]. Moreover, to sustain heme in solution, heme arginate was used in the current study to measure absorption[35]. This study has identified HO-1 as a key candidate in the regulation of heme iron transport in the gastrointestinal tract of mice. Increased HO-1 expression in Hfe KO mice contributes to enhanced heme iron absorption.

Enhanced heme iron intake by homozygous Hfe subjects may contribute to body iron overload and early manifestation of phenotypic traits. This may have implications for dietary recommendations on heme intake by HH subjects to avert tissue iron loading. Moreover, since high intake of red meat has been associated with an elevated amount of iron in the body and increased risk of metabolic diseases, an emerging consensus, in general, suggests reduced red meat consumption by the populace.

COMMENTS

Background

Hemochromatosis patients are characterized with high level of heme- and inorganic iron absorption from the diet coupled with excessive iron accumulation in parenchyma cells of the liver and the heart due to low hepcidin expression.

Research frontiers

Enhanced heme iron intake by homozygous Hfe subjects may contribute to body iron overload and early manifestation of phenotypic traits. Moreover, since high intake of red meat has been associated with elevated amount of iron in the body and increased risk of metabolic diseases, an emerging consensus, in general, suggests reduced red meat consumption by the populace.

Innovations and breakthroughs

These results indicate that loss of Hfe protein results in increased dietary heme iron absorption that further contributes to the iron loading of the liver and other tissues of mice. This may have implications for dietary recommendations on heme intake by HH subjects to avert tissue iron loading.

Applications

Implications for dietary recommendations on heme intake by Hfe subjects to modulate iron loading are important clinical considerations.

Terminology

HH: Hemochromatosis; Hfe: Hemochromatosis gene; Flvcr1: Feline Leukemia Virus Subgroup C Cellular Receptor 1; HO-1: Hemoxygenase-1; Abcg2: ATP-Binding Cassette, Subfamily G, Member 2; MR: Mucosal retention; MT:Mucosal transfer,TMU:Total mucosal uptake.

Peer-review

It is a very well written manuscript investigating the influence of Hfe mutation on Fe labeled duodenal heme absorption in mice and showing that heme absorption was enhanced from both duodenal tied-loop segments and by oral gavage methods.

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CASE REPORT

Role of plasmapheresis in early allograft dysfunction following deceased donor liver transplantation

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Author contributions: All the authors contributed to the manuscript.

Institutional review board statement: Approval for this case report was obtained from the Institutional review board.

Informed consent statement: All involved persons (subjects or legally authorized representative) gave their informed consent (written or verbal, as appropriate) prior to study inclusion.

Conflict-of-interest statement: The above doctors have no conflicts of interest or financial ties to disclose.

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Abstract

The role of plasmapheresis in liver failure and hepatic encephalopathy is undefined and its use as a strategy to salvage patients with severe allograft dysfunction after liver transplantation remains investigational. We present a case of early allograft dysfunction following deceased donor liver transplantation (DDLT) where plasmapheresis was effective as a bridge to recovery and possibly avoiding a retransplantation. A 16 years old boy, known to have decompensated Wilson's disease underwent DDLT at our Public Sector Hospital. He received a healthy liver from a brain-dead donor, whose liver was considered too large for the boy. The graft was reduced *in situ* to a left lobe graft. Surgery was uneventful and the recipient was well for the initial 96 h. On Doppler and further computed tomography scan, a partial portal vein thrombus was noted. He was reexplored and a Fogarty endothombecteomy was performed. Following the second surgery, he developed severe allograft dysfunction with a peak bilirubin of 40 mg/dL. He underwent imaging to rule out technical causes for the dysfunction, followed by a liver biopsy, which revealed acute cellular rejection. Multiple cycles of plasmapheresis were initiated. Over the next two weeks, the graft demonstrated a gradual recovery. He was discharged on the 30th postoperative day, with a serum bilirubin of 5.5 mg/dL. He remains well on follow-up, with the liver function tests improving further. Our report demonstrates the beneficial effect of plasmapheresis, which appears to be an effective treatment option for

early allograft dysfunction following liver transplantation and may obviate the need for retransplantation.

Key words: Liver transplantation; Allograft dysfunction; Plasmapheresis

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Core tip: We demonstrate the beneficial effects of plasmapheresis, which appears to be an effective treatment option for early allograft dysfunction following liver transplantation and may obviate the need for retransplantation.

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INTRODUCTION

With expanding indications and increasing demand for liver transplantation (LT) donor organ shortage is a major limitation. Early allograft dysfunction (EAD) is not an uncommon entity, especially in transplantation with organs from marginal donors[1]. The incidence of EAD varies between 1.4%-23%, with a median range of 5%-6%[1-3]. This wide range of incidences is attributable to the myriad of definitions which exist for EAD although most definitions are a combination of elevated bilirubin, international normalized ratio (INR), transaminases, and hepatic encephalopathy.

EAD leads to increased morbidity and may result in mortality and liver support therapies need to be instituted[2-5]. In severe forms, retransplantation may be the only treatment modality. If the duration of early graft dysfunction passes uneventfully, the patient often recovers spontaneously[1-6]. Measures like liver support devices lessen the hepatic metabolic burden and may help in the recovery of graft function[2-4]. Plasmapheresis has been used in acute liver failure, but its role in supporting dysfunctional liver allografts remains unclear[7,8].

In this brief report, we present a case of allograft dysfunction following deceased donor liver transplantation (DDLT) where the graft was salvaged using multiple cycles of plasmapheresis.

CASE REPORT

A 16-year-old boy weighing 33 kg, with decompensated Wilson's disease underwent DDLT at our Public Sector Hospital. He received a healthy liver from a 34-year-old brain-dead donor. The donor had an initial Sodium value of 194 meq/L, which was controlled and brought down to 164 meq/L at the time of organ retrieval. The donor had one episode of significant hypotension. As the donor liver was considered too large for the boy, it was reduced in situ into a left lobe graft.

The recipient operation was uneventful with a total blood loss of 1000 mL. The end lactate was 4.2 mmol/L from a peak of 10.2 mmol/L in the anhepatic phase. The total cold ischemia time was 210 min. The graft had an accessory artery from the left gastric artery, taken as a cuff from the celiac axis and anastomosed to the common hepatic artery of the recipient. The surgery was uneventful and the recipient was well initially; being extubated on the 1st postoperative day (POD). Immunosuppression was initiated with steroids (Methyl-Prednisolone 0.25 mg/kg per day) and calcineurin inhibitors (Tacrolimus 0.03 mg/kg per day) from POD 1. On the 5th POD, his drain output increased from 600 mL in 24 h to 1700 mL and his serum bilirubin which had dropped to 3.8 mg/dL, went upto 9.3 mg/dL. Doppler showed poor flow in the portal vein. On further imaging, he was noted to have a partial portal vein thrombus. He underwent emergency re-exploration when a Fogarty endothrombectomy was done and the graft was revascularised with an iliac vein interposition graft for the portal venous anastomosis. During the second surgery, the graft was noted to be very stiff. Following this, he developed severe allograft dysfunction (rising serum bilirubin > 10 mg/dL over 3 consecutive days in the absence of biliary complications). Over the next 5 d his bilirubin increased up to 23.5 mg/dL, while his transaminases remained normal. He underwent repeat imaging which ruled out technical causes for the dysfunction including a patent portal vein. Liver biopsy was performed which was suggestive of moderate acute cellular rejection, there was no evidence of antibody mediated rejection. He received pulsed steroid therapy (Methyl-Prednisolone 20 mg/kg per day on consecutive three days). Despite the steroid pulse, the graft dysfunction did not abate and the hyperbilirubinemia persisted on an upward trend, peaking at 40.8 mg/dL on the 15th POD. In an effort to salvage the graft, plasmapheresis was initiated on the 15th POD.

Plasmapheresis was done on 5 consecutive days using continuous flow centrifugal technology based Spectra Optia Apheresis system (Terumo BCT, Denver, CO, United States). Acid citrate dextrose-A anticoagulation and dual vascular access were used. Patient's total blood volume (BV) was calculated as per Nadler's formula and Plasma volume (PV) was calculated according to the formula PV = BV × (1 - Hematocrit)[8]. 1.0 PV was processed in each session with 100% replacement using 5% albumin solution and blood group specific fresh frozen plasma. The inlet: Anticoagulant ratio was kept 1:12 to 1:15 and blood flow rate kept between 45-50 mL/min. Baseline calcium was monitored before each procedure and 20 mL 10% calcium gluconate was given prophylactically during the procedure to prevent citrate toxicity. Continuous monitoring of pulse and blood pressure was carried out during the procedure to prevent any adverse

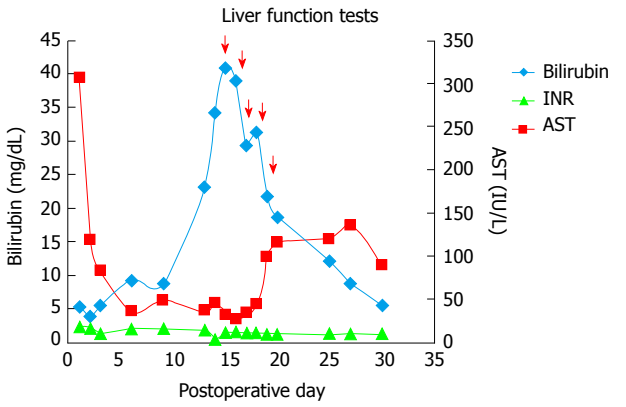

Figure 1 Liver function tests and its response to plasmapheresis. Red arrow: Plasmapheresis; Bilirubin in mg/dL; AST: Aspartate transaminase in IU/L; INR: International normalized ratio.

events related to the procedure. No serious adverse effects were observed during the procedure. Complete blood count, INR, liver function tests, renal function tests, arterial ammonia, arterial blood gas analysis, were performed every 12 hourly irrespective of the timings of the plasmapheresis.

His bilirubin showed a steady fall and by the 5th cycle of plasmapheresis it had dropped to 15 mg/dL (Figure 1). In the interim, he had an episode of fever with chills, and grew *K. pneumonia* in his blood culture. This was successfully treated with appropriate antibiotics (Piperacillin-Tazobactum). No obvious source for the infection could be discerned. He was discharged on the 30th POD being asymptomatic, tolerating oral diet well, with stable vital signs and with a serum bilirubin of 5.5 mg/dL. He remains well on follow-up, with the latest liver function tests showing a total bilirubin of 1 mg/dL, 4 mo after transplantation.

DISCUSSION

Although the pathophysiological basis for early allograft dysfunction has not been wholly elucidated; it appears to be a critical interplay between donor factors, recipient characteristics, and intra-operative events[1,2,4,5].

Despite a few studies including one by Park *et al*[4] having shown plasmapheresis to be beneficial in severe graft dysfunction; the role of plasmapheresis remains undefined in graft dysfunction[1-4]. The mechanisms by which plasmapheresis is beneficial hasn't been completely elucidated, but it does remove the plasma containing free and protein-bound toxic substrates and infuse fresh plasma, as well as clotting factors and albumin, thus functioning as a liver support; creating a milieu conducive to liver regeneration[1-6]. Plasmapheresis is an important adjunct in the treatment of hepatic encephalopathy as it improves blood-clotting, hyperbilirubinemia, and hyperammonemia; acting as a bridge to LT[7,8].

In a series from Japan, all 46 patients with liver failure following LT improved with plasmapheresis[5]. In another recent study by Choe *et al*[3] consisting of 143 patients with EAD of whom 107 underwent Plasmapheresis. There was a significant improvement in the 1-mo and 1-year survival of this subgroup of patients as compared to those who did not undergo plasmapheresis. A report from Johns Hopkin also suggested that plasmapheresis may aid in the recovery of primary allograft nonfunction following liver transplantation[9,10].

As demonstrated in our patient, a single plasmapheresis session cannot be expected to provide a definite beneficial effect in patients with a failing liver graft[2,3]. Repeated sessions appear necessary to achieve cumulative effects. The timing and interval of plasmapheresis must be adjusted on a case-by-case basis, by daily determination of patient's general condition and liver graft function[2-4]. In a dysfunctional liver, the liver enzymes often fluctuate, depending on the condition of the liver graft, and hence cannot be used to assess the effectiveness of plasmapheresis. Prothrombin time is readily affected by the plasma infusion and is also not a predictable marker of the effectiveness of plasmapheresis[3,11]. Serum bilirubin appears to be the most reliable parameter to base decision regarding the initiation, continuation and termination of plasmapheresis[2,4,11].

In countries, where retransplantation may not be a feasible option due to the lack of availability of donor grafts and/or the huge financial burden involved, plasmapheresis appears to be a readily available artificial liver support system with the added advantage of being economical, simple and easy to use.

Apart from benefiting the patient, effective management of early allograft dysfunction helps a unit improve its overall efficiency and sets a benchmark for excellence in care by showing an enhancement in healthcare delivery in general.

In conclusion, our report demonstrates the beneficial effect of plasmapheresis, which appears to be an effective treatment option for early allograft dysfunction following DDLT and may obviate the need for retransplantation.

COMMENTS

Clinical diagnosis
Early liver allograft dysfunction.

Differential diagnosis
Arterial complications, venous complications, biliary complications, rejection, infection.

Laboratory diagnosis
Early liver allograft dysfunction.

Imaging diagnosis
Early liver allograft dysfunction.

Pathological diagnosis
Early liver allograft dysfunction.

Treatment
Plasmapheresis.

Term explanation

Plasmapheresis - total plasma exchange.

Experiences and lessons

Useful not to disregard a simple but very effective procedure such as plasmapheresis in treating early allograft dysfunction.

Peer-review

The paper is good, although it doesn't seem to get different conclusion from the larger case series already published.

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CASE REPORT

Management of unstable angina in a patient with Haemophilia A

Andreina Carbone, Tiziana Formisano, Francesco Natale, Maurizio Cappelli Bigazzi, Donato Tartaglione, Enrica Golia, Felice Gragnano, Mario Crisci, Renato Maria Bianchi, Raffaele Calabrò, Maria Giovanna Russo, Paolo Calabrò

Andreina Carbone, Tiziana Formisano, Francesco Natale, Maurizio Cappelli Bigazzi, Donato Tartaglione, Enrica Golia, Felice Gragnano, Mario Crisci, Renato Maria Bianchi, Raffaele Calabrò, Maria Giovanna Russo, Paolo Calabrò, Chair of Cardiology, Second University of Naples, AORN dei Colli, Monaldi Hospital, 80131 Naples, Italy

Author contributions: Carbone A, Formisano T, Natale F, Tartaglione D, Golia E and Gragnano F designed the report; Cappelli Bigazzi M and Crisci M performed clinical evaluation and interventional procedure; Gragnano F and Bianchi RM collected the patient's data; Carbone A, Calabrò R, Russo MG and Calabrò P analyzed data and wrote the paper.

Institutional review board statement: This case report was exempt from the Institutional Review Board standards at Second University of Naples.

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Abstract

Hemophilia A is an X-linked recessive disorder characterized by a deficiency of coagulation factor VIII (FVIII) and therefore by a greater risk of bleeding during percutaneous interventional procedures and during the dual antiplatelet therapy (DAPT) in patients with ischemic heart disease. Information regarding safe percutaneous procedures in hemophiliacs is limited. Since the introduction of FVVIII concentrates, the life expectancy of hemophiliac patients has improved and consequently, the rate of ischemic heart disease in this population is increased. Frequently the replacement therapy can trigger the onset of an acute coronary syndrome. We report a case of a patient with mild Hemophilia A, who presents with unstable angina, treated successfully with coronary angioplasty and drug eluting stent implantation without replacement of FVVIII, treated with long term DAPT without major bleeding after six months of follow up.

Key words: Hemophilia A; Unstable angina; Dual antiplatelet therapy; Drug eluting stent; Coagulation factors replacement therapy

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Core tip: Hemophilia is a rare condition, but in some

cases, could create difficulties in the management of other disease, such as acute coronary syndrome and unstable angina. Data in literature regarding this condition are lacking. This case report would be an example of the management of patients with mild deficit of Factor VIII activity according to the recent consensus.

Carbone A, Formisano T, Natale F, Cappelli Bigazzi M, Tartaglione D, Golia E, Gragnano F, Crisci M, Bianchi RM, Calabrò R, Russo MG, Calabrò P. Management of unstable angina in a patient with Haemophilia A. *World J Hematol* 2017; 6(2): 28-31 Available from: URL: http://www.wjgnet.com/2218-6204/full/v6/i2/28.htm DOI: http://dx.doi.org/10.5315/wjh.v6.i2.28

INTRODUCTION

Hemophilia A is an X-linked recessive disorder with frequency of approximately 1 in 8500 live births, characterized by a deficiency of coagulation factor VIII (FVIII) and therefore by a greater risk of bleeding. Hemophilia A is defined "mild" when the activity of FVIII is greater than 5% (> 0015 IU/mL) and presents episodes of bleeding after hematological stress (surgery, childbirth, trauma). It is a moderate disorder for levels of FVIII activity between 1% and 5% (0.01-0.05 IU/mL)[1]. The severe form, in which the activity of FVIII is < 1% (< 0.01 IU/mL), represents 50% of cases and is characterized by spontaneous bleeding in the joints and muscles and high risk of intracranial hemorrhage[1]. Since the introduction of FVIII concentrates in 1960 and the preventive treatment in 1970 the life expectancy of this patients, in developed countries, has improved. Consequently, the rate of ischemic heart disease in this population is increased. Kulkarni *et al*[2] assessed the prevalence of ischemic heart disease in 3422 American patients with hemophilia, analyzing discharge records from the hospital between 1993 and 1998. The prevalence of coronary heart disease was 0.05% in patients under 30 years of age and 15.2% in those aged 60 or more[2], similar to the general population[3]. Girolami *et al*[4,5] have shown that most of thrombotic cardiovascular events in hemophiliac patients occur during the infusion of recombinant FVIII or DDAVP.

The first line therapy of Acute Coronary Syndromes is the percutaneous transluminal coronary angioplasty (PTCA) and stent implantation. In hemophiliacs, invasive treatment is more dangerous because they have increased risk of bleeding during the procedure and with the use of anticoagulants and antiplatelet agents. The management of unstable angina in this population is suggested by a Consent Document of the World Federation of Hemophilia[6]. Experts recommend levels of FVIII activity of 80%, during the PTCA, and in 48 h, achieved through the infusion of recombinant FVIII.

We show a case of a patient with mild Hemophilia A who presents with unstable angina, not triggered by the infusion of clotting factor concentrate, treated successfully with coronary angioplasty and drug eluting stent (DES) implantation without replacement of FVIII.

CASE REPORT

A 55 years old age man with mild deficit of FVIII activity (FVIII activity of 50%), HCV-related liver disease, tobacco use, hypertension, dyslipidemia, family history of cardiovascular disease, prostate cancer in treatment with anti - androgen hormone therapy, came to our attention for worsening of constrictive chest pain at rest persisting for a month, for which he performed ECG exercise test positive. The patient did not report neither history of coronary heart disease nor intra-articular or intramuscular hemorrhage. He was admitted to the Department of Cardiology for practicing coronary angiography and eventually angioplasty and coronary stenting. During hospitalization, despite optimal anti-ischemic drug therapy (bisoprolol 2.5 mg, nitroglycerin patch of 10 mg, atorvastatin 80 mg, telmisartan 80 mg; Cardioaspirin 100 mg, the patient experienced recurrent episodes of constrictive chest pain with no changes of electrocardiogram and negative cardiac marker of necrosis. The level of FVIII activity, before the procedure, was 50% defining a very mild deficit. Catheterization was performed through the right radial artery with 6 F sheath. The coronary angiogram revealed the presence of 70% calcified stenosis of the left anterior descending artery at its middle part, involving the bifurcation of the first diagonal branch, with a calcific stenosis at its middle portion (Figure 1). During the procedure Bivalirudin was administered as a bolus and Clopidogrel loading dose of 600 mg was given before the procedure. PTCA and stenting with 2 Zotarolimus eluting stents DES 2.75 mm × 18 mm and 3.0 mm × 26 mm was performed using Minicrush technique (Figure 2A). Lastly, we performed a post-dilation with kissing balloon technique with non-compliant balloons, 5 mm × 15 mm (on the LAD) and 3.0 mm × 15 mm (on the diagonal branch). The final angiographic result was excellent with TIMI flow 3 (Figure 2B). The hemostasis at the puncture site was performed using Radistop, which was removed after eight hours without evidence of bleeding or hematoma. After the procedure, the patient was monitored in the Cardiology ward and the clinical conditions were good and hemodynamically stable during the hospitalization. In particular, there were no bleeding or further episodes of chest pain. The patient was discharged after 72 h of observation on DAPT (Aspirin 100 mg/d; Clopidogrel 75 mg/d) for one year, in addition to anti ischemic therapy previously reported. At six months follow up, the patient did not report any major bleedings and was asymptomatic.

DISCUSSION

PTCA in hemophiliacs has a greater risk of bleeding than in general population for many reasons. Arterial puncture

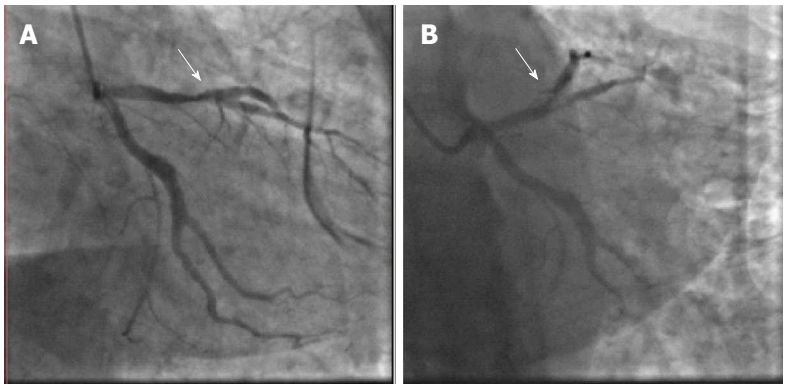

Figure 1 Left coronary angiogram. Preintervention angiogram of the left anterior descending artery revealed the presence of 70% calcified stenosis of its middle part (A), involving the bifurcation of the first diagonal branch, with a calcific stenosis at its middle portion (B). Arrow indicates the site of the lesions.

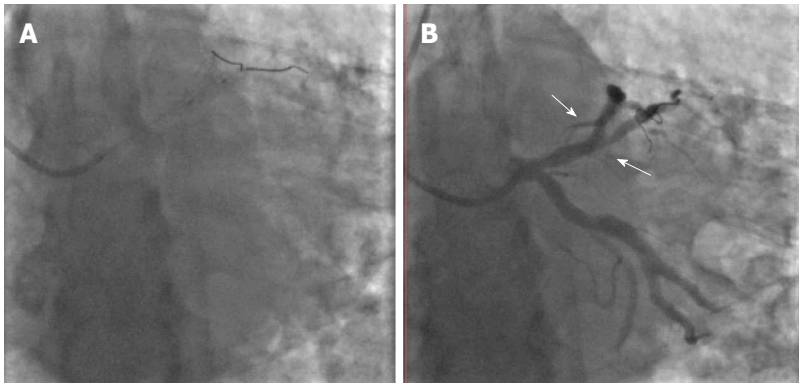

Figure 2 Stents implantation and final result. Angiogram (A) shows PTCA and stenting with 2 Zotarolimus eluiting stents DES 2.75 mm × 18 mm and 3.0 mm × 26 mm performed using Minicrush technique; B: Post-intervention angiogram of the LAD. Arrows indicate site of stents placement. PTCA: Percutaneous transluminal coronary angioplasty; DES: Drug eluting stent; LAD: Left anterior descending.

is related to high risk of local complications, reduced by recombinant FVIII administration, by radial artery puncture and using effective hemostasis system for the puncture site. The management of unstable angina in hemophiliacs is suggested by a Consent Document of the World Federation of Hemophilia[6]. Experts recommend levels of FVIII activity of 80%, during the PTCA, and in the following 48 h, achieved through the infusion of recombinant FVIII, but this increases the risk of acute thrombosis in patients with unstable plaques. Many cases of myocardial infarction have been described during administration of coagulation factor concentrates, prothrombin complex and desmopressin[5,7]. Moreover, Girolami *et al*[4,5] have shown that most of thrombotic cardiovascular events in hemophiliac patients occur during the infusion of recombinant FVIII or desmopressin.

In the reported case, we decided to not pretreat the patient with replacement therapy, taking in account the clinical history free of major bleeding and the level of FVIII activity. To improve procedural safety, we undertook angiography using a radial approach, minimizing the risk of local complications. As anticoagulant during the interventional procedure we used Bivalirudin, a direct thrombin inhibitor, associated with lower rate of bleeding and with positive results in hemophiliac patient. Antiplatelet agents are needed to prevent in-stent thrombosis. The current guidelines suggest 1 year of dual antiplatelet therapy (DAPT) for DES and one month for bare metal stent[8]. Considering that Haemophilia is not associated with any platelet defect, antiplatelet agents should be given according to guidelines: indeed, acute stent thrombosis in patients with coagulation defects not receiving DAPT after stenting have been described in literature[9]. In the past BMS were preferred to DES for the shorter duration of DAPT[10]. New generation of DES do not need long-term DAPT, making treatment duration similar to that required for BMS. Moreover, drug-coated stent demonstrates superior safety and efficacy with one month of DAPT in patients at high bleeding risk[10].

In summary, we described the case of a patient with mild Haemophilia A presenting with unstable angina, treated with PTCA and stenting of the LAD at a bifurcation site with DES. The procedure was carried out safely, using the new generation DES. After six months of follow up the patient was in therapy with DAPT and had not experienced any complications and had no FVIII replacement.

COMMENTS

Case characteristics
A 55-year-old age man with mild deficit of factor VIII (FVIII) activity came to the authors' attention for worsening of constrictive chest pain at rest persisting for a month.

Clinical diagnosis
Unstable angina in patient with mild hemophilia.

Differential diagnosis
Acute myocardial infarction, atypical chest pain.

Laboratory diagnosis
Deficit of FVIII activity.

Imaging diagnosis
The coronary angiogram revealed the presence of 70% calcified stenosis of the left anterior descending artery.

Pathological diagnosis
Unstable angina due to significant stenosis of one epicardial coronary artery.

Treatment
Angioplasty and stenting of the coronary stenosis, dual antiplatelet treatment for one year.

Related reports
Hemophilia A is an X-linked recessive disorder characterized by a deficiency of coagulation FVIII and therefore by a greater risk of bleeding. Angioplasty and stenting is more dangerous in this population and also the use of anticoagulants and antiplatelet agents.

Term explanation
Angioplasty, also known as balloon angioplasty and percutaneous transluminal angioplasty, is a minimally invasive, endovascular procedure to widen narrowed or obstructed arteries.

Experiences and lessons
Guidelines about the management of acute coronary syndrome in hemophiliacs don't exist, and their treatment should be tailored. Mild hemophilia should be not pretreat with recombinant factor therapy, considering the low bleeding risk.

Peer-review
This case report is very interesting and clinically relevant.

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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

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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

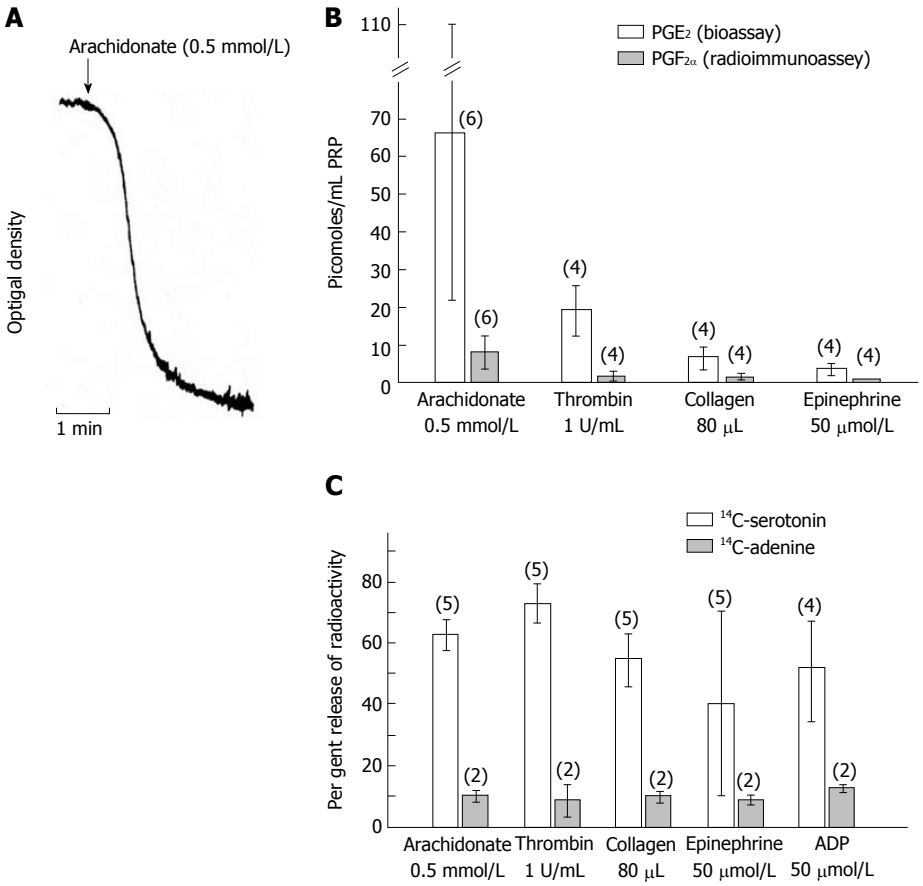

Figure 1 Arachidonic acid-induced human platelet aggregation and prostaglandin formation (Silver *et al*[3] 1973). Arachidonate acid (AA), 0.5 mmol/L induces a normal platelet aggregation curve (A). AA induces secretion of large amounts of prostaglandins after platelet aggregation, but little or no prostaglandins 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%,

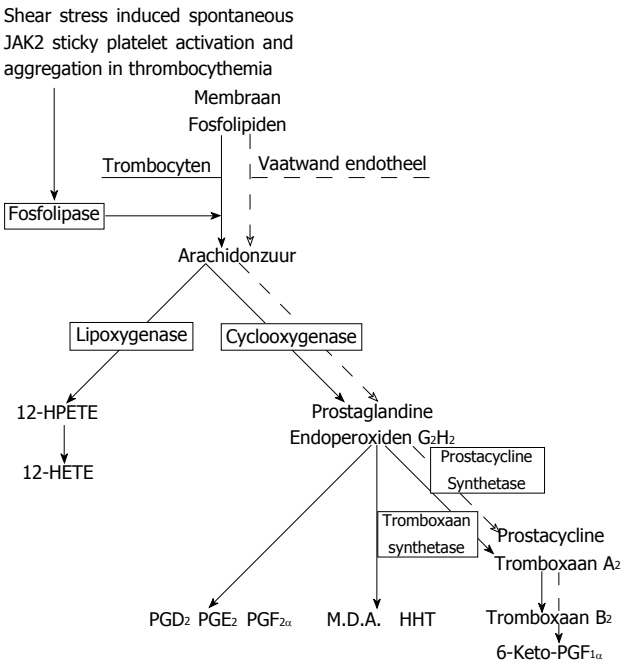

Figure 2 Dutch design by Michiels and Van Vliet on membrane phospholipid - arachidonic acid metabolism in platelets as compared to endothelial cells conceptualize in 1976, three years before the Moncada and Vane[13] publication in the *NEJM*. Arachidonic acid (AA) is metabolized by lipoxygenase into (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

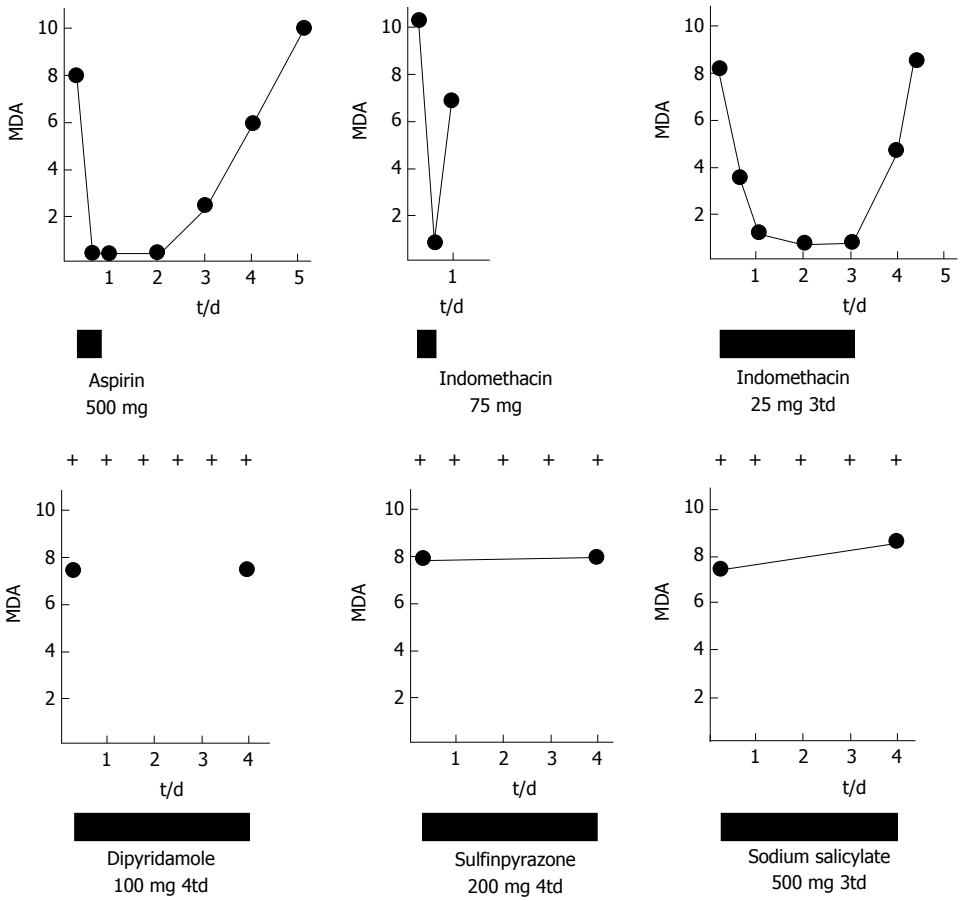

Figure 3 The effect of platelet aggregation inhibiting drugs on stimulated platelet aggregation, erythromelalgia and malondialdehyde concentration in N-ethylmaleimide stimulated platelet rich plasma. The effect of aspirin (acetylsalicylic acid), indomethacin, dipyridamol, sulfinpyrazon and sodium salicylate 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.

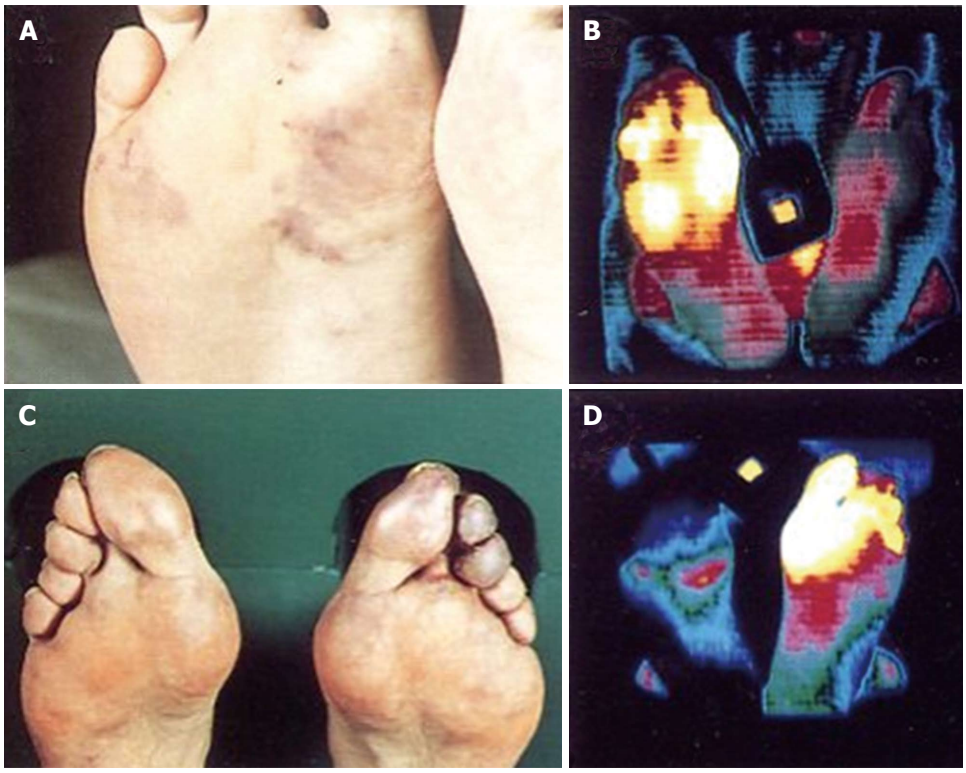

Figure 4 Isothermpgrams of two essential thrombocythemia patients with erythromelalgia in toes and fore foot sole. Typical mottled red blue congestion and thermographic visualization of erythromelalgia in the fore foot and toes. Skin surface temterature: blue 24 ℃-25 ℃; green 26 ℃-27 ℃; purple 28 ℃-29 ℃; 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,

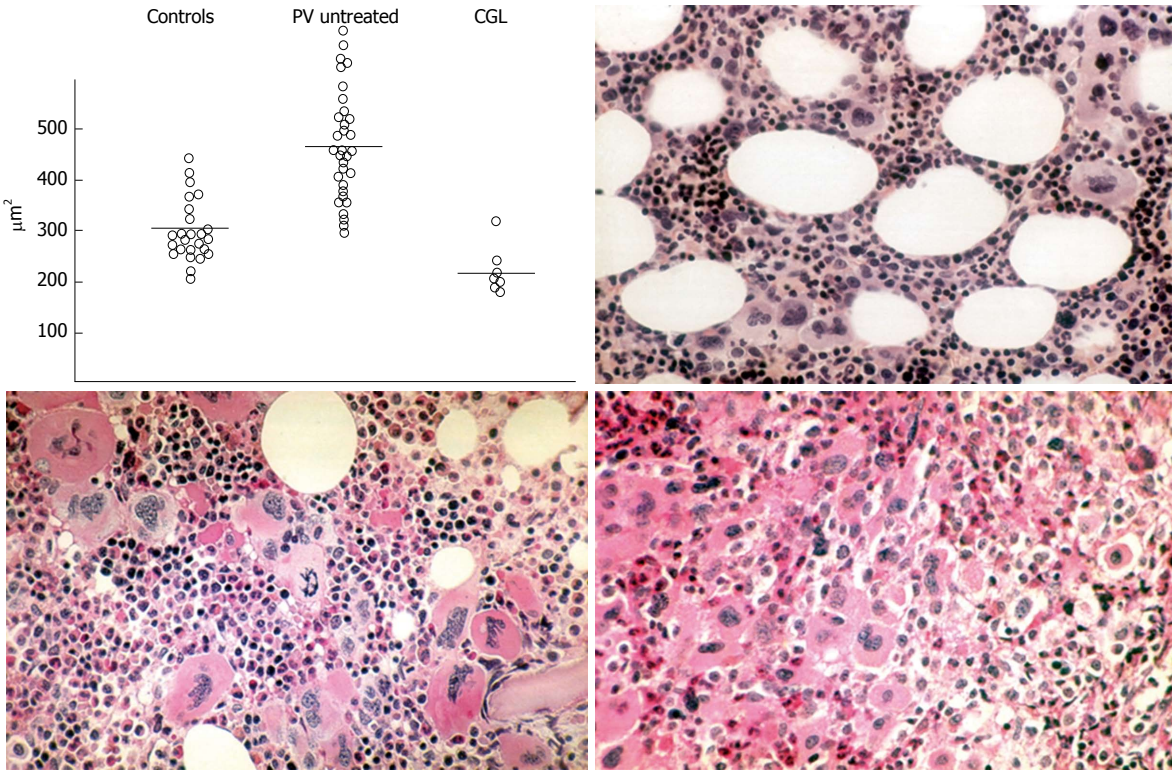

Figure 5 Planimetry of megakaryocyte sizes (μm^2) from bone marrow smears in controls, polycythemia vera and chronic granulocytic leukemia upper left: Normal size megakaryocytes in controls; large megakaryocytes in untreated polycythemia vera and small sized megakaryocytes in chronic granulocytic leukemia (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

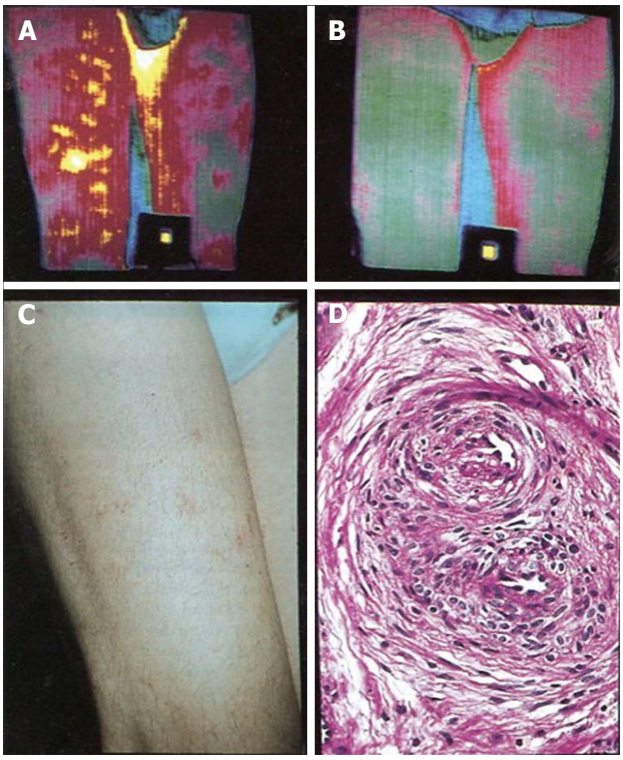

Figure 6 Isothermpgrams of upper legs showing "superficial thrombophlebitis" (A) in the right upper leg, which completely disappeared after treatment with aspin once daily (B) and superficial thrombophlebitis manifested as red painful indurated hot spots, erythromelalgia of the skin (C) in the upper 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

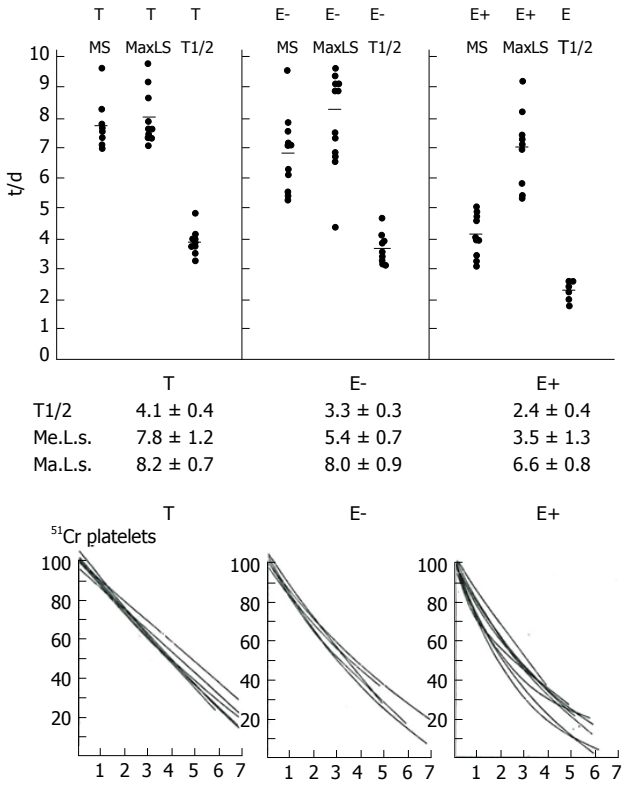

Figure 7 Platelet survival times and platelet disappearance curves according to Branehög *et al*[50] in 10 thrombocythemia patients complicated by erythromelalgi (E+), 10 asymptomatic thrombocythemia patients (E-), and 11 asymptomatic patients with thrombocytosis (T). Curvilinear platelet survival curves 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

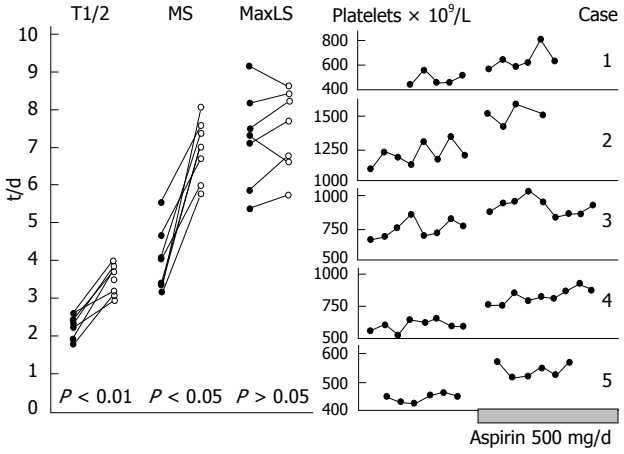

Figure 8 Platelet survival curves in seven E+ thrombocythemia patients before and after aspirin 500 mg/day, and peripheral blood platelet counts before and after maintenance aspirin treatment 250 mg/d. MS: Mean survival; T1/2; MaLS: Maximal life span according to Branehög *et al*[50].

(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

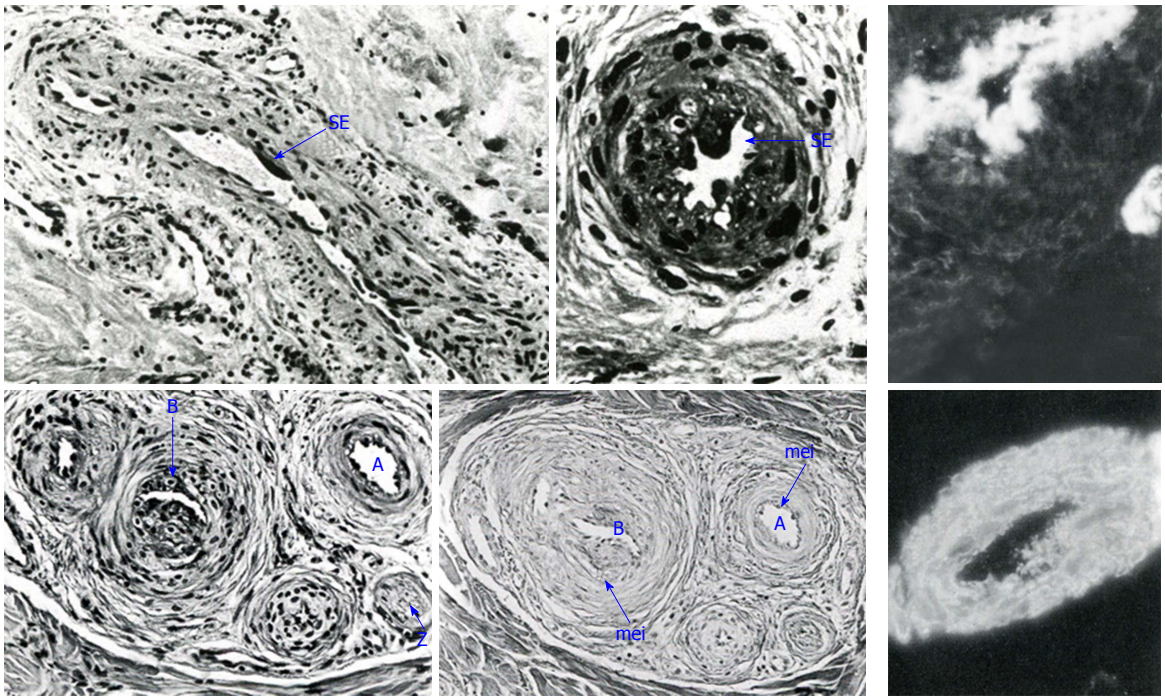

Figure 9 Arterioles with swelling of endothelial cells, proliferation of cells of the inner layer below the media and normal venules, capillaries and nerves (upper and left), and elastica von Gieson stain showing a normal membrana elestica interna (mei) in a normal arteriole (A). Source Michiels 1981. 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.

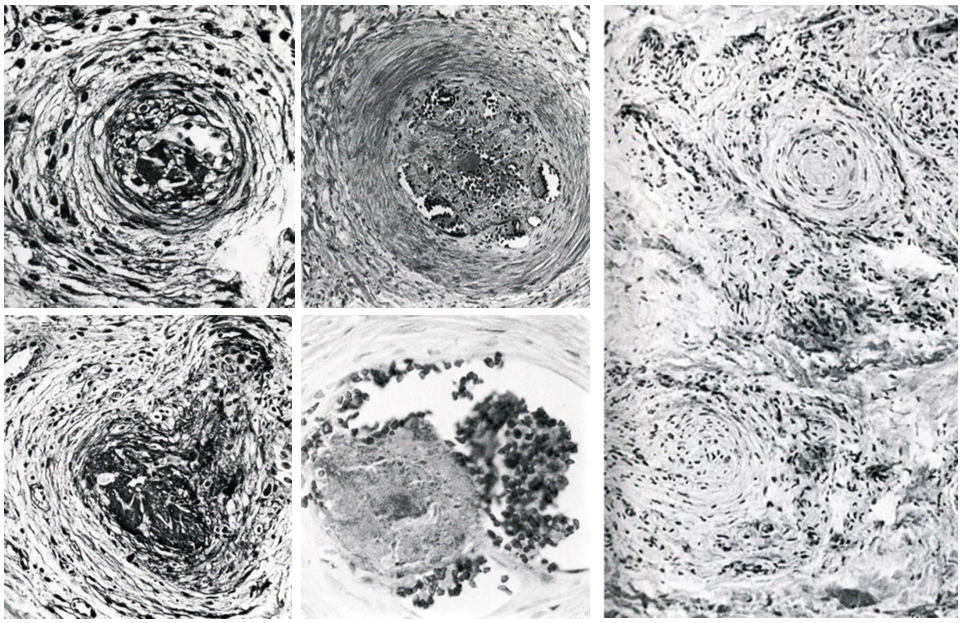

Figure 10 Thrombotic occlusion of arterioles on top of fibrouscular intimal proliferation. Thromboangiitis oblitterans (left panel) and recanalisation of arterioles showing vessel wall fibosis of arterioles in two cases of erythromelalgia complicated by acrocyanosis and digital gangrene (middle panel). 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-

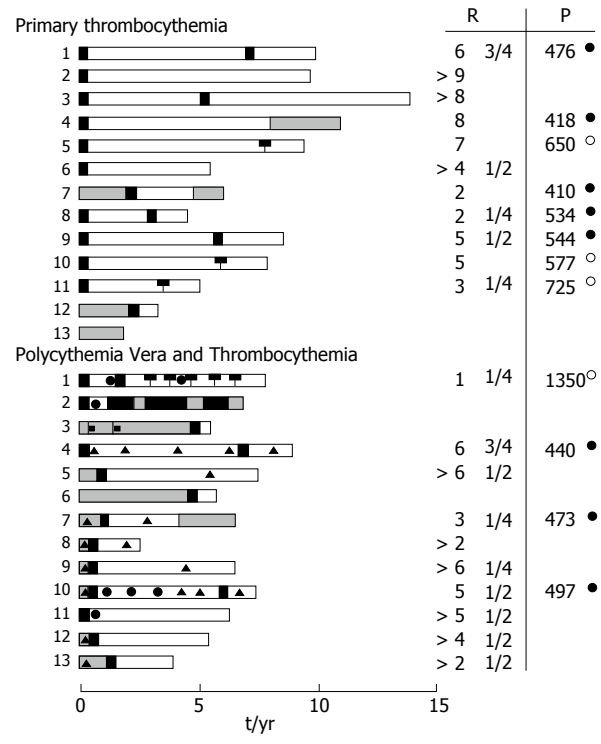

Figure 11 Results of treatment in 13 patients with primary or essential thrombocythemia and 13 patients with polycythemia vera and thrombocythemia. Source Michiels *et al*[53], 1985. Busulfan induced remission of thrombocythemia (platelet counts < 350 × 10^9/L) lasted 2 to 9 years (long busulfan remitters) 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

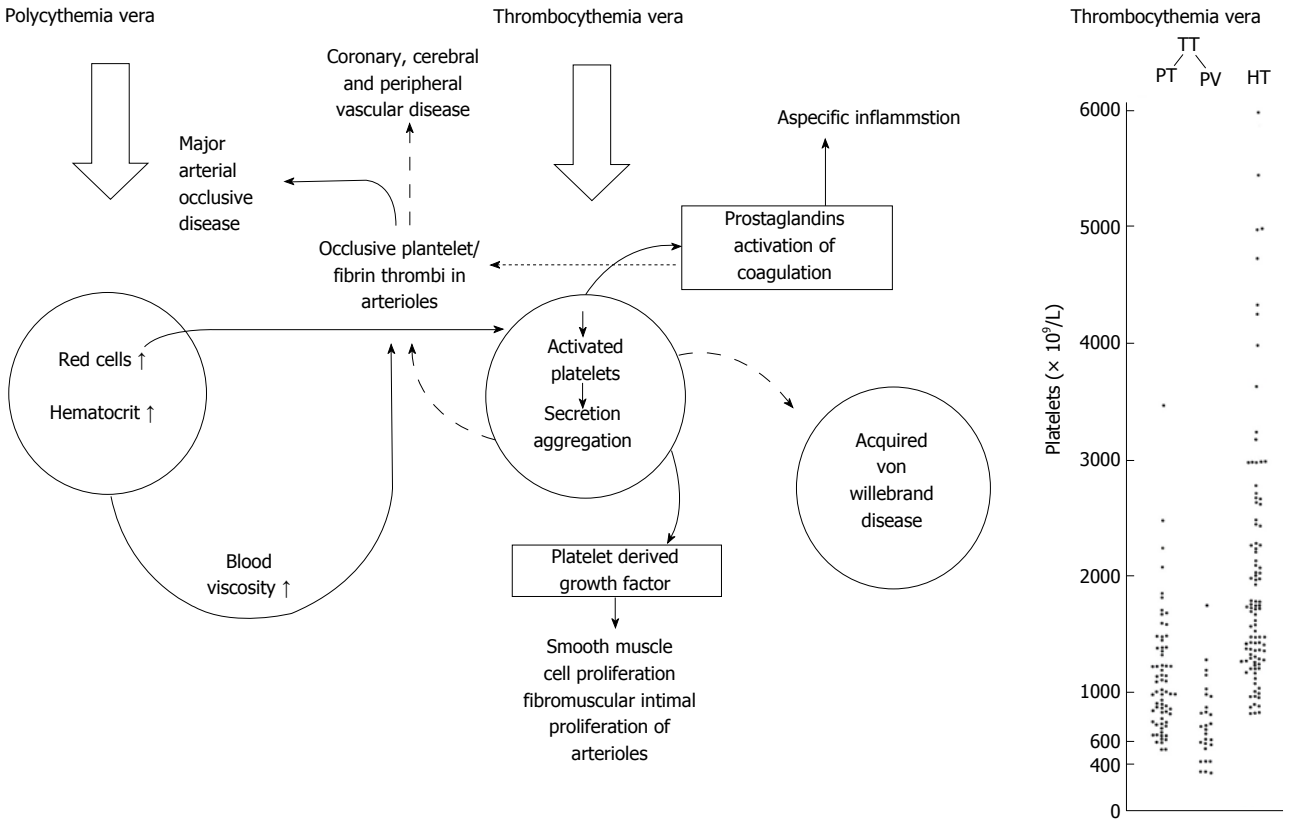

Figure 12 Pathophysiology of erythromelalgia as multicellular processes caused by platelet mediated microvascular erythromelalgic arteriolar inflammation and thrombosis in myeloproliferative thrombocythemia vera and major arterial thrombotic disease in polycythemia vera. Shear induced production of prostaglandin 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

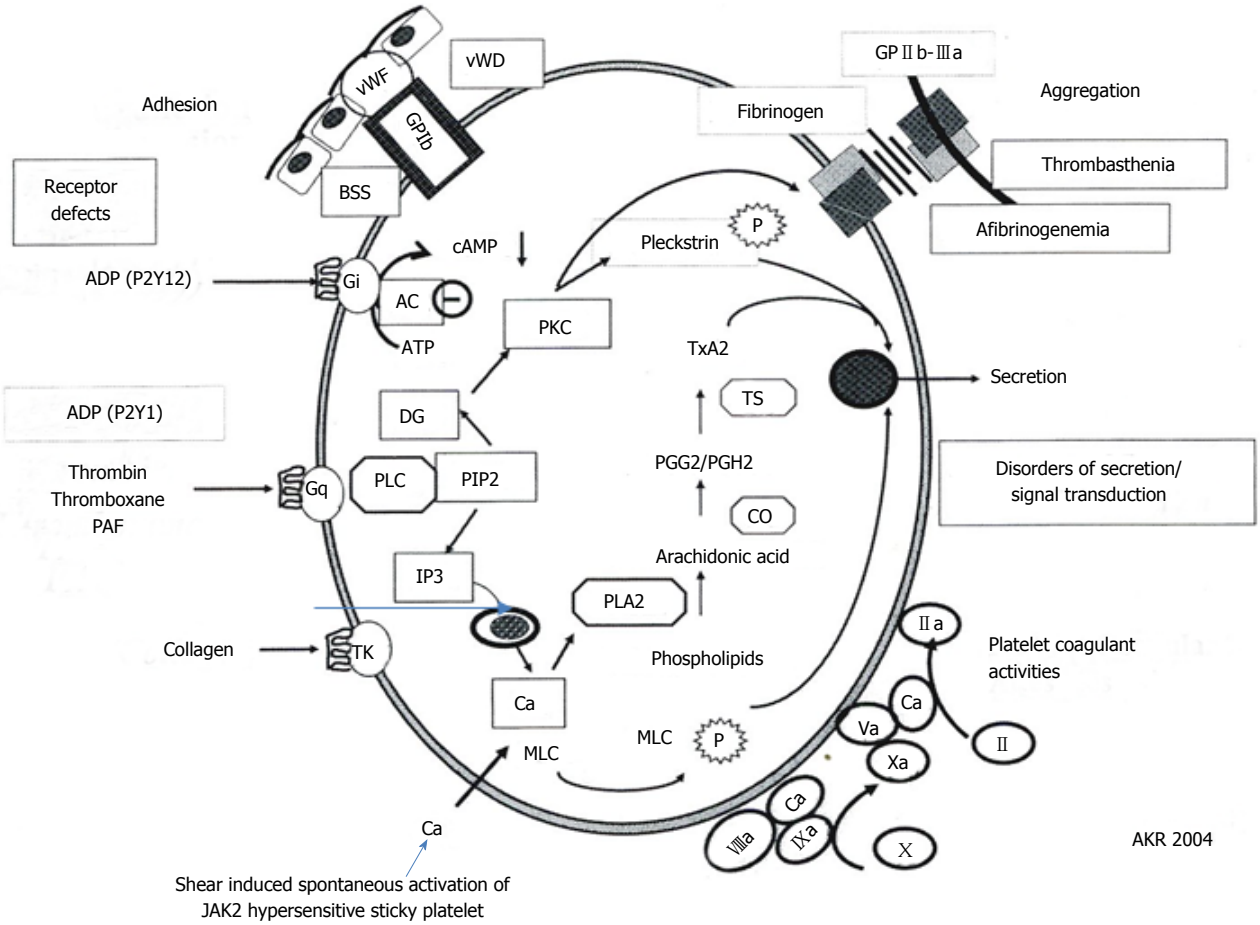

Figure 13 A schematic representation of platelet adhesion through von Willebrand factor and glycoprotein Ib disturbances in Bernard Soulier syndrome, von Willebrand disease and aggregation disturbances in Glanzmann's thrombasthenia, and receptor defects of ADP (P2PY12, P2Y1), thrombin, thromboxane, platelet 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

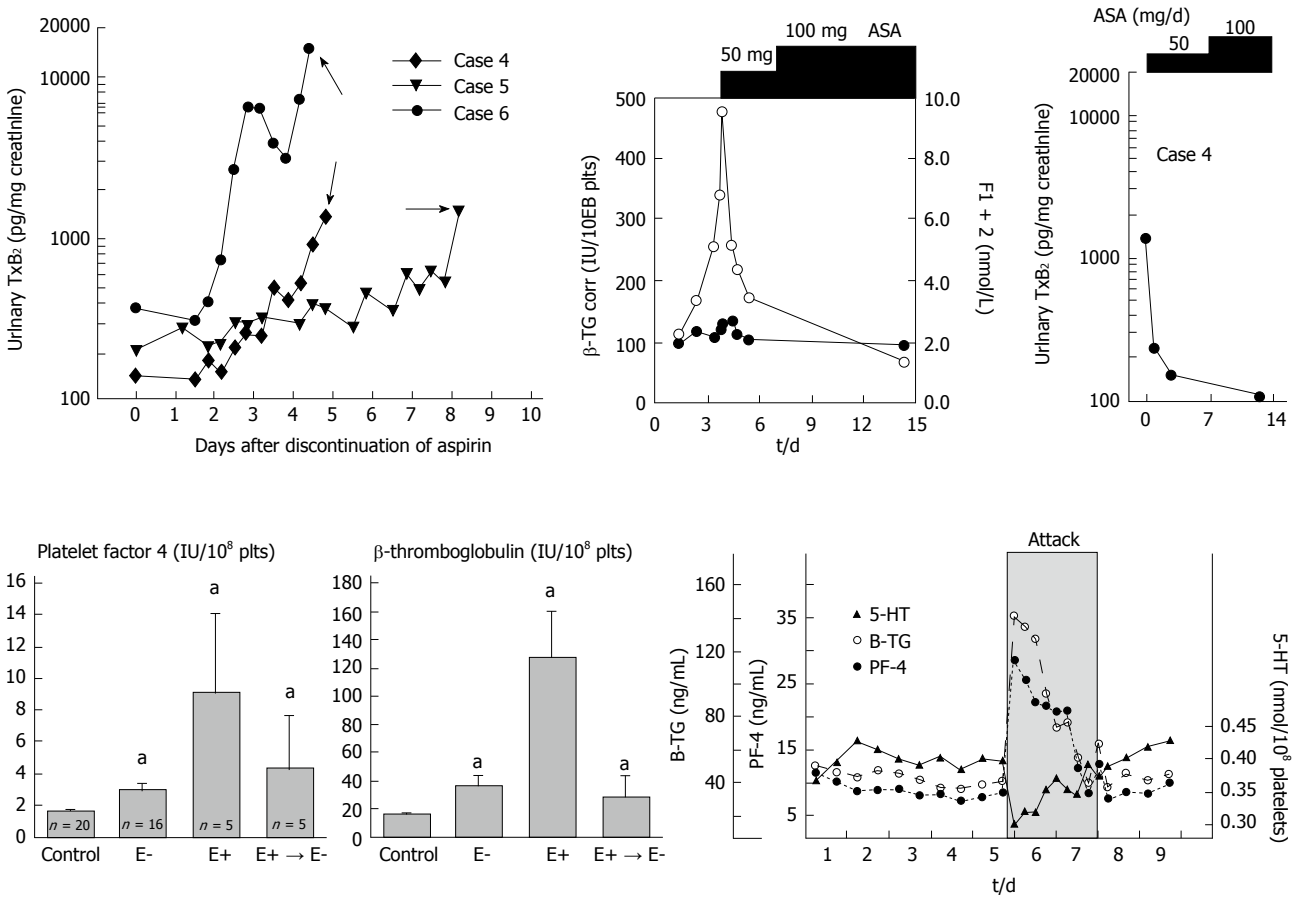

Figure 14 Vaso-active substances, prostaglandins endoperoxides and other factors released during JAK2 mutated platelet aggregation account for the inflammatory symptoms in JAK2-thrombocythemia of ET and PV patients. Upper part: Simultaneous study of clinical signs and symptoms of erythromelalgia, platelet 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

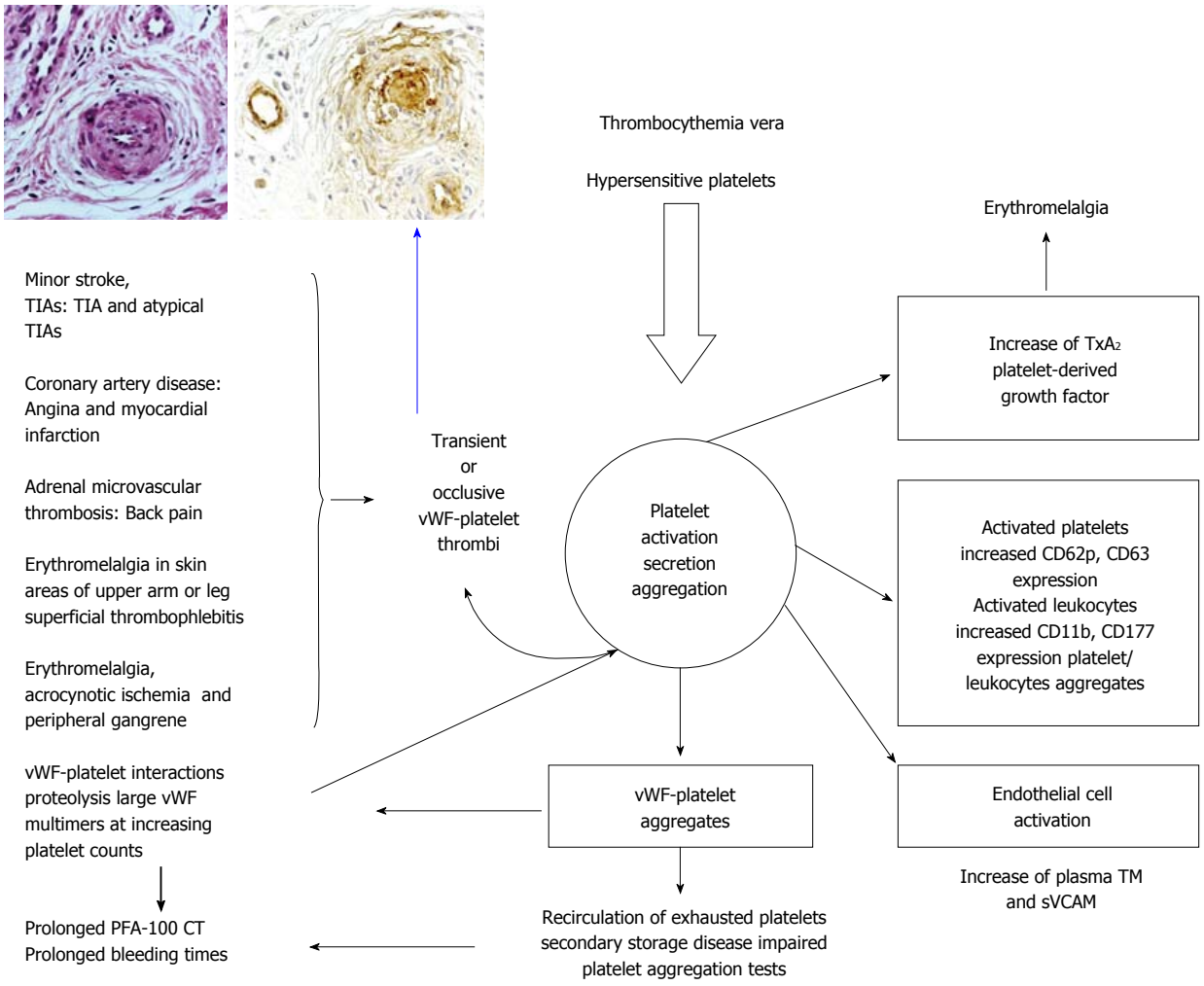

Figure 15 Shear induced platelet activation of constitutively JAK2^{V617F} hypersensitive sticky platelet with increased CD62p and CD 63 expression) in thrombocythemra vera of JAK2^{V617F}-positive ET and PV patients is the cause of a broad spectrum of platelet-mediated arteriolar inflammatory and thrombotic 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

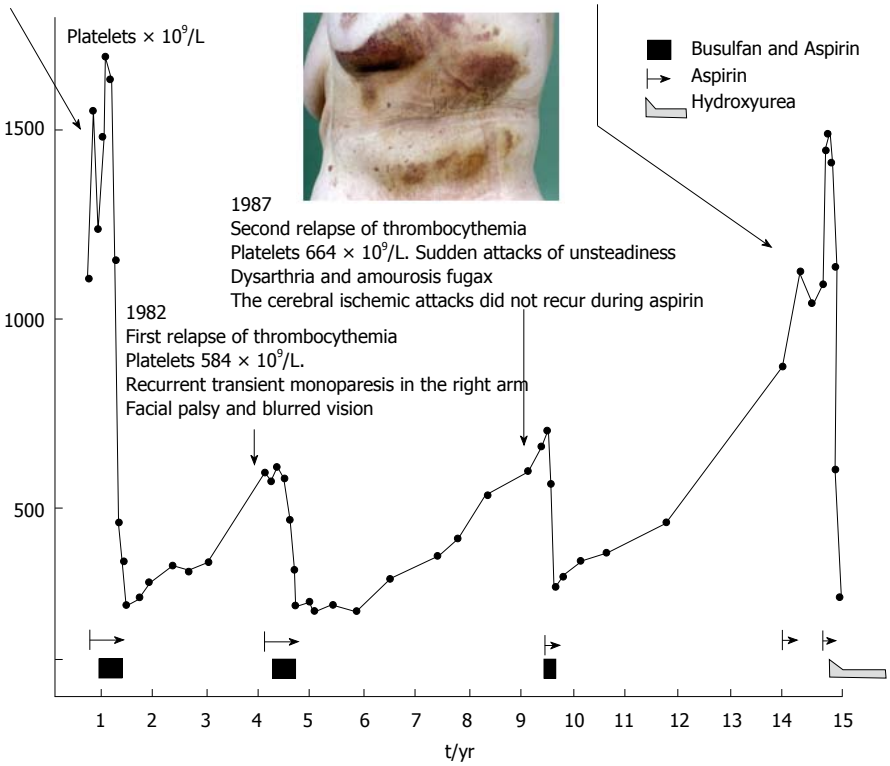

Figure 16 Longterm observations in a 72-year-old woman with a three years history of burning painful red or blue toes and forefoot sole of the right foot (erythromelalgia) presented in December 1978 with spontaneous hemorrhages of large ecchymoses on the chest and subcutaneous hematotomas. Paradoxical 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

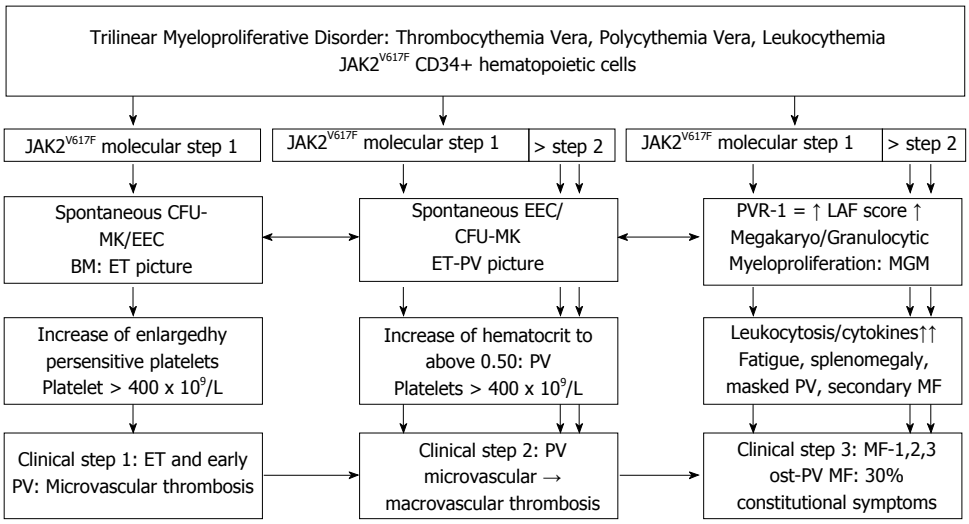

Figure 17 The molecular etiology of JAK2^{V617F} heterozygous mutated essential thrombocythemia (essential thrombocythemia picture Step 1) and evolution into combined herozygous/homozygous or homozygous JAK2^{V617F} mutated sequential stages of prodromal polycythemia vera classical PV (ET/PV pictures)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

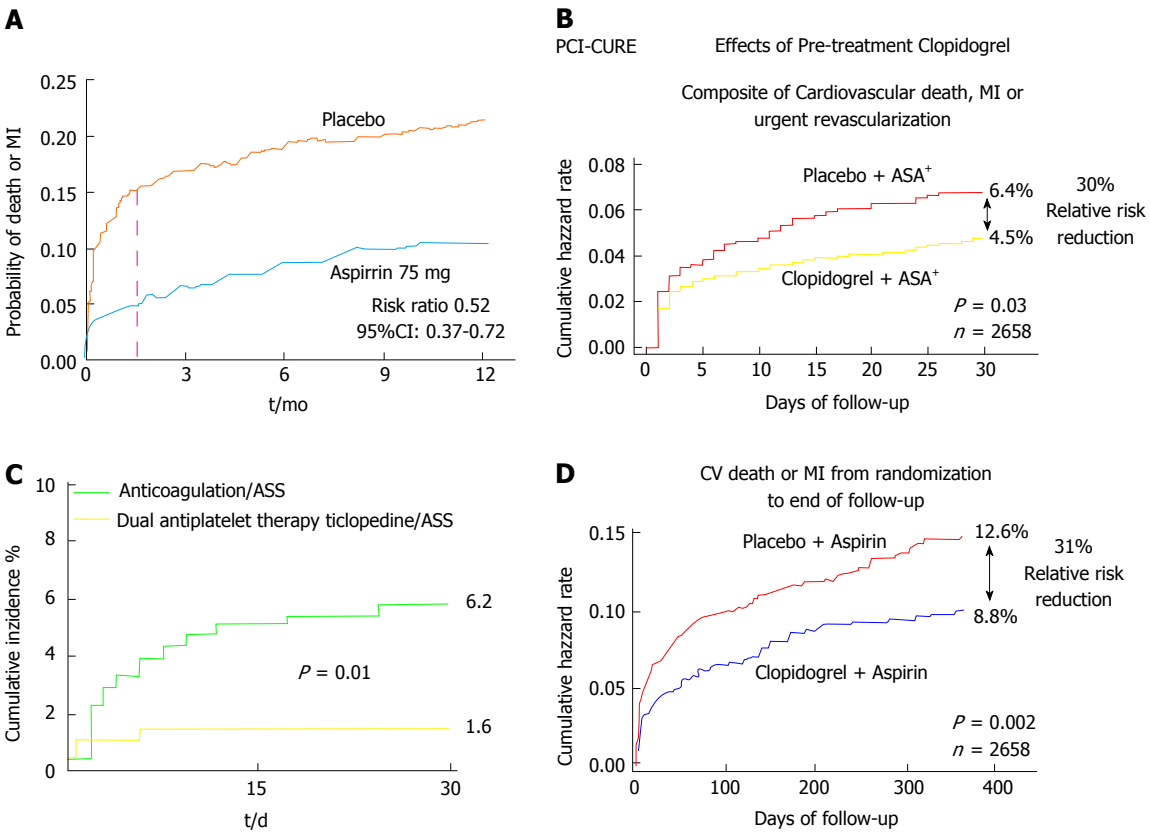

Figure 18 Low dose aspirin 75 mg OD *vs* placebo in 796 patients with unstable angina or non-Q myocardial infarction in the presence of arteriosclerotic vascular pathology was effective to reduce the probability of death or myocardial infarction during one year follow-up. A: Aspirin 75 mg OD *vs* placebo 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

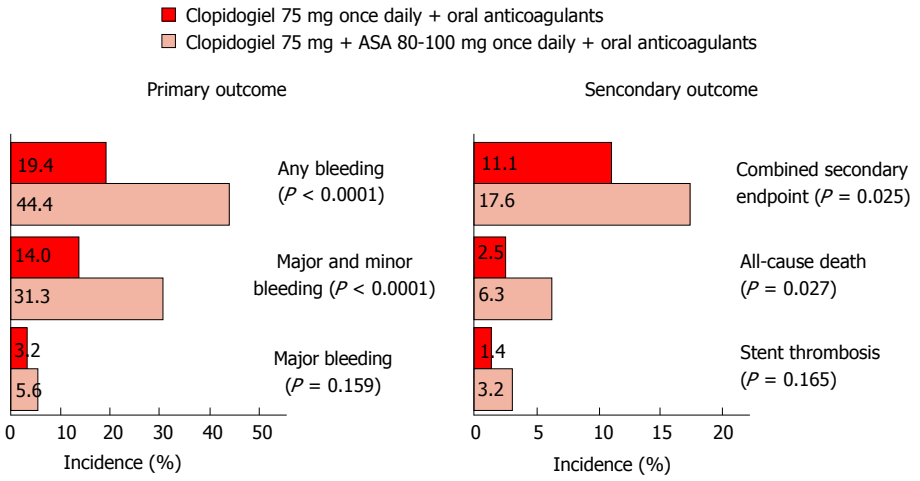

Figure 19 Dual clopidogrel and coumarin compared to triple clopidogrel coumarin and aspirin in 573 patients undergoing PCI receiving oral anticoagulation for another reason in the WOEST study was equal in terms of death, reoclusion and revascularization but superior for dual clopidogrel/coumarin in terms 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

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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

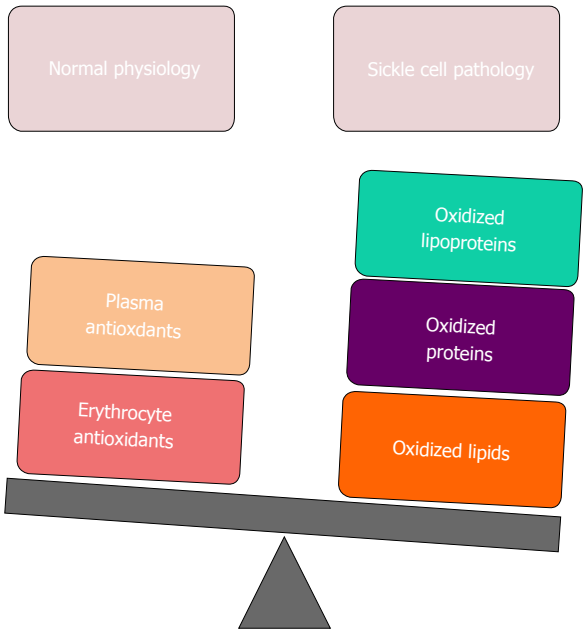

Figure 1 The balance between antioxidants and oxidants in sickle cell disease is altered towards the increase of oxidative stress and production of oxidized lipids, proteins and lipoproteins.

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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