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A portrait photograph of Prof. Dr. Gert H. Rothmann. He is a middle-aged man with dark hair, wearing glasses, a white shirt, a patterned tie, and a dark suit jacket. The photo is set against a blue background and has an orange border at the top. A small logo is visible in the bottom left corner of the photo area.

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A portrait of a middle-aged man with short, graying hair, wearing round gold-rimmed glasses. He is smiling slightly and looking directly at the camera. He is wearing a dark suit jacket over a white shirt and a patterned tie. The tie features a repeating geometric pattern in dark and light colors. The background is a solid, dark color. The lighting is soft, highlighting his facial features.


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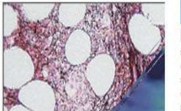
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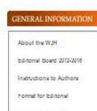


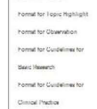
























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MINIREVIEW

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Bone marrow cell death and proliferation: Controlling mechanisms in normal and leukemic state

Kurata M, Suzuki S, Abe S, Onishi I, Kitagawa M

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Bone marrow cell death and proliferation: Controlling mechanisms in normal and leukemic state

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Abstract

Bone marrow cell death and proliferation are regulated by multiple factors including genetic and epigenetic alterations of hematopoietic cells, crosstalk of hematopoietic cells with bone marrow mesenchymal cells through direct cell-cell interaction or cytokine/chemokine production, vascularity of the bone marrow, and interactions of sympathetic nerve system with hematopoiesis. Cell proliferation usually predominates over cell death in neoplastic processes such as leukemia and myeloproliferative neoplasms, while apoptotic processes also have a significant role in the pathogenesis of myelodysplastic syndromes. Recently, hematopoietic stem cells (HSCs) and leukemia stem cells (LSCs) have been identified and their characters on self renewal process, differentiation, cell dynamics and drug resistance have been implicated. Although most leukemia cells are initially sensitive to chemo- or radiotherapy, LSCs are resistant and considered to be the basis for disease relapse after initial response. HSCs and LSCs may use similar interactions with bone marrow microenvironment. However, bone marrow microenvironment called niche should influence the normal as well as malignant hematopoiesis in different manners. Recent studies

have expanded the number of cell types constituting bone marrow niche and made the issue more complex. Since the majority of excellent and contributing studies on bone marrow niches have been performed in animal models, niches in human tissues are beginning to be localized and characterized. In this article, we summarize the relation of hematopoietic cells with niches and hope to point a hint to the novel strategy for treatment of malignant proliferation of hematopoietic cells.

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Key words: Bone marrow; Hematopoietic stem cells; Niche; Apoptosis; Leukemia

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INTRODUCTION

Cell dynamics of hematopoietic cells/leukemic cells may be regulated by multiple factors in the bone marrow. Recently, interactions of hematopoietic stem cells (HSCs) with the bone marrow microenvironment have been extensively studied mainly using animal models with genetic modification. Many signaling pathways such as CXCR4/CXCL12, Jagged/Notch, Wnt, Ang-1/Tie2, SCF, very late antigen-4 (VLA-4), VCAM-1, and TGF have been shown to regulate the self-renewal, differentiation, proliferation, and apoptosis/senescence of HSCs^[1-11]. Mechanisms of crosstalk between hematopoietic cells and the bone marrow microenvironment would be closely associated with the pathogenesis of hematologic diseases including malignancies. Although leukemia cells are thought to harbor cell-autonomous mutations, the interaction with the microenvironment should play an important role

in the regulation of leukemia cell dynamics^[12]. This article reiterates the importance of mechanisms affecting the functions of the bone marrow microenvironment on the proliferation/apoptosis of hematopoietic/leukemia cells.

HSCS AND THEIR NICHE

The HSCs have two opposing characters - maintenance of an undifferentiated state analogous to pluripotent stem cells, and the execution of tissue-specific hematopoietic functions^[13]. In the bone marrow, characters of HSCs are maintained within a special microenvironment called a niche. The concept of niches was proposed in the 1970s; however, the nature and function of these stromal structures remains unclear. HSCs are in contact with bone-lining osteoblastic cells, but only a part of the HSCs seem to be located in this endosteal niche region. Instead, many HSCs are found in association with the sinusoidal endothelium, referred to as the vascular niche. However, osteoblast depletion results in extramedullary hematopoiesis, suggesting that a vascular niche alone is not sufficient to maintain hematopoiesis^[14]. Endosteal and vascular niches might regulate different HSC populations, although recent data depicts a more complicated feature, with functional crosstalk between cells in these two regions^[2,15,16].

Recently it was suggested that primitive mesenchymal cells, including CXCL12-abundant reticular cells^[17-20], nestin-expressing cells with sympathetic nerve system^[21-23], nonmyelinating Schwann cells^[24] and macrophages^[25,26] act as bone marrow niche-associated cells. The interactions between CXCR4 and CXCL12 (SDF-1) are especially important in the localization and retention of HSCs in the bone marrow. Furthermore, chemokine interactions through CXCL12 can cause the up-regulation of vascular cell adhesion molecule-1 and VLA-4 expression^[27]. CXCL12 also has an essential role in colonization of the bone marrow by HSCs during early development, because CXCL12-deficient embryos have severely reduced HSC numbers and a disturbed function^[28]. Further studies on the interplay of such regulatory forces as “cell fate and localization determinant” will likely shed light on the pathogenesis of hematological diseases.

BONE MARROW NICHES IN HEMATOPOIETIC PATHOLOGY

Recent animal model studies have provided insights into the role of aberrant microenvironment signaling leading to the pathogenesis of hematological diseases. Perturbations in niche signaling in murine models^[29] can mimic idiopathic myelofibrosis, leading to enhanced stem cell mobilization and the creation of alternate niches^[30]. The deletion of the *Dicer1* gene specifically in mouse osteoblasts (endosteal niches) disrupted the integrity of hematopoiesis. Myelodysplastic syndromes (MDS)-like phenotypes emerged in spite of the fact that hematopoietic cells had an intact *Dicer1*^[31]. Therefore, perturbation of specific mesenchymal stromal cells can cause disorder in the differentiation, proliferation, and apoptosis of hematopoietic

cells. In this mouse model, acute myeloid leukemia (AML) emerged with several acquired genetic abnormalities, supporting the concept of niche-induced leukemogenesis. Also, in human samples, the impaired expression of *DIC-ER*-associated genes was demonstrated in mesenchymal stromal cells from MDS patients^[32].

Concerning the bone marrow cell dynamics in MDS, excessive apoptosis of hematopoietic cells was observed to be induced by the bone marrow microenvironment^[33-48]. The apoptosis was mediated by paracrine as well as autocrine factors, implicating both medullary stromal cells and hematopoietic cells in the pathology of the disease. Pro-inflammatory cytokines such as tumor necrosis factor in the bone marrow microenvironment are mainly paracrine mediators of apoptosis. As autocrine stimulation mechanisms, it has recently been shown that the deregulation of ribosome biogenesis can initiate a stress response in hematopoietic cells through the p53-mediated signaling pathway. Thus, both the stromal cells of the bone marrow microenvironment and hematopoietic cells themselves possess a common and characteristic biology in this heterogeneous disease entity.

In human samples, the microenvironment has also been studied in multiple myeloma^[49]. Self-renewal pathway activation in the niche (such as the canonical Wnt pathway) has been postulated to result in enhanced myeloma cell survival^[50]. Another example of microenvironment-associated disease is the WHIM (Warts, Hypogammaglobulinemia, Infections, and Myelokathexis) syndrome. This rare syndrome is known to exhibit congenital neutropenia often caused by mutations in CXCR4 with increased sensitivity to CXCL12^[51].

LEUKEMIA STEM CELLS AND NICHES

Like many cancers, AML has been identified as a cell autonomous disorder. Namely, the genetic events leading to transformation of the normal hematopoietic cell are found within leukemia cells and are necessary and sufficient for the generation of leukemia. However, many studies have demonstrated evidence of functional heterogeneity among AML cells. In particular, there seems to be a subpopulation of AML cells referred to as “leukemia stem cells” (LSCs) that alone have long-term repopulating potential and the ability to propagate and maintain the AML phenotype^[52]. To understand the difference of LSCs from HSCs, clarification of the crosstalk between LSCs and niches would be very important. Niche-mediated mechanisms promote the engraftment and survival of LSCs. The biology of LSCs has been determined by studies of primary human AML cells transplanted into NOD-SCID or NOD-SCID IL2Rγ^{null} mice^[53,54]. In these xenograft models, AML cells have a phenotypic hierarchy, which parallels that of normal hematopoietic stem/progenitor cells^[1,55-58]. For example, the CD34⁺CD38⁻ AML population could engraft efficiently in NOD-SCID mice, but the more differentiated CD34⁺CD38⁺ and CD34⁻ AML cells were unable to engraft and yield colony-forming progenitors. Human AML cells preferentially engrafted in

the bone marrow endosteal region in NOD-SCID IL2R^{null} mice, and remained adjacent to bone marrow osteoblasts for up to 4 mo following transplantation^[54]. Homing to the microenvironment appears important in sustaining the survival of LSCs. Moreover, these cells were highly enriched for quiescent cells and were resistant to cytosine arabinoside chemotherapy^[59].

CROSSTALK OF LSCS WITH NICHES

Next, the specific mechanisms of niches regulating LSC fate should be clarified^[60]. For example, the interaction between CXCR4 and CXCL12 is known to regulate normal HSC proliferation and survival^[61-63]. What is the case in LSCs? Actually, CXCR4 is also expressed on primary leukemic cells, and high-level expression of CXCR4 on AML cells is a negative prognostic factor of relapse-free and overall survival^[64,65]. Treatment with the CXCR4 antagonist was shown to inhibit stromal cell-induced proliferative signals in AML cells and to increase the sensitivity to chemotherapy, resulting in the extended survival of AML-transplanted mice compared with treatment with chemotherapy alone^[66]. These data suggest that LSCs possess a CXCR4-dependent homing capacity that is analogous to normal hematopoietic cells, and antagonism of the CXCR4/CXCL12 axis is a logical therapeutic strategy for the treatment of acute leukemia.

However LSCs are rather heterogeneous. Even in LSCs, different patterns of interactions with niches were identified^[67]. The homing of pre-LSCs was similar to long-term HSCs, while the homing of established LSCs was most similar to that of committed myeloid progenitor and distinct from HSCs.

After homing to the bone marrow niches, leukemia cells are retained in these niches through cellular adhesion molecules such as VLA-4 and LFA-1 integrins^[68]. Increased VLA-4 expression has been demonstrated to correlate with increased bone marrow blast counts in AML^[69]. Furthermore, VLA-4-highly expressing AML cells show relative resistance to chemotherapy-induced apoptosis, and the administration of VLA-4 neutralizing antibodies inhibits this resistance^[70].

On the other hand, the leukemia cells were able to directly modulate the niche at the expense of normal hematopoietic stem and progenitor cells by down-regulating CXCL12 levels in areas of leukemia infiltration^[71]. It has been shown that stem cell factor, a niche regulator, was secreted by the leukemia cells, leading to the abnormal retention and engraftment of normal HSCs in the microenvironment with leukemic infiltration. Thus, identifying the mechanisms involved in the generation of various signals by HSCs, LSCs, and niches might provide new insights into the pathogenesis of acute leukemia as well as MDS and myeloproliferative neoplasms.

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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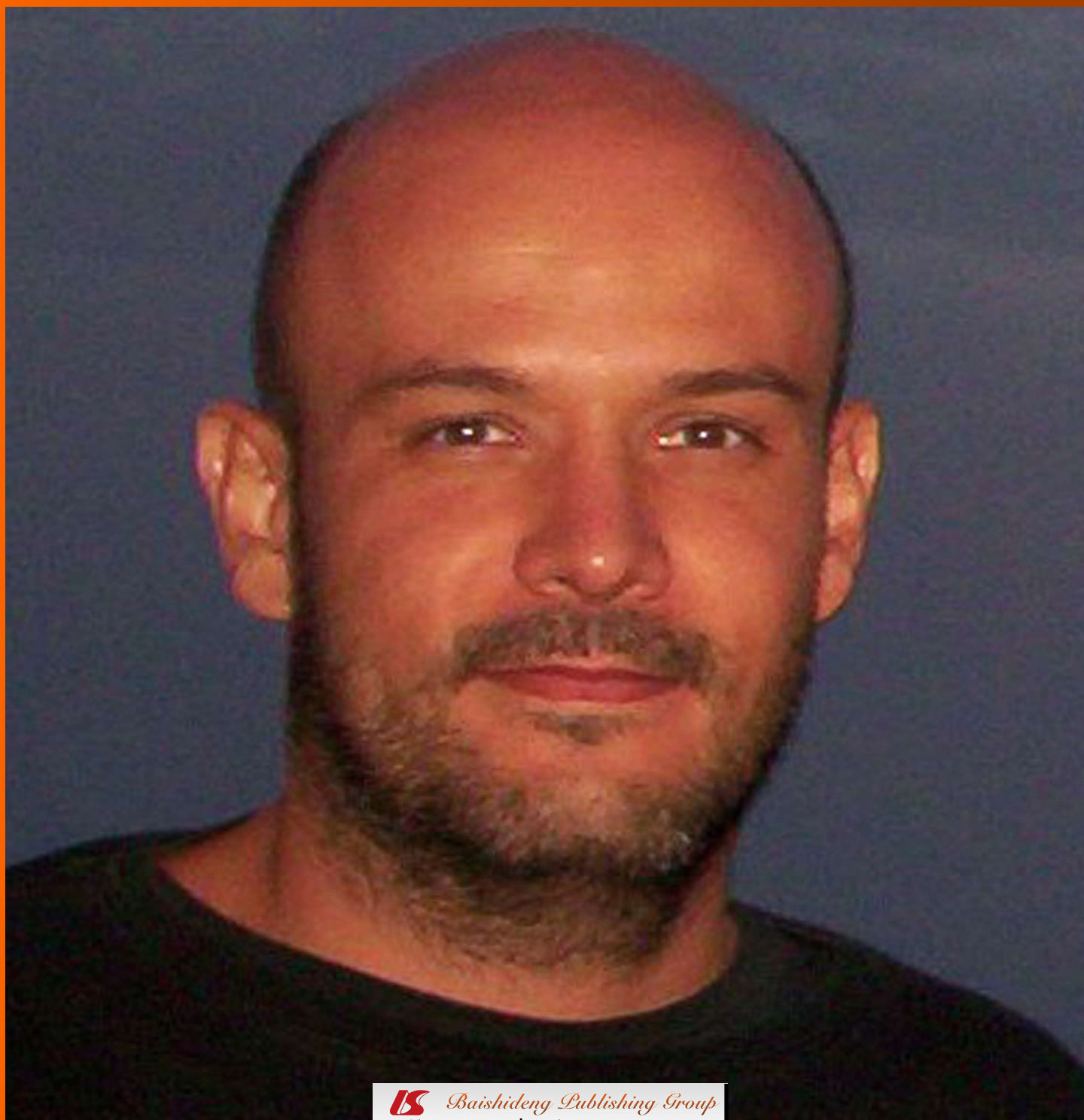
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Contribution of new immunoglobulin-derived biomarkers in plasma cell dyscrasias and lymphoproliferative disorders

Marie-Christine Kyrtsionis, Dimitrios Maltezas, Efstathios Koulieris, Tatiana Tzenou, Stephen J Harding

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Abstract

New assays for serum immunoglobulin (Ig) free and heavy chain quantification were developed for routine clinical practice. Serum free light chain (sFLC) assay was shown to improve detection, management and prognostication in all plasma cell dyscrasias. More precisely, sFLC measurements proved to be prognostic for the progression of monoclonal gammopathy of undetermined significance and smoldering multiple myeloma (MM), became markers of response and survival in amyloid light-chain amyloidosis and contributed to accurate follow-up of patients with light chain and non secretory MM. In addition, sFLC and they ratio (sFLCR) were shown useful for the prognosis and monitoring of intact Ig myeloma; their evaluation was incorporated in the new uniform response criteria. sFLC or sFLCR were also observed abnormal in B-cell non-Hodgkin lymphoma/chronic lymphocytic leukemia (CLL). Moreover, increased sFLC levels, summated sFLC or abnormal sFLCR predict shorter overall survival in early-stage CLL while increased sFLC constituted an independent, adverse

prognostic factor for event-free and overall survival in diffuse large B-cell lymphoma and Waldenstrom's macroglobulinemia. Clinical applications of heavy Ig chain separately (HLC) measurements are more recent and mainly concern MM in which HLC deriving ratios correlated with parameters of disease activity and constituted an adverse survival marker.

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Key words: Immunoglobulin quantification; Freelite™; Hevylite™; Diagnosis; Monitoring; Prognosis

Core tip: Recently manufactured assays allow the quantification of serum immunoglobulin (Ig) free light chain (sFLC) or of κ or λ restricted heavy Ig chain separately (HLC). These measurements, or the calculation of their corresponding ratios, were shown useful for routine clinical practice in Hematology. sFLC measurements added important prognostic information for monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma (MM), amyloid light-chain (AL) amyloidosis, Waldenstrom's macroglobulinemia and chronic lymphocytic leukemia while they contributed to accurate follow-up of MGUS, MM and AL amyloidosis patients. HLC measurements are more recent and mainly concern MM in which they constituted a prognostic marker.

Kyrtsionis MC, Maltezas D, Koulieris E, Tzenou T, Harding SJ. Contribution of new immunoglobulin-derived biomarkers in plasma cell dyscrasias and lymphoproliferative disorders. *World J Hematol* 2013; 2(2): 6-12 Available from: URL: <http://www.wjgnet.com/2218-6204/full/v2/i2/6.htm> DOI: <http://dx.doi.org/10.5315/wjh.v2.i2.6>

INTRODUCTION

During the past decade, new assays for serum immuno-

globulin (Ig) free and, more recently, heavy chain quantification were developed for routine practice^[1].

Igs are produced during B lymphocyte development where they are initially expressed on the surface of the cell. Production of Igs continues throughout B-cell development and terminal differentiation into plasma cells where it is greatest; lymphoplasmacytes and plasma cells normally secrete Ig.

Igs are symmetrical and are made up of mirror imaged identical light and heavy chains. There are five classes of heavy chain, γ , α , μ , δ and ϵ with two classes of light chain κ and λ ; with approximately $\times 2$ greater κ production compared to λ . B-cells and plasma cells produce an excess of serum free light chains (sFLC) during normal Ig synthesis that enter the blood and the extravascular compartment. This production is rapidly cleared (2-6 h) and metabolized by the kidney although trace quantities (1-10 mg/L) can be found in the urine. In patients with plasma cell dyscrasias (PCD) and B-cell lymphoproliferative disorders (LPD) homogeneous FLC are produced by the malignant clone. sFLC are important biomarkers and may be present in serum in very large excess. Their quantification was not possible before the development of immunoassays utilizing specific polyclonal sheep antisera against κ and λ epitopes that are not visible when the FLC are bound to their heavy chain partners^[2].

These assays have revolutionized the ability to detect and quantify sFLC with a sensitivity of less than 0.5 mg/L. Furthermore, the calculation of a κ/λ ratio (sFLCR), which incorporates both monoclonal Ig production and polyclonal Ig suppression, offers additional prognostic information^[3].

Monoclonal Ig heavy chain is routinely detected by serum protein electrophoresis (SPEP), identified by immunofixation and quantified by SPEP-densitometry or nephelometry. Guidelines recommend SPEP to monitor monoclonal Ig concentrations as markers of response and relapse. However, SPEP quantification can be inaccurate at low concentrations (< 3 g/L) and can be difficult in patients where the monoclonal Ig co-migrates with other proteins, commonly seen in IgA and IgM isotypes. In such instances guidelines recommend the use of total Ig nephelometric assays, which do not distinguish between the monoclonal and polyclonal Ig's and will be insensitive as the Ig concentration approaches the normal range. Furthermore, SPEP linearity at high concentrations and the variable catabolism of monoclonal IgG can make assessment of the serum load inaccurate. Production of immunoassays targeting the unique junctional epitope between the light chain and heavy chain constant region of Ig enables separate quantification for the different heavy Ig classes bounded to their respective light chain (HLC) *i.e.*, IgG κ , IgG λ , IgA κ , IgA λ , IgM κ and IgM λ . Measuring the molecules in pairs then produces a ratio of the involved/uninvolved-polyclonal Igs (HLCR)^[1-4].

The diagnostic and prognostic utility of these tests in the management of PCD and B-cell LPD is established by now in some disease entities and under evaluation in others^[5]. Herein, we will describe their main contributions

in these disorders that, with regard to sFLC measurements, are depicted in Figure 1.

CONTRIBUTION OF FLC AND HLC MEASUREMENTS IN PCD AND LPD

Monoclonal gammopathy of undetermined significance

Monoclonal gammopathy of undetermined significance (MGUS) is a preneoplastic condition, defined as serum monoclonal protein < 30 g/L, $< 10\%$ clonal plasma cells in the bone marrow (BM) and no evidence of end organ damage. sFLCR is abnormal in approximately 1/3 of MGUS patients. It was shown that abnormal sFLCR constitutes an independent factor for disease progression^[6]. Using three risk factors, abnormal sFLC, presence of non-IgG MGUS and monoclonal protein ≥ 15 g/L, a powerful stratification model to predict disease evolution to multiple myeloma (MM) was produced^[7].

Preliminary HLC results in MGUS patients suggest that isotype specific HLC-IgG pair suppression is an indicator of susceptibility to evolve to myeloma; however the same was not observed for HLC-IgA^[8].

MM

MM is characterized by BM plasma cell infiltration and the presence of serum/urine monoclonal Ig. Clinical manifestations vary widely. The disease may be indolent or extremely severe and accompanied by significant morbidity. Survival ranges from a few months to more than a decade.

Serum FLC quantification is useful for diagnosis, response evaluation, monitoring and prognostic purposes.

Serum FLC measurements are more accurate than quantification of urine proteinuria in light chain myeloma (LCM) while in the majority of non secretory MM, low levels of abnormal serum FLCs are actually found (oligosecretory disease). In both aforementioned MM subgroups, sFLC fluctuations can be used for disease monitoring^[9]. Figure 2 shows the clinical course of an LCM patient along with sFLC fluctuations; it is interesting to observe that this patient would have been characterized "non-secretory" because she has no serum monoclonality and never presented albuminuria or positive urine immunofixation in spite of increased sFLC and it would have been impossible to monitor her disease without sFLC measurements.

In an attempt to improve criteria of response to treatment^[10], sFLCR was incorporated to the MM uniform response criteria^[11] and its normalization along with immunohistological confirmation of clonal disease absence, defined a deeper response, the stringent complete remission (sCR). A better evaluation of the depth of response is important as the quality of response influences treatment free and overall survival after treatment^[12]. However, the impact of sCR compared to CR and very good partial remission in terms of progression free and overall survival has not been fully proven yet.

With regard to prognosis, sFLC and sFLCR were

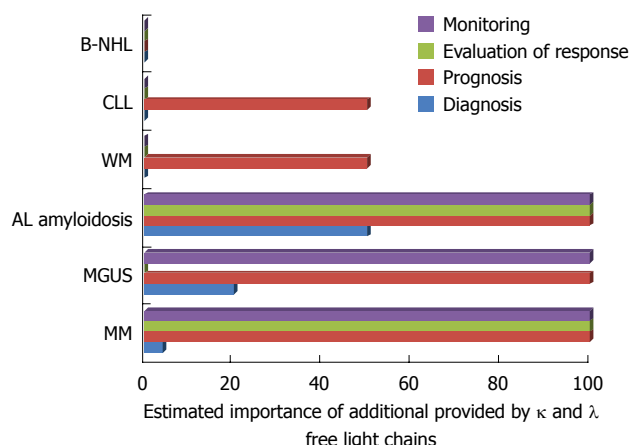


Figure 1 Estimated importance of additional information provided by free κ and λ free light chains for the diagnosis, prognosis, evaluation of response and monitoring of multiple myeloma, monoclonal gammopathy of undetermined significance, amyloid light-chain amyloidosis, chronic lymphocytic leukemia, non-Hodgkin lymphomas and Waldenstrom's macroglobulinemia. MM: Multiple myeloma; MGUS: Monoclonal gammopathy of undetermined significance; CLL: Chronic lymphocytic leukemia; B-NHL: B-cell non-Hodgkin lymphomas; WM: Waldenstrom's macroglobulinemia; AL: Amyloid light-chain.

shown predictive of outcome in all MM subcategories. Patients with smoldering myeloma and abnormal sFLCR (< 0.125 or > 8) were shown to have an increased progression risk^[13]. An adverse outcome was observed in patients with overt MM and sFLCR $>$ median or < 0.03 or > 32 ^[14,15] while the combination of sFLCR and other markers of disease activity (LDH, β_2 -microglobulin, genetic abnormalities) provided powerful prognostic models^[16]. There have also been proposals of sFLCR incorporation in to the International Score System^[15,17], that remain to be validated in larger patient cohorts and in the new agents era.

sFLC measurements during follow-up of patients are useful, not only for the evaluation of response as already mentioned, but also because, with the improvement of treatment modalities resulting in prolonged survival, unusual relapses may be observed. Light chain escape is a transformation that may occur, characterized by a shift in secretion from intact Ig to LC only in a subset of patients^[18,19].

The rationale for HLC measurements in MM is quite obvious. Serum monoclonal Ig quantification is part of MM diagnostic criteria and it is also used for monitoring response and relapse. However, MM aggressiveness was not found related to the amount of Ig secretion^[20] as detected by classical densitometry or nephelometry total Ig quantification although Ig amount was a risk factor of Durie and Salmon's staging system^[21]. It is indeed attractive to study whether the specified and precise quantification of the monoclonal component renders it a prognostic indicator.

New assays (Hevylite) that allow the separate quantification of the involved IgG κ , IgG λ , IgA κ and IgA λ , along with their deriving ratios: IgG κ /IgG λ , IgG λ /IgG κ , IgA κ /IgA λ and IgA λ /IgA κ ^[1,22], were recently introduced; their utility was shown by several groups. It was preliminary reported that increased HLC-IgG and -IgA ratios

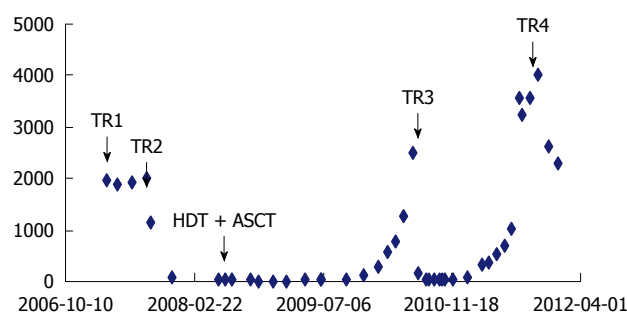


Figure 2 κ free light chain fluctuations in a light-chain κ multiple myeloma patient, during disease course. κ -free light chain fluctuations in an LC- κ multiple myeloma patient that presented mild anaemia and bone pains and no serum or urine paraprotein by serum protein electrophoresis and immunofixation. TR: Treatment; HDT + ASCT: High dose treatment and autologous stem cell transplantation.

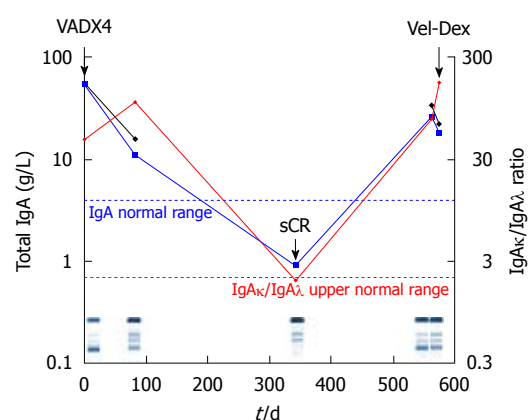


Figure 3 Heavy chain fluctuations in the course of an immunoglobulin A multiple myeloma patient. An immunoglobulin (Ig)A κ multiple myeloma patient achieved stringent complete remission (sCR) after second line treatment with velcade-Dexamethasone. At relapse, he was retreated with velcade-Dex. A second response by serum protein electrophoresis (SPE) was recorded after 8 d but the patient continued to clinically decline and bone marrow aspirations showed considerable plasma cell infiltration. The IgA κ /IgA λ ratio normalised at sCR, became abnormal – relapse and increasingly abnormal during the second clinical relapse in contrast to the SPE results. Total IgA (blue line), IgA normal range (upper limit, blue dashed line), monoclonal protein by SPE densitometry (black line), IgA κ /IgA λ ratio (red line) and IgA κ /IgA λ ratio normal range (upper limit, red dashed line).

were predictive of a shorter progression-free survival^[23] and overall survival^[24,25].

Two recent studies showed the first that HLCR correlated with parameters of disease activity and tumor burden including anemia, sFLCR, increased β_2 -microglobulin and marrow infiltration of more than 50%, while HLCR values above median constituted an independent predictor of adverse survival^[26]. In addition, the same study found that the depression of uninvolved polyclonal Igs and increased HLCR were related to a shorter time to treatment^[26]. The second study evaluated MM patients in complete remission after autologous stem-cell transplantation and showed that an increased κ/λ ratio of the uninvolved isotype was associated with a longer progression-free and overall survival^[27].

Serum HLC measurements may also offer additional

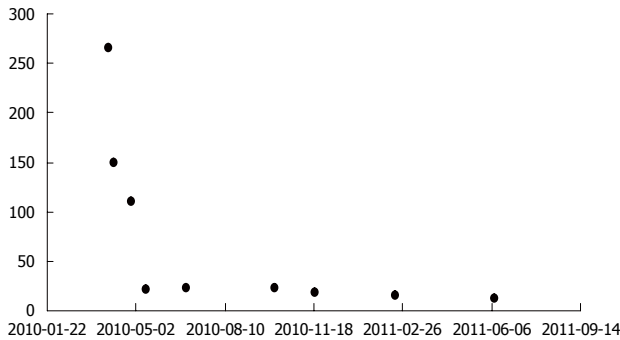


Figure 4 Lambda free light chain fluctuations in a amyloid light-chain-amyloidosis patient, in response to treatment. Total immunoglobulin (Ig)G and IgA were decreased, immunofixation showed only κ monoclonality. λ -FLC fluctuations in response to treatment, in a LC- λ AL amyloidosis patient with bone marrow, kidney and stomach involvement FLC: Free light chain; AL: Amyloid light-chain.

information during follow-up, compared to classical total Ig quantification (Figure 3).

Systemic amyloid light-chain amyloidosis

Systemic amyloid light-chain (AL) amyloidosis is due to the deposition of misfolded monoclonal light chains or their fragments in tissues or organs, leading to their dysfunction^[28]. The diagnosis is frequently difficult to make and in the absence of characteristic amyloidosis signs or of serum intact Ig paraprotein, and physicians should be sensitized to AL amyloidosis eventuality in order to detect it. In such a context sFLC measurements are useful and will be found increased in up to 94%-98% of patients, even in the absence of any Ig monoclonal peak on serum electrophoresis or immunoelectrophoresis. Indeed, diagnosis should be subsequently biopsy proven.

In addition, sFLC serum concentrations allow easy monitoring of response to treatment^[29]. Figure 4 shows serum FLC fluctuations in response to treatment in a patient with AL amyloidosis presenting BM, stomach and renal involvement. The case is however extreme because usually sFLC values are much lower in this disease.

sFLC levels at diagnosis constitute an adverse marker of survival in AL amyloidosis^[30]. The addition of cardiac biomarkers (cardiac troponin T and N-terminal pro-B-type natriuretic peptide) to sFLC levels was highly predictive of patients' survival^[31] and a new prognostic staging system was built^[32].

Preliminary data on HLC measurements in AL amyloidosis appear promising. In a subset of AL amyloidosis patients with no detectable serum or urinary monoclonal bands and a normal sFLC ratio, the HLC ratio was abnormal in 19% of cases, identifying 2 IgA κ , 3 IgA λ , and 4 IgG κ clones^[33].

Solitary plasmacytoma

Focal infiltration by monoclonal plasma cells in the absence of systemic disease is observed when solitary plasmacytomas are formed. They represent 3%-5% of PCD; they may arise from bone or be extramedullary, extra osseous.

Bone solitary plasmacytomas present an increased tendency to evolve to MM; sFLC measurements help monitoring these patients and increased sFLCR was shown to constitute an independent risk prognostic factor of evolution^[34,35].

Waldenstroms' macroglobulinemia

Waldenstroms' macroglobulinemia (WM) is a lymphoplasmacytic lymphoma characterized by the presence of a serum IgM monoclonal component. The disease is rare and presents a wide range of clinical manifestations including fatigue, hyperviscosity symptoms, lymphadenopathy, organomegaly, peripheral neuropathy and other. Asymptomatic patients do not require treatment and usually enjoy a prolonged survival, while patients with aggressive symptomatic disease should be immediately treated with chemotherapy.

There are so far only preliminary results on the contribution of sFLC and HLC levels in WM patients at diagnosis. It was shown that sFLC may be increased and, in such case, correlate with markers of disease activity, such as increased β 2M, anemia^[36] and low serum albumin levels. Patients with elevated sFLC presented shorter time to treatment^[37] and adverse outcome^[38]. Increased HLC-IgM were also found correlated with markers of disease activity such as BM infiltration of more than 50% and low serum albumin levels while high HLCR correlated with shorter time to treatment^[39].

Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in the Western world and presents a variable outcome. More than two third of the patients are asymptomatic at the time of diagnosis and may not require treatment for months and even years. Currently available disease prognostic markers, including Rai and Binet staging systems and underlying molecular alterations do not apply perfectly for asymptomatic patients and other prognostic tools are under investigation. For the majority of CLL patients, life expectancy largely depends on time to first treatment^[40].

It was shown that increased sFLC are observed in almost half of CLL cases, and that sFLCR abnormalities are present in a significant proportion of patients and identify those at risk of progressive disease^[41,42].

More recently, increased polyclonal sFLC were also found to constitute an adverse marker for time to first treatment in CLL^[43]. This finding was confirmed by Morabito *et al*^[44] that evaluated the sum of absolute κ and λ sFLC and found that the prognostic impact of sFLC ($\kappa + \lambda$) value above 60.6 mg/mL was superior compared to FLCR; thus, a model, based on four variables, namely sFLC ($\kappa + \lambda$) more than 60.6 g/L, Binet staging, ZAP-70, and cytogenetics was built and separated 4 patients' groups with different time to treatment.

B-cell lymphomas

sFLC may potentially be increased in any B-cell lympho-

mas (B-NHL). In a series of 208 patients with various B-NHL, sFLC were found increased in 13% (26/202)^[45].

Increased sFLC has been shown associated with adverse outcome in patients with diffuse large B-cell lymphoma^[46] and predictive of increased risk of non-Hodgkin's lymphoma development in human immunodeficiency virus infected patients^[47]. The contribution of sFLC measurements is currently being investigated in other B-cell NHL also^[48].

CONCLUSION

In all malignant B-cell disorders and especially in true PCD, FLC measurements help patients' accurate diagnosis, prognosis and monitoring. Ongoing studies on the contribution of the more recent HLC assays, show that they will most probably acquire the same importance.

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Emerging immunological concepts in the pathogenesis of myelodysplastic syndromes

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Abstract

The involvement of T-lymphocytes in the pathogenesis of myelodysplastic syndromes (MDS) is now well documented by relevant clinical and experimental findings. This brief review will focus on the T-cell repertoire pattern typical of MDS patients as well as on the potential role exerted by specific T-cell subsets in this context. Future investigations should further explore the specific role played by different T-cell subsets in the bone marrow milieu typical of MDS, further clarifying which of the described changes represent either an epiphenomenon or rather a real causative factor in the pathogenesis of these disorders.

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Key words: Myelodysplastic syndromes; T-cells; T-cell receptor repertoire; Regulatory T cells; Immunotherapy

Core tip: T-lymphocytes are deeply involved in the pathogenesis of myelodysplastic syndromes (MDS); patients with MDS display a typical T-cell repertoire pattern; specific T-cell subsets, such as regulatory T-cells and Th17 T-cells, play a specific role in this context.

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INTRODUCTION

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal haematologic diseases, characterized by dysplastic haemopoiesis and by a variable degree of peripheral cytopenia. Although their pathogenesis is dominated by recurrent molecular, cytogenetic and epigenetic defects, also immune abnormalities have been often advocated in this scenario. In fact, specific laboratory and clinical immune manifestations have been described in a large percentage of patients^[1] and, starting from the crucial demonstration that erythroid precursors of MDS patients can be inhibited *in vitro* by autologous T-cells^[2], a number of experimental data underline the possible involvement of different T-cell subpopulations in the MDS pathogenesis^[3,4]. This hypothesis has been essentially addressed by studies investigating either the profile of the T-cell receptor (TCR) repertoire, especially within the third complementarity determining region (CDR3), or the potential role of specific T-cell subsets such as for instance regulatory T-cells (Treg) and CD3⁺ CD4⁺ IL-17 producing (Th17) T-cells.

PATHOGENESIS OF MDS

The analysis of the TCR repertoire has now shown to offer relevant insights in a variety of haematologic malignancies^[5]. Studies focussing on MDS patients have essentially demonstrated an increase in the frequency of expanded lymphocyte subpopulations expressing homogeneous TCR repertoire profiles, which tend to assume an oligoclonal pattern especially in CD8⁺ cells^[6,7]. Among the different techniques, the so called spectratyping, which has identified specific CDR3 length distributions in different immune-mediated conditions^[8,9], has been the most widely

applied. The potential functional role of these lymphocyte expansions has been specifically addressed by a study showing that expanded CD8⁺ T-cells selected by MDS patients were able to inhibit the cell growth of a dysplastic clone harbouring trisomy 8 in co-culture^[10].

Considering their subtle ability to influence both autoimmunity and tumour progression, Treg have been strongly investigated in MDS patients. Noteworthy their frequency was correlated with several indicators of disease activity, such as bone marrow blast infiltration, high International Prognostic Scoring System score and history of progression^[11]. Moreover, they were also shown to display a polyclonal CDR3 profile, as already observed in the post-allograft setting^[12], and to belong to the naive subset, especially in high-risk patients^[11]. Even more importantly significant differences were shown between low and high risk disease. In fact, Treg were shown to be impaired in their function and bone marrow homing in early stage MDS, whereas they retained their function and were expanded in late stage disease. These findings suggest that an impairment of Treg suppressive function and bone marrow trafficking could be involved in the autoimmune mechanisms typical of early stage MDS. On the contrary, the increased Treg activity observed in higher risk patients could be the expression of an impairment of anti-tumour immunity, potentially facilitating the progression towards a more aggressive disease^[13]. More recently, a flow-cytometric approach based on the concomitant expression of CD25 and CD127, was able to show also in low risk MDS patients an increased frequency of Treg, pointing at a potential impairment of anti-tumor immunity even in the early stage of these disorders^[14]. On the whole, these data could imply the involvement of Treg in the modulation of anti-tumour immunity and, consequently, of MDS progression.

Other T-cell subsets have been explored in this context, among which CD4⁺ CD8⁺ double-positive T-cells, a subset of differentiated effector memory cells with potential antiviral and immune modulating functions, showing an abnormal distribution in MDS patients, especially in those with more advanced disease^[15]. Much more importantly, Th17 cells appeared to be over-represented in low risk compared with high risk MDS, being its frequency inversely correlated with that of Treg. Noteworthy, the increased Th17/Treg ratio observed in low risk patients was shown to be associated with increased bone marrow apoptosis, thus potentially explaining the increased risk of autoimmunity and the better response to immunosuppressants observed in this patient subgroup^[16].

Also natural killer (NK) cells were demonstrated to display functional defects in MDS patients, even more pronounced in patients with higher risk disease^[17]. More importantly, NK cells were shown to modulate cytotoxicity against dysplastic hematopoietic precursors^[18]. Also myeloid derived suppressor cells, which play a potential role in regulating immune tolerance even in the context of neoplastic disorders^[19], would deserve to be explored in MDS patients.

All these laboratory findings have been corroborated by relevant therapeutic experiences, starting from the well known response to antithymocyte globulin described in a relevant fraction of MDS patients^[20], which is also paralleled by specific immunological effects, such as loss of the lymphocyte-mediated inhibition of colony forming unit-granulocyte macrophage and alterations in the TCR repertoire profile^[21]. A number of other immunomodulating agents, among which thalidomide, infliximab, SCIO-469-a p38 α -mitogen-activated protein kinase inhibitor and cyclosporin A^[22], have shown different degree of efficacy when offered to MDS patients. Also therapeutic strategies based on more complex immunological approaches have shown a potential benefit in MDS patients, among which vaccination programs exploiting the immunogenicity of Wilms tumor gene product 1-peptide^[23,24] as well atransplantation strategies based on reduced intensity or non myeloablative conditioning regimens, which typically rely on immune tolerance modulation^[25].

CONCLUSION

Even though a number of clinical and laboratory findings point at the central role of molecular defects in the MDS biology, all the above mentioned data highlight the relevant involvement of different immunological players, among which undoubtedly T-lymphocytes. Future investigations should further explore the specific role played by different T-cell subsets in the bone marrow milieu typical of MDS, further clarifying which of the described changes represent either an epiphenomenon or rather a real causative factor in the pathogenesis of these disorders.

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Circulating microparticles and microRNAs as players in atherosclerosis

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est in assessing the feasibility of therapeutic regulation of miRNAs to manipulate them with a special focus on cardiovascular disease. We speculate on the future developments of MPs which contain miRNA as new therapeutic targets for proliferative vascular diseases such as atherosclerosis.

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Key words: Microparticles; MicroRNA; Atherosclerosis

Core tip: Circulating microparticles (MPs) and microRNAs (miRNA) play an important role in atherosclerotic disease. MPs contain the major source of systemic RNA including microRNA of which aberrant expression appears to be associated with stage and progression of atherosclerosis. We speculate on the future developments of MPs which contain miRNA as new therapeutic targets for proliferative vascular diseases such as atherosclerosis.

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Abstract

Microparticles (MPs) are extracellular membrane vesicles released from normal, apoptotic and pathological cells following a process of detachment from cells of origin. MPs are typically defined by their size, exposure of phosphatidylserine, the expression of surface antigens, proteins and genetic material, originating from their donor cells, and as important vehicles of intercellular communication across numerous biological processes. MPs contain the major source of systemic RNA including microRNA (miRNA) of which aberrant expression appears to be associated with stage and progression of atherosclerosis. The involvement and influence of miRNA during the onset and progression of atherosclerotic disease have generated a lot of inter-

ATHEROSCLEROSIS

Atherosclerosis is one of the most important and common cause of death and disability in the world and remains an occult but important precursor of significant cardiovascular events. Yet atherosclerosis is a systemic disease with important consequences in many other regional circulations, including those supplying the brain, kidneys, mesentery, and limbs. Atherosclerosis as a chronic inflammatory disease of the arterial wall is closely related to subendothelial lipoprotein retention, endothelial activation and migration of the immune cells to the inflamed intima

which result in formation of fatty streaks and subsequent atheromas^[1,2]. Monocytes are prominently involved in initiation, progression and complication of the atherosclerotic lesions. They enter atherosclerotic plaque and transform to macrophage-like, lipid-loaded foam cells^[3]. Also, it is now increasingly admitted that cell-derived microparticles (MPs) may contribute to the initiation, progression and clinical complications of cardiovascular diseases and they appear interesting biomarkers to predict this pathology^[4]. In addition, the ratio of circulating MPs to endothelial progenitor cells may be a new and valuable cellular marker of vascular dysfunction in atherosclerosis^[5].

MP

MPs were described as small (0.1-1 μm), pro-inflammatory vesicles released by various cell types (*e.g.*, leucocytes, endothelial cells, platelets, monocytes) in a tightly regulated process. MP vesiculation occurs as a cellular response to various physiological conditions including: apoptosis, senescence, cellular activation, shearing stress and biochemical triggers (such as cytokines and chemotherapeutics)^[6]. They contain cytoplasm and surface markers of their cells of origin^[7,8]. Once released into the circulation, MPs bind and fuse with their target cells through receptor-ligand interactions, thereby acting as biological vectors mediating vascular inflammation and coagulation^[9]. Therefore, MPs have been shown to play a fundamental role in several cardiovascular diseases^[10,11]. Increasing evidence indicates that inflammatory and pro-coagulatory effects of MPs on their target cells are caused by a specific lipid composition (*e.g.*, byphosphatidylserine) as well as by the transfer of inflammatory cell components from their cells of origin^[7]. However, it was also shown that MPs transport messenger RNAs (mRNAs) thereby affecting protein expression of their target cells^[12]. Recent progress in the understanding of microRNA (miRNAs) has prompted the questions of whether MPs also affect their target cells *via* transferring endogenous miRNAs and whether these MPs have different miRNA patterns than their maternal cells.

miRNA

miRNAs are a class of small (about 22 nucleotides long), non-coding RNA that bind to mRNAs thereby acting as endogenous post-translational gene regulators^[13]. Since more than 1000 different human miRNAs have already been discovered, the interaction between miRNAs and mRNAs is highly complex and currently not completely understood. miRNAs are potent and crucial regulators of important cellular processes such as differentiation, growth, and survival. miRNAs regulate gene expression through binding to 3' UTRs of target mRNAs whereby inducing either messenger RNA degradation or inhibition of protein translation. However, approximately one-third of human protein-encoding genes are miRNAs regulated, underlining the extraordinary impact of miRNAs on protein expression^[14,15].

Recent data indicate that miRNAs play a vital role in many cardiovascular diseases and can be found in cardiac tissue as well as in circulating blood, opening the possibility to use them as diagnostic surrogate markers^[16-19]. They were reported alterations in the expression of specific miRNAs in human atherosclerotic plaques which suggest that miRNAs may have an important role in regulating the evolution of atherosclerotic plaque toward instability and rupture^[20,21]. Bidzhekov *et al.*^[22] identified miRNAs co-expressed in plaque tissue and classical monocytes (miR-99b, miR-152), or non-classical monocytes (miR-422a) which serve as therapeutic targets for treating inflammatory vascular diseases. Also, miR-126 is highly expressed in the heart and vasculature of zebrafish^[23] and miR-221 positively regulates smooth muscle proliferation and neointimal formation^[24,25]. The effects of miR-221 are strengthened by the concurrent upregulation of miR-21 in neointimal lesions^[26]. miR-21, which is a ubiquitously expressed prosurvival miRNA, was shown to inhibit PTEN expression under these conditions^[26]. Other miRNAs that may also contribute to vessel formation include miR-130a, which is upregulated in endothelial cells during tubulogenesis and facilitates the process by targeting and inhibiting the expression of antiangiogenic transcription factors GAX and HOXA5^[27]. In contrast, overexpression of miR-92a in endothelial cells blocked tubulogenesis and was associated with reduced cell migration and adhesion, but did not affect viability or proliferation^[28]. On the other hand miRNA-145 has been studied for its therapeutic properties in atherosclerotic disease. It was found that miRNA is abundant in arteries^[26] and in differentiated vascular smooth muscle cells^[29].

CONCLUSION

Furthermore, it was promoted the idea that MPs represent transport vehicles for a large number of specific miRNAs in circulation^[30,31]. Moreover, it was showed that embryonic stem cell-derived MPs contain abundant miRNAs and that they can transfer a subset of miRNAs to mouse embryonic fibroblast *in vitro*^[32]. Additionally, MPs released from mesenchymal stem cells protect from acute kidney injury induced by ischaemia reperfusion injury and from subsequent chronic renal damage^[33]. Other studies demonstrated that the expression of miRNAs and RNAs in secreted vesicles does not necessarily reflect the intracellular expression of RNAs^[34,35]. Hergenreider *et al.*^[36] suggest that extracellular vesicles from Krüppel-like factor 2 (KLF2)-transduced cells contain a specific combination of miRNAs, including miR-143/145, which mediate the biological properties. Thus, vesicles from KLF2-transduced endothelial cells, but not from controls, reduce atherosclerotic lesion formation *in vivo*, in a miR-143/145-dependent manner and they may provide a promising strategy to combat atherosclerosis. Also, stem cell-derived MPs appear to be naturally equipped to mediate tissue regeneration under certain conditions^[37]. Therefore, the extracellular vesicles are considered potent sources of genetic information transfer between mammalian cells and

tissues resulting in both beneficial (cell communication, stem cell plasticity and repair of injured tissues) and potentially detrimental (spread of disease) outcomes^[37].

These data open new research perspectives on the use of MPs to transfer miRNAs-based information from stem cells/precursors/cells to target differentiated cells. Further studies on MPs biology and function may help elucidate the exact role that MPs as gene therapy tools.

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Aspirin responsive platelet thrombophilia in essential thrombocythemia and polycythemia vera

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Abstract

Essential thrombocythemia (ET) and polycythemia vera (PV) frequently present with erythromelalgia and acrocyanotic complications, migraine-like microvascular

cerebral and ocular transient ischemic attacks (MIAs) and/or acute coronary disease. The spectrum of MIAs in ET range from poorly localized symptoms of transient unsteadiness, dysarthria and scintillating scotoma to focal symptoms of transient monocular blindness, transient mono- or hemiparesis or both. The attacks all have a sudden onset, occur sequentially rather than simultaneously, last for a few seconds to several minutes and are usually associated with a dull, pulsatile or migraine-like headache. Increased hematocrit and blood viscosity in PV patients aggravate the microvascular ischemic syndrome of thrombocythemia to major arterial and venous thrombotic complications. Phlebotomy to correct hematocrit to normal in PV significantly reduces major arterial and venous thrombotic complications, but fails to prevent the platelet-mediated erythromelalgia and MIAs. Complete long-term relief of the erythromelalgic microvascular disturbances, MIAs and major thrombosis in ET and PV patients can be obtained with low dose aspirin and platelet reduction to normal, but not with anticoagulation. Skin punch biopsies from the erythromelalgic area show fibromuscular intimal proliferation of arterioles complicated by occlusive platelet-rich thrombi leading to acrocyanotic ischemia. Symptomatic ET patients with erythromelalgic microvascular disturbances have shortened platelet survival, increased platelet activation markers β -thromboglobulin (β -TG), platelet factor 4 (PF4) and thrombomodulin (TM), increased urinary thromboxane B2 (TXB2) excretion, and no activation of the coagulation markers thrombin fragments F1+2 and fibrin degradation products. Inhibition of platelet cyclooxygenase (COX1) by aspirin is followed by the disappearance and no recurrence of microvascular disturbances, increase in platelet number, correction of the shortened platelet survival times to normal, and reduction of increased plasma levels of β -TG, PF4, TM and urinary TXB2 excretion to normal. These results indicate that platelet-mediated fibromuscular intimal proliferation and platelet-rich thrombi in the peripheral, cerebral and coronary end-arterial microvasculature are responsible for the erythromelalgic ischemic complica-

tions, MIAs and splanchnic vein thrombosis. Baseline platelet P-selectin levels and arachidonic acid induced COX1 mediated platelet activation showed a highly significant increase of platelet P-selectin expression (not seen in ADP and collagen stimulated platelets), which was significantly higher in JAK2^{V617F} mutated compared to JAK2 wild type ET.

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Key words: Erythromelalgia; Migraine-like cerebral transient ischemic attacks; Platelets; β -thromboglobulin; Thrombomodulin; Thrombosis; Aspirin; Anticoagulation; Arterial platelet thrombophilia; Essential thrombocythemia; Polycythemia vera

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INTRODUCTION

Dameshek *et al*^[1] observed specific clinical features at the time of presentation in 20 newly diagnosed polycythemia vera (PV) patients seen between 1928 and 1937, 75 years ago. The spectrum of clinical manifestations were headache, usually quite severe, in 17; attacks of migraine in 3; vertigo in 14; visual disturbances, particularly spots before the eyes and colored scotomas in 6; paresthesias, numbing and tingling in toes and fingers in 12; circulatory disturbances of the legs, feet or hands in 6; and various types of major thromboses (cerebral, coronary, venous, arterial) in 9 cases. Many cases were never correctly diagnosed but masqueraded under the diagnosis of various types of peripheral vascular disease, including thromboangiitis obliterans (Burger's disease), atypical erythromelalgia, hypertension, symptoms referable to several organ systems or atypical symptoms difficult to classify. Since the sensations of pain, warmth and tingling of the legs are common to both polycythemia and the rather poorly defined disorder of erythromelalgia (Brown^[2]), the possibility of polycythemia should be suspected when the diagnosis of erythromelalgia is made or suspected. Dameshek *et al*^[1] noted that the lack of large vessel involvement in PV and the extremely high platelet counts suggested the possibility of "platelet polycythemia" or "thrombophilia" with multiple small peripheral vascular thromboses.

Aspirin sensitive erythromelalgia is characterized by warm, red, congested extremities and painful burning sensations and causally linked to thrombocythemia in various myeloproliferative disorders (MPD), typically occur in patients with essential thrombocythemia (ET) and PV and has never been observed in patients with reactive thrombocytosis^[2-6]. Acroparesthesias characterized by

tingling, pins and needles sensations and numbness in the toes or fingers usually precede the disabling red, swollen, burning distress. Warmth intensifies the discomfort and cold provides relief^[5]. The burning distress is always associated with local warmth, swelling with mottled redness and blue spots. Intense burning, throbbing and aching with peeling of the skin of the affected toes or fingers that become cold, black and ischemic come at a more advanced stage^[7-12]. If left untreated, digital acrocyanotic ischemia and gangrene of toes and fingertips do occur. Smith *et al*^[3] first described that aspirin promptly relieved erythromelalgic pain for approximately 3 d. This long-lasting effect of a single dose of aspirin is so specific for erythromelalgia that it can be used as a diagnostic criterion^[3-6]. If left untreated, erythromelalgia usually leads to painful acrocyanosis and gangrene of one or more toes or tips of the finger^[4,12]. When erythromelalgia progresses to ischemic acrocyanosis or gangrene of a toe or fingertip, the peripheral arterial pulsations are usually normal. Anticoagulation with coumarin in cases with erythromelalgia complicated by digital necrosis had no therapeutic effect, whereas subsequent treatment with aspirin 500 mg/d not only relieved the erythromelalgic pain, but also markedly improved the digital ischemic circulation disturbances (thromboangiitis obliterans)^[4,5].

The long-lasting relief of erythromelalgia for several days in ET and PV patients by one dose of aspirin 500 mg is correlated with inhibition of ADP, epinephrine or collagen induced platelet aggregation that persists for several days. This is the result of irreversible inhibition of platelet cyclooxygenase (COX1), as can be measured by the malondialdehyde (MDA) in platelet rich plasma (PRP)^[5,6,13-19]. N-ethylmaleimide added to PRP maximizes the arachidonic COX1 pathway to form unstable prostaglandin endoperoxides intermediates, thromboxane A2 (TXA2) and its stable end products, thromboxane B2 (TXB2) and MDA. The analgesics sodium salicylate, glafenin, paracetamol and acetaminophen, as well as the platelet inhibiting agents dipyridamole and sulfinpyrazone, in adequate dosages for at least 1 wk had no appreciable effect on erythromelalgia or platelet COX1 activity as measured by MDA production in PRP^[4-6]. Erythromelalgia in ET and PV is caused by platelet mediated arteriolar thrombosis and inflammation that can only be relieved by selective inhibition of platelet COX1^[4,5]. This platelet-mediated thrombotic disease (platelet thrombophilia) in the end-arterial circulation of thrombocythemia (ET and PV) patients, first described by Michiels *et al*^[5], is confirmed by the Rotterdam MPD Study Group in a series of prospective histopathological, platelet kinetic, experimental and aspirin intervention studies in symptomatic and asymptomatic ET patients^[20-36].

HISTOPATHOLOGY OF ERYTHROMELALGIA IN THROMBOCYTHEMIA

Very little was known about the histopathology of eryth-

Table 1 A Platelet kinetic, hemostatic and thromboxane B2 studies in normal individuals (control), asymptomatic essential thrombocythemia patients (E-), essential thrombocythemia patients suffering from erythromelalgia (E+) and the effect of acetylsalicylic acid (aspirin) in E+ patients

Study population	Control healthy	ET		
		E-	E+	E+ treated
Aspirin treatment	No	No	No	Yes→E
Platelet kinetic study ^[22]				
Patients (n)	6	10	10	7
Platelet survival (d)	8.0 ± 0.4 ¹	6.6 ± 0.3 ¹	4.2 ± 0.4 ¹	6.9 ± 0.4
Hemostatic studies ^[24]				
Patients (n)	20	16	5	5
Platelet count (× 10 ⁹ /L)	256 ± 10	671 ± 66	689 ± 105 ¹	857 ± 52
Platelet activation markers				
Thrombomodulin (ng/mL)	40 ± 2.1	73 ± 4 ¹	90 ± 10 ¹	64 ± 12
PF4 (IU/10 ⁸ plts)	1.6 ± 0.1 ¹	2.9 ± 0.5 ¹	9.1 ± 5.0 ¹	4.3 ± 3.3
β-TG (IU/10 ⁸ plts)	16 ± 1.2	37 ± 6.6	128 ± 33	29 ± 15
Coagulation activation markers				
F1+2 (nmol/L)	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.4	1.1 ± 0.3
FDP (ng/mL)	669 ± 31	707 ± 51	702 ± 83	-

¹Significant difference. Data are expressed as mean ± SE. PF4: Platelet factor 4; β-TG: β-thromboglobulin; F1+2: Prothrombin fragment 1 + 2; FDP: Fibrin degradation products; ET: Essential thrombocythemia; E+: Erythromelalgia present; E-: Erythromelalgia absent. Originated from^[22,24].

romelalgia in thrombocythemia as the opportunity to examine tissue from the involved extremities was not available in the literature. From 1977 to 1981, Michiels^[4] (Departments of Hematology and Pathology, Erasmus University, Rotterdam) performed skin punch biopsies from erythromelalgic areas in thrombocythemia patients. Histopathological studies from skin areas of erythromelalgia in ET and PV patients revealed characterization by specific arteriolar vascular changes in the reticular dermis, usually without involvement of venules, capillaries or nerves^[4,5,20,21]. In recently relapsed erythromelalgia, endothelial cells are swollen with large nuclei and the vessel lumen is narrowed by proliferation of smooth muscle cells with vacuolization and swelling of the cytoplasm and deposits of intracellular material^[4,5,20]. The internal elastic lamina appears to be split between proliferated smooth muscle cells, giving rise to the appearance of fibromuscular intimal proliferation^[20]. The arterioles with proliferative thickening of their walls may be occluded by fresh thrombi if left untreated. Ultimately, the arterioles may become completely fibrosed. Specific immunohistochemical staining of skin biopsies from recently relapsed erythromelalgic cyanotic skin areas showed fresh thrombi occluding these arterioles, which stained strongly for von Willebrand factor (vWF), opposed to only a weak staining for fibrin, indicating a platelet-rich thrombus (Michiels *et al.*^[4,5], van Genderen *et al.*^[24], Figure 1). Michiels^[4] conceptualized that the symptoms of platelet-mediated erythromelalgia, including migraine-like cerebral ischemic attacks and visual disturbances, are the result of platelet activation and aggregation *in vivo*, which preferentially takes place in the arterioles^[5,20]. The high shear rate of the blood flow in the end-arterial circulation very likely contributes to this localization of platelet mediated arteriolar thrombosis and inflammation in thrombocythemia^[4,5,20]. In this process of intravascular platelet activation and aggregation, vasoactive

substances like prostaglandins and other factors released by activated and leukocytes account for the inflammatory symptoms and platelet derived growth factor (PDGF) for the fibromuscular intimal proliferation of arterioles in erythromelalgic areas (Figures 2 and 3). Intravascular platelet aggregates or thrombi clearly lead to acrocyanotic microvascular occlusions. If left untreated, erythromelalgia will lead to irreversible end arterial occlusion and gangrene of toes (diagnosed as thromboangiitis obliterans by Dameshek *et al.*^[1]) or fingertips (Figure 3).

INVOLVEMENT OF PLATELETS AS THE CAUSE OF ERYTHROMELALGIA IN ET

To further demonstrate the involvement of platelets in the pathogenesis of erythromelalgic acrocyanosis of ET patients, we performed prospective platelet kinetic studies using Cr-labeled autologous platelets in 10 asymptomatic and in 10 symptomatic thrombocythemia patients complicated by erythromelalgia and in a control group of 6 cases of reactive thrombocytosis (Table 1)^[22]. Platelet survival times were significantly shortened (4.2 ± 0.4 d) in symptomatic thrombocythemia complicated by erythromelalgia compared with asymptomatic thrombocythemia patients (6.6 ± 0.3 d, *P* < 0.001) and patients with reactive thrombocytosis (8.0 ± 0.4 d, *P* < 0.001). After treatment of erythromelalgia with aspirin 500 mg per day in 7 symptomatic thrombocythemia patients, platelet survival increased significantly from 4.0 ± 0.3 to 6.9 ± 0.4 d (*P* < 0.001), thereby causing a significant rise in the platelet count of about 200 × 10⁹/L in each of the aspirin treated thrombocythemia patients (Table 1)^[22]. Coumadin failed to improve platelet survival or symptoms of erythromelalgia in 2 thrombocythemia patients^[22]. This clearly documents the active involvement and causative role of platelets in the etiology of erythromelalgia

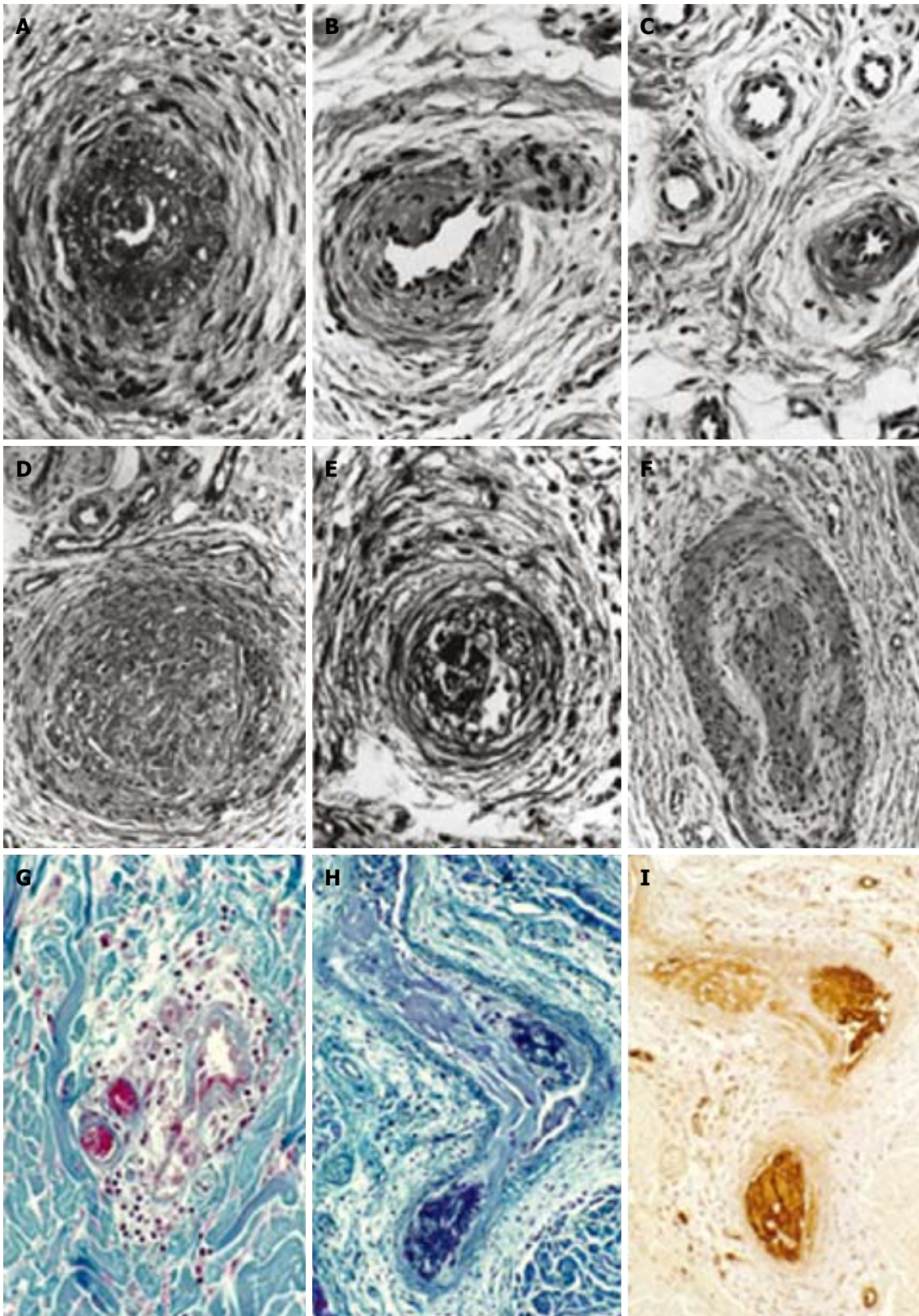


Figure 1 The histopathological appearances of a skin punch biopsy from areas of recently relapsed erythromelalgia 1 wk after aspirin discontinuation arteriolar I fibromuscular intimal proliferation, swollen endothelial cells, slight intravascular and perivascular infiltration with inflammatory cells, some perivascular fibrosis and no occlusive arteriolar thrombi. The histopathological appearances from relapsed erythromelalgia complicated by blue ischemic spots 3 wk after aspirin discontinuation show arterioles with pronounced fibromuscular intimal proliferation without occlusive thrombi (A-C) and fibromuscular intimal proliferation with occlusive thrombotic arteriolar lesions (D-F) perivascular oedema, vascular and perivascular fibrosis and infiltration by inflammatory cells (thromboangiitis obliterans). Originated from Michiels *et al*^[20]. Biopsy from red-bluish discolored erythromelalgic skin area showing arteriolarthrombi with a weak fibrin staining (H) as compared to a positive fibrin staining from a patient with primary antiphospholipid syndrome and recurrent arterial skin thromboses (G). The occlusive arteriolar thrombi revealed a strong staining for von Willebrand factor (I) indicating platelet rich thrombi. This documents that thrombi from skin areas of erythromelalgia complicated by acrocyanosis are indeed platelet-rich and fibrin-poor thrombi. Originated from van Genderen *et al*^[24].

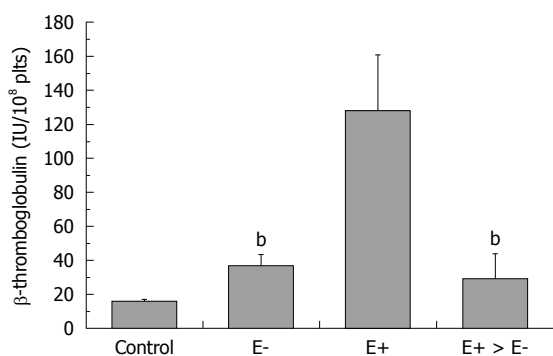


Figure 2 β -thromboglobulin levels in healthy control, and in essential thrombocythemia (E) either asymptomatic (E-), complicated by erythromelalgia (E+) and after treatment of E+ with aspirin (E+ $\rightarrow$ E-)^[24]. ^b $P < 0.01$, significant differences between E- and E+ $\rightarrow$ E- as compared to E+.

[and very likely also transient ischemic attacks (TIAs)] as a pathognomonic microvascular thrombosis (platelet thrombophilia) in ET and PV patients (Figures 1 and 3).

Bellucci *et al.*^[23] showed that increased levels of plasma β -thromboglobulin (β -TG) in 16 asymptomatic ET persisted if the platelet count normalized by cytostatic reduction of the platelet count. In this study, increased β -TG levels were interpreted as platelet activation and endothelial cell damage may be present since thrombomodulin (TM) levels tended to be increased in ET patients^[23]. van Genderen *et al.*^[24] performed a prospective intervention study and measured plasma β -TG and platelet factor 4 (PF4) as markers of platelet activation, TM as a marker for endothelial cell activation, and activation markers of coagulation including prothrombin fragments (F1+2) and fibrin degradation products in controls, asymptomatic ET and symptomatic ET complicated by erythromelalgia. Compared to 20 controls, PF4 and β -TG levels were significant higher in 16 asymptomatic ET patients (Table 1, Figure 2). Compared with 16 asymptomatic ET patients and 20 control subjects, erythromelalgia in 5 symptomatic ET patients was characterized by significant and much higher β -TG and TM levels, but no significant differences were detected in either F1+2 or FDP levels (Table 1, Figure 2)^[24]. Treatment of erythromelalgia with aspirin (500 mg for at least 7 d) resulted in the disappearance of erythromelalgic signs and symptoms, which was paralleled by a significant increase in platelet count and a significant decrease in β -TG and TM levels (Table 1, Figure 2)^[24]. From the increase in plasma levels of β -TG and TM and its correction by aspirin, we concluded that increased interactions of activated hypersensitive platelets and endothelial cells do occur as the initiating process of fibromuscular intimal proliferation seen in the very early stage of platelet-mediated inflammatory symptoms of erythromelalgia (Figures 1 and 3). The generation of thrombin appears not to be essential for the etiology and subsequent formation of platelet-rich thrombi in erythromelalgic acrocyanosis (platelet thrombophilia), thereby giving a plausible explanation for the inefficacy of coumadin-derivatives (vitamin K antagonists) and heparin in

the prevention and treatment of erythromelalgia in ET and PV patients.

In a collaborative study, Wehmeier, Schroer and Michiels (Duesseldorf-Rotterdam) prospectively investigated the effect of anagrelide on clinical symptoms and *in vivo* platelet activation, as measured by plasma levels of TXB2 and PDGF in 17 ET patients, while on no treatment and after correction of platelet count by anagrelide treatment (unpublished data 2000). The mean value of plasma PDGF levels in 17 untreated ET patients (4.8 ng/mL) was significantly higher compared to normal controls (2.77 ng/mL, range 0.99-4.66 ng/mL, $P = 0.0005$) and returned to normal (2.13 ng/mL) during anagrelide treatment. Twelve of 17 (70%) untreated ET patients had plasma PDGF levels above the upper limit of normal. The plasma PDGF level was still above normal in 1 case of ET during anagrelide treatment. The mean value of plasma PDGF expressed in ng per 10⁵ platelets (ng/10⁵ pL) was significantly lower in the untreated ET patients (0.48 ng/10⁵ pL) compared to normal controls (0.92 ng/10⁵ pL) and increased to a level (0.60 ng/10⁵ pL) during anagrelide treatment, which is not significantly different from controls. The increased plasma level and decreased platelet content of PDGF may indicate that circulating activated platelets in untreated ET had released some of their PDGF content^[5,20]. The mean value of plasma TXB2 levels in 17 untreated ET patients was 22-fold higher (13.8 ng/mL) and significantly increased compared to normal controls (0.63 ng/mL, range 0.34-0.97 ng/mL, $P = 0.0002$). This very high mean value of plasma TXB2 significantly decreased to 3.2 ng/mL ($P = 0.004$) during anagrelide treatment, which is still significantly above the normal values of 0.63 ng/mL ($P = 0.04$). The mean value of plasma TXB2 expressed in ng per 10⁵ platelets (ng/10⁵ pL) was 4-fold and significantly higher (1.0 ng/10⁵ pL) in the untreated ET patients compared to normal controls (0.25 ng/10⁵ pL, $P = 0.04$). The mean value of plasma TXB2 (0.86 ng/10⁵ pL) was significantly lower in 17 ET patients during anagrelide treatment compared to the period of no treatment, but still remained 3.4-fold and significantly higher compared to normal controls (0.25 ng/10⁵ pL, $P = 0.008$).

Unequivocal convincing evidence for platelet activation *in vivo* during erythromelalgic attacks is provided by measuring platelet-specific proteins or stable degradation products of platelet-derived TXB2 excretion in urine^[25-28]. The endogenous synthesis of TXA2 by platelets, estimated by the measurement of their main stable TXB2 metabolites in urine, was found to be increased in ET and PV patients^[29,30]. The increased biosynthesis of platelet derived TXB2, as measured by urinary excretion of TXB2 in ET and PV patients, may reflect spontaneous platelet activation *in vivo*. As the erythromelalgic microvascular thrombotic predisposition in ET is the consequence of the thromboxane-mediated routes of platelet activation^[31], a prospective intervention study was designed to demonstrate whether increased urinary TXB2 excretion as a

Table 2 Clinical manifestations and sequence of aspirin-responsive microvascular cerebral ischemic attacks in each of 17 patients with essential thrombocythemia

	Patient number																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Thrombotic events sequence	MI CS	VS E	CS VS E	VS CS	MI VS CS AP	VS E	CS AP	CS VS	CS VS	E VS CS E MI	CS AP	CS E	E CS	CS E	CS E	VS CS	CS E
Time lapse of sequential thrombotic events (yr)	2	5	2	3	5	4	1	3	1	7	4	3	2	1	3	1	4
Cerebral and visual symptoms																	
Transient hemiparesis	+				+						+	+		+	+	+	
Unstable gait	+		+	+	+		+	+	+	+	+	+		+		+	+
Dysarthria			+		+		+		+			+	+	+			+
Transient monocular blindness		+							+							+	
Scintillating scotomata		+		+	+	+											
Blurred vision		+	+	+	+	+		+	+	+						+	+
Headache	+	+	+	+	+	+		+	+					+	+	+	
Number of attacks	S	M	S	S	M	S	S	F	S	F	S	S	F	S	F	M	S

Because of ignorance of a causal relationship between microvascular cerebral ischemic attacks and essential thrombocythemia by the referring doctors, the time lapses between sequential microthrombotic events were prolonged ranging from 1 to 7 years. MI: Myocardial infarction; CS: Cerebral symptoms; VS: Visual symptoms; AP: Angina pectoris; E: Erythromelalgia. Number of attacks: F: Few; S: Several 4-10; M: More than 10. Source Michiels *et al*^[31].

Table 3 Incidence of thrombotic and bleeding complications in the prospective 1975-1996 Rotterdam study of 68 essential thrombocythemia patients during a median follow-up of 6.7 years according to treatment strategy *n* (events/100 persons per year)

Treatment strategy	Duration of follow-up (person/yr)	Thrombotic complication	Bleeding complications
Asymptomatic (14 patients)			
Watchful waiting	127	27 (33.3)	2 (1.6)
Symptomatic (54 patients)			
Low dose aspirin	139	5 (3.6)	10 (7.2)
Platelet reduction	113	10 (8.9)	2 (1.8)
Low dose aspirin + platelet reduction	40	0 (0)	4 (10)
Total	419	42 (100)	18 (100)

Originated from^[61,71].

reflection of increased platelet thromboxane formation precedes the occurrence of microvascular thrombosis in ET patients complicated by erythromelalgia after discontinuation of aspirin^[32]. Within 10 d after discontinuation of aspirin, 3 ET patients developed arterial microvascular thrombosis of the extremities (erythromelalgia), which was preceded by a 3 to 30 fold increase in urinary TXB2 end products excretion (1100 to 15000 pg/mg creatinine) compared with 3 ET patients (100 to 800 pg/mg creatinine), who remained asymptomatic after discontinuation of aspirin^[32]. The increased urinary TXB2 end products excretion and the clinical signs of erythromelalgia could be inhibited to normal (50 to 300 pg/mL creatinine) by a platelet-specific low dose aspirin regimen of 50 mg daily without affecting vascular endothelial cyclooxygenase^[32]. These observations clearly indicate that activated platelets in the end-arterial circulation are the main source of increased TXB2 generation in symptomatic ET patients complicated by erythromelalgia, which provides the rationale of a very low dose of aspirin 50 mg daily or 100 mg every other day for the prevention of microvascular circulation disturbances in symptomatic thrombocythemia patients with ET or PV.

MIGRAINE-LIKE MICROVASCULAR CEREBRAL ISCHEMIC ATTACKS IN ET

From 1978 to 1993, we documented 59 consecutive cases of ET and 26 cases of PV associated with thrombocythemia. The specific manifestations in 55 symptomatic cases were erythromelalgia and its acrocyanotic ischemic complications in 43, TIAs in 23 and coronary artery disease in 9 patients and 30 were asymptomatic^[33]. Of the 23 patients with TIAs, six out of 26 (23%) had PV and 17 out of 59 (29%) ET. The spectrum of TIAs and visual disturbances in these 17 ET patients in our first report (Table 2, Michiels *et al*^[33]) has been described and interpreted by the neurologist Dr. Koudstaal (Department of Neurology, Erasmus University Medical Center, Rotterdam). Erythromelalgia (E), visual symptoms (VS) and/or angina pectoris (AP) followed or preceded the microvascular cerebral symptoms (CS in Table 2)^[33]. These cerebral and visual ischemic symptoms are interpreted by the vascular neurologist Dr. Peter Koudstaal (Table 2) in 1993^[33] and by the neurologists Koudstaal *et al*^[37] (Table 3) as migraine-like microvascular cerebral ischemic attacks

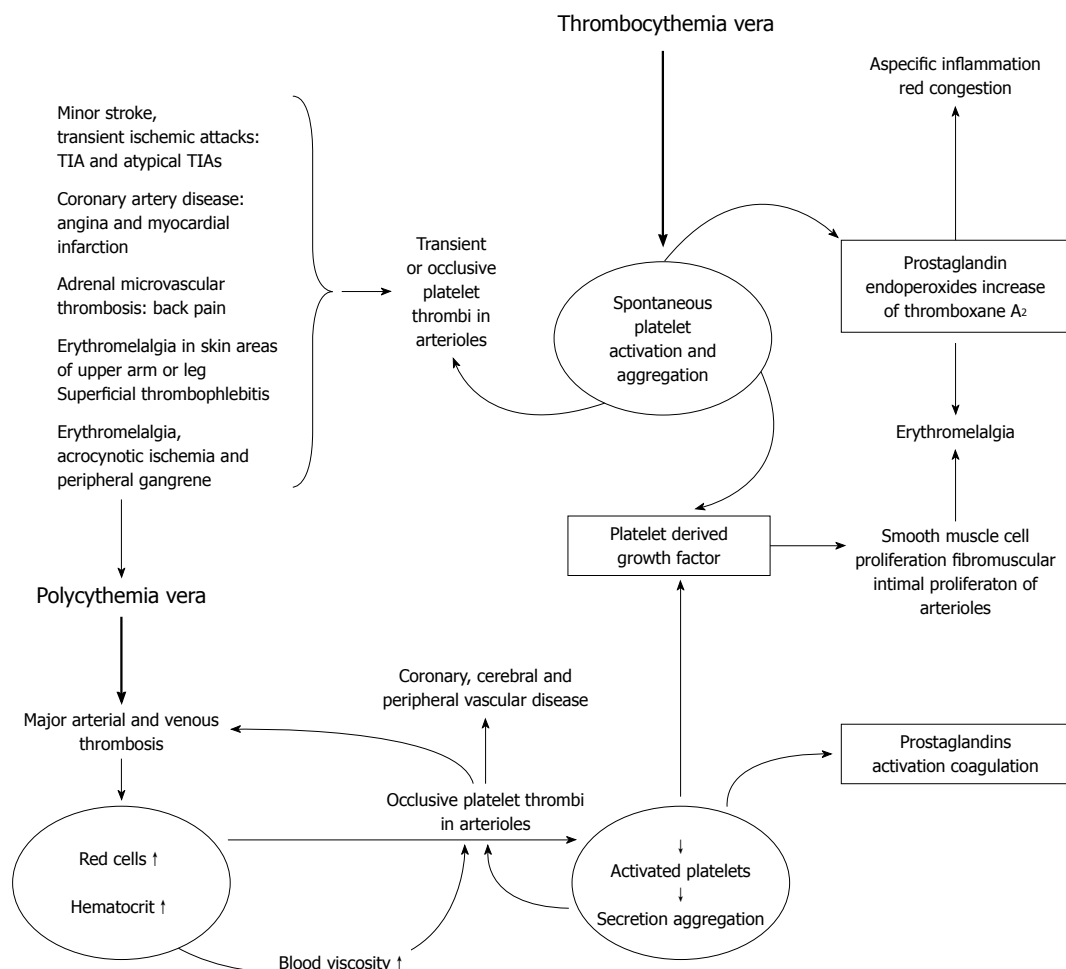


Figure 3 Etiology and pathophysiology of platelet-mediated fibromuscular intimal proliferation and platelet thrombi in erythromelalgia and its acrocyanotic complications in thrombocythemia vera (essential thrombocythemia and polycythemia vera) and etiology of major thrombosis on top of erythromelagic microvascular circulation disturbances in polycythemia vera.

(MIAs, Tables 2 and 4). Risk factors for vascular disease were present in only 2 and the peripheral arterial pulses were normal in all. Screening with brain computed tomography (CT), carotid duplex ultrasonography and extensive cardiac investigations at the time of MIA or visual disturbances in all 17 patients did not reveal any obvious co-existent cause of the microvascular ischemic event in any patient. In two studies of 22 ET patients, the spectrum of MIAs revealed a specific pattern different from TIAs in atherothrombotic disease: 11 had focal symptoms (transient monocular blindness in 3, transient mono- or hemiparesis in 6 and both of these in 1, migraine accompaniments in 1 and partial stroke in 1) and nonfocal symptoms occurred in 14 (transient postural instability in 13, dysarthria in 8 and scintillating scotomas in 5). These transient focal and nonfocal neurological and VS all had a sudden onset, occurred with a march rather than all at one time, lasted for a few seconds to several minutes and were usually associated with or followed by a dull or pulsatile headache^[33,34]. This clinical presentation of MIAs show a striking similarity with migraine accompaniments and supports the crucial role of platelets in the pathogenesis of migraine-like MIAs in ET (Figure 3). Fourteen evaluable ET

patients with MIAs remained asymptomatic while treated with aspirin (250 to 500 mg daily) for 1 to 9 years with increased platelet counts between 520 to $1250 \times 10^9/L$ ^[33]. Recently, Billot *et al*^[34] prospectively studied the neurological disorders in 37 ET patients, of whom 11 (30%) presented with neurological symptoms. Among them, 4 had thrombotic events (TIA in 3, cerebral venous thrombosis in 1), 7 complained of transient or fluctuating subjective symptoms, including cephalgia, dizziness, instability, visual disturbances and transient loss of consciousness (MIAs). Brain magnetic resonance imagery failed to detect any substratum with subjective symptoms. JAK2^{V617F} mutation was found in 9 of 11 patients with neurological symptoms *vs* 14 of 26 ET patients without symptoms. In a recent study, we observed similar MIAs due to disturbances of the cerebral or ocular microcirculation as the main presenting feature in 10 JAK2^{V617F} positive patients with early myeloproliferative disease ET in 6 and PV in 4^[36]. The ages of the patients at the time of first symptoms and increased platelet count ranged from 21 to 53 years for ET and from 39 to 50 years for PV. The MIAs in these 10 JAK2^{V617F} mutated MPN patients ranged from attacks of transient blindness, diplopia

Table 4 Nature of neurological and visual, aspirin responsive migraine-like microvascular cerebral ischemic attacks in 56 reported cases of essential thrombocythemia *n* (%)

ET-related clinical manifestations	Relative incidence
Acroparesthesia or numbness	13 (24)
Painful toes and/or cyanosis (Erythromelalgia)	12 (21)
Transient ocular attacks	
Visual scotomas	11 (20)
Amaurosis fugax	5 (9)
Diplopia	3 (5)
Hemianopsia	2 (4)
Blurred vision	14 (25)
Transient ischemic attacks TIAs or hemiparesis arm and/or leg	17 (30)
Atypical TIAs total	31 (55)
Aphasia	3 (5)
Dysarthria	12 (21)
Unsteadiness or unstable gait	16 (29)
Functional symptoms	
Pulsatile headache	26 (46)
Syncope	3 (5)
Vertigo	2 (4)
Dizziness	3 (5)
Seizures	3 (5)
Organic mental syndrome	1 (2)

ET: Essential thrombocythemia; TIAs: Transient ischemic attacks. Originated from^[33,35,37].

and scotomas to migraine-like attacks followed by throbbing headaches, nausea, vomiting or even seizures. The time lapse between the first symptoms of MIAs and the delay in diagnosis of ET in 5 patients ranged from 4 to 12 years, indicating that its causal relationship with early stage JAK2^{V617F} mutated in ET and PV are in most cases still overlooked because of unfamiliarity with the typical clinical appearances and the absence of any abnormality on a cerebral CT scan.

The neurologists Koudstaal *et al*^[37] reviewed the literature and analyzed the spectrum of erythromelalgia and MIAs in 56 patients with ET in 4 studies^[33,35,37,38], of which the nature of neurological and VS are shown in Table 4. Fröhli *et al*^[39] documented the prophylactic efficacy of aspirin on vascular complications in 22 patients with ET (*n* = 9) or thrombocythemia associated with PV in remission after bloodletting (*n* = 13) at platelet counts between 400 to 1000 × 10⁹/L. Aspirin (250 mg/d) in 22 symptomatic patients during a total period of 45 patient-years resulted in the disappearance of digital microvascular ischemia in 11 and of MIAs, including unstable gait, dizziness and blurred vision, which were completely abolished in 12 patients. Discontinuation of aspirin was followed by prompt recurrence of the microvascular circulation disturbances.

Scheffer *et al*^[40] analyzed our 9 cases of coronary events as the presenting symptom of ET (Department of Cardiology, Erasmus University, Rotterdam), including myocardial infarction in 4 and unstable AP in 5. The 5 ET patients with unstable angina had coronary disease (one vessel disease in 2 and two vessel disease in 3) un-

derwent coronary bypass surgery. During continuous treatment with low doses of aspirin (500 mg/d) there was no recurrence of vascular events in 4 patients for 1 to 5 years at platelet counts of 650, 800, 860 and 1000 × 10⁹/L. Five patients in maintained complete remission of ET (platelet counts < 350 × 10⁹/L) for more than 1 to 6 years after treatment with busulphan remained asymptomatic^[40].

ACTIVATED PLATELET, LEUKOCYTES AND ENDOTHELIAL CELLS IN ET AND PV

The concept of platelet-mediated inflammatory and thrombotic processes in the end-arterial circulation of the brains, heart, adrenal, skin and extremities in thrombocythemia vera is completely in line with the recent demonstration of *in vivo* activation of platelets, leukocytes and endothelial cells in ET and PV patients (Figure 3)^[23,24,29,30,32]. The increased risk of erythromelalgic microvascular and major arterial thrombosis in ET and PV is associated with *in vivo* activation of leukocytes (increased CD11) expression and leukocyte alkaline phosphatase (LAP) score, together with increased plasma markers indicating platelet activation, proteolysis of high vWF multimers and endothelial damage^[24,41,42]. Increased CD62 and CD63 antigens and levels of soluble vascular adhesion molecule 1 (sVCAM-1) reflecting *in vivo* platelet activation has been demonstrated in 52 patients with ET and PV, both before and after cytoreductive treatment compared with 22 healthy controls^[42]. In these 52 ET and PV patients, sVCAM-1 expression was increased before and after treatment compared with 22 healthy controls and 17 non-thrombocythemic patients with acute cerebrovascular ischemia and normal platelet counts. Cella *et al*^[43] found increased levels of soluble P-, E- and L-lectins in PV and ET patients compared to controls. In particular, PV patients showed higher levels of P-selectin as well as E-lectin, both possibly released from activated endothelium cells (EC) as the consequence of increased platelet-EC interactions, resulting in increased TM levels. These findings indicate that spontaneous *in vivo* platelet activation in MPD patients not on aspirin in ET and PV result in endothelial damage, probably through release of angiogenic factors and *in vivo* activation of platelets and leukocytes^[41,42].

All PV and the majority of our symptomatic ET patients [erythromelalgic thrombotic thrombocythemia (ETI)] have high LAP scores^[35]. Westwood *et al*^[44] and Johansson *et al*^[45] found low serum erythropoietin (EPO) levels and EPO independent erythroid colony formation (EEC⁺) in about half of the ET patients. According to Tom Pearson, such cases of EEC⁺ ET with low serum EPO levels are to be regarded as prodromal PV patients^[46,47]. It became evident from 2005 that the majority of EEC⁺ ET patients with low serum EPO carry the JAK2^{V617F} mutation^[48]. EEC-positive ET is associated with a higher risk of developing microvascu-

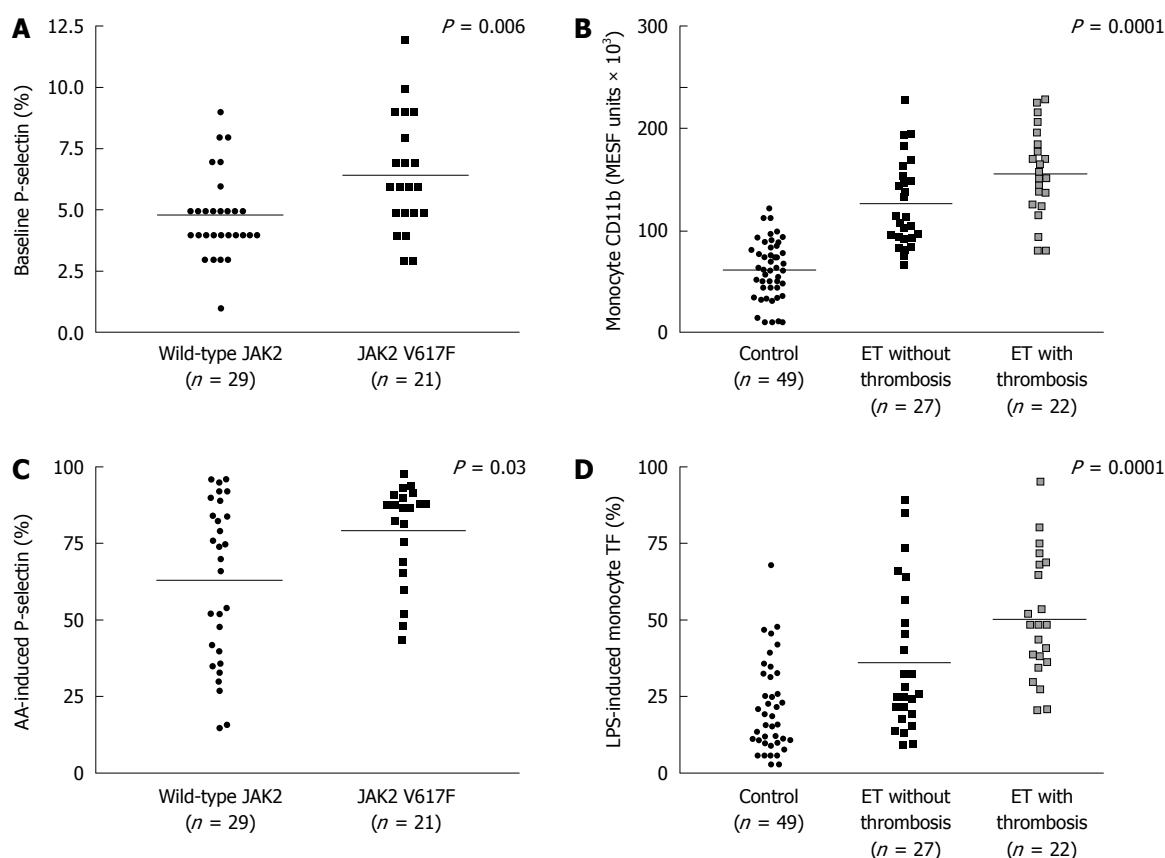


Figure 4 Baseline P-selectin levels (A), and arachidonic acid-induced P-selectin levels (C) in JAK2V617F mutated essential thrombocythemia and JAK2 wild type essential thrombocythemia; monocyte CD11b (B), and LPS-induced monocyte tissue factor expressions (D) in controls, essential thrombocythemia without thrombosis and essential thrombocythemia with thrombosis. Majority of essential thrombocythemia (ET) patients were on myelosuppressive therapy (mainly hydroxyurea), and a minority used aspirin at time of laboratory investigations. AA: Arachidonic acid.

lar and major thrombotic complications compared to EEC-negative ET^[45,49]. Early or initial (prodromal) PV, according to ECP criteria defined by Michiels *et al.*^[50,51] between 2002 and 2004 (http://www.mpn-stichting.nl/doctors_brochure_2004.pdf), is typically featured by a PV picture in the bone marrow, has positive results for EEC, low serum EPO levels, slightly increased values for hematocrit up to 0.50, and a much higher thrombotic risk compared to EEC-negative ET^[45,48-51].

Falanga *et al.*^[52] showed in 2000 that patients with ET ($n = 37$) and PV ($n = 34$) have increased peripheral blood mononuclear cell (PMN) activation parameters (PMN membrane CD11b and LAP antigen expression, cellular elastase content, plasma elastase and myeloperoxidase levels) evaluated simultaneously with the levels of plasma markers of endothelial damage (thrombomodulin and vWF antigen) and hypercoagulation (thrombin-antithrombin complex, prothrombin fragment 1 + 2 and D-dimer). These data nicely confirm our findings that ET and PV are at increased risk for platelet-mediated thrombosis, which is associated with *in vivo* platelet, leukocyte and EC activation and associated with laboratory signs of coagulation system activation as well^[52]. Falanga *et al.*^[53] further studied 37 ET patients with JAK2^{V617F} mutation, 38 JAK2 wild type ET and 50 healthy controls. Leukocytes, PMN counts and platelets from overall ET

patients, compared to controls, expressed significantly higher membrane tissue factor (TF) and P-selectin ($P < 0.01$ = activation marker) and lower platelet surface markers CD41 and CD42b ($P > 0.01$ very likely down regulated after activation). TF antigen levels and membrane TF appeared significantly higher in the V617F JAK2 carriers compared to JAK2 wild-type^[52,53]. The presence of circulating platelet/PMN aggregates was significantly greater in the JAK2-mutation carriers than in JAK2 wild-type ET and controls ($P < 0.05$). PMN surface activation and inflammatory markers, *i.e.*, CD14, TF, CD11b, and LAP-scores, were all significantly higher in ET *vs* control subjects, with CD14 and LAP being the highest in ET carrying the JAK2^{V617F} mutation. Finally, a significant increase in plasma hypercoagulation markers was found in ET patients and the only difference was higher plasma thrombomodulin levels in ET carrying the JAK2^{V617F} mutation, reflecting subclinical increased platelet-endothelial cell interactions ($P < 0.01$)^[52,53].

Cervantes and his team performed a very interesting study on increased platelet and leukocyte activation in 22 ET patients with thrombosis, including erythromelalgia, TIA, stroke, myocardial infarction, 27 ET patients without thrombosis and in 49 controls^[54]. The majority of the 49 ET patients received myelosuppressive therapy (hydroxyurea, anagrelide or P32) in 95% of ET with thrombosis and in

83% of ET without thrombosis. Only 33% and 15% of ET patients with and without thrombosis used aspirin. The key findings are the following. Firstly, baseline platelet P-selectin levels are higher in ET compared to controls. Secondly, arachidonic acid (AA) is transformed by platelet COX1 into prostaglandin metabolites, including TXA2 (a potent platelet activator), and AA-induced platelet aggregation induced a highly significant increase of platelet P-selectin expression [$P \geq 0.0001$, which is not seen in ADP and collagen stimulated platelets (Figure 4)]^[54]. Both baseline and AA induced platelet P-selectin expressions were significantly higher in JAK2^{V617F} mutated ET compared to JAK2 wild type ET. These observations are completely in line with the existence of hyper-reactive platelets in ET and PV caused by the JAK2^{V617F} gain of function mutation. Monocyte CD11b expression and PLS-induced monocyte TF expression is the highest in ET with thrombosis (Figure 4). Cervantes and his team did not study untreated symptomatic ET patients at the time of presentation and the effect of aspirin treatment alone and the effect of aspirin on top of myeloproliferative treatment in JAK2^{V617F} mutated and JAK2 wild type ET. Such prospective studies need to be done to differentiate between the role of platelets and the contribution of leukocyte activation in platelet and leukocyte mediated inflammatory and thrombotic processes. The sustained relief of erythromelalgia and MIAs by aspirin is correlated with inhibition of ADP, epinephrine, collagen and AA-induced platelet aggregation, which is the result of irreversible inhibition of platelet COX1, as can be measured by the MDA in PRP^[5,6,13-19]. Ticlopedine (and also clopidogrel, prasugrel) inhibits the ADP platelet membrane receptor but not the AA induced platelet COX1 activation. This is the reason why platelet ADP receptor inhibitors (ticlopedine, clopidogrel, prasugrel) are shown by us as ineffective for the cure and prevention of platelet mediated microvascular disturbances in ET^[5,31]. It is predicted that aspirin, but not ADP-receptor inhibitors, will suppress the AA-induced platelet P-selectin expression and that aspirin will not affect the increased monocyte CD11b expression and the LPS induced monocyte TF expression. This hypothesis is drawn from our clinical observation that aspirin does not affect the increased LAP-score and does not affect symptoms like pruritus, which is much more prevalent in JAK2^{V617F} hetero/homozygous mutated PV than in heterozygous JAK2^{V617F} mutated ET. A key experiment may be to assess both platelet and leukocyte activation markers in those JAK2^{V617F} mutated prodromal and overt PV patients before and after a warm shower that elicits pruritus.

Anagrelide is a selective inhibitor of megakaryocyte differentiation^[55], in particular endoreduplicative activity^[56], but the number of megakaryocytes is not reduced^[57]. Using immunohistochemistry, Thiele studied CD61⁺ megakaryopoiesis in ET before and after anagrelide treatment^[55,58,59]. Before therapy, the megakaryocytes in a typical case of ET (platelet count $9.7 \times 10^{11}/L$) are dispersed and loosely clustered with predominance of large and giant

Table 5 Incidence of thrombosis in polycythemia vera and essential thrombocythemia patients

	PV		ET	
	GISP	Marchioli	Cortelazzo	Carobbio
Patients number	1213	1638	100	439
Age group (yr)				
< 40	1.8	2.1	1.7	NA
40-60	2.8	NA	6.3	NA
> 65	5.1	4.9	15.1	2.3
Previous Thrombosis	4.8	5.0	NA	2.3

According to age and previous vascular event in different Italian retrospective polycythemia vera and essential thrombocythemia studies anno 2008. ET: Essential thrombocythemia; PV: Polycythemia vera; NA: Not available. Originated from Landolfi *et al*^[62].

forms (Figure 5). Following anagrelide monotherapy for about 1 year (platelet count near to normal, $4.5 \times 10^{11}/L$), the number of megakaryocytes is not significantly decreased but there is a prevalence of small forms (Figure 5). This left-shifting of megakaryocyte ploidy and/or differentiation by anagrelide is probably generated by an arrest of endomitotic activity of the disease-specific large to giant megakaryocytes in ET (Figure 5). By this mechanism, anagrelide selectively lowers platelet production without a significant effect of bone marrow erythropoiesis and granulopoiesis, and without an increased risk of myelofibrosis and leukemia.

LOW, INTERMEDIATE AND HIGH THROMBOTIC AND HEMORRHAGIC RISK STRATIFICATION IN ET

In a historical cohort of 100 ET patients, Cortelazzo *et al*^[60] could distinguish ET patients at low or high risk for major thrombotic complications. Cortelazzo *et al*^[60] did not regard erythromelalgic microvascular disturbances. The idea of high risk factors for thrombosis in ET by Cortelazzo *et al*^[60] in their retrospective study of 100 ET patients (Table 5) should be re-interpreted in view of the Rotterdam experiences on the effectiveness of aspirin for thrombosis prevention in ET (Table 3)^[61]. In the Bergamo study, the risk for a thrombotic complication is slightly increased at ages younger than 40 years and significantly increased at the age of > 60 years, but the moderately increased thrombotic risk of 6.3% in the age group of 40 to 60 years is not significantly different from the thrombotic risk of 15% in the age group of > 60 years (Table 5). In subsequent large retrospective studies in PV and ET, the incidence of major thrombosis at age above 65 years is much lower (2.3% to 5.1%, Table 5)^[62]. All well known risk factors for vascular thrombotic disease, such as smoking, hypertension, diabetes and hypercholesterolemia, did not contribute to a further increase of thrombotic complications in ET in this study. The type and number of thrombotic events in 20 of 100 ET patients in this

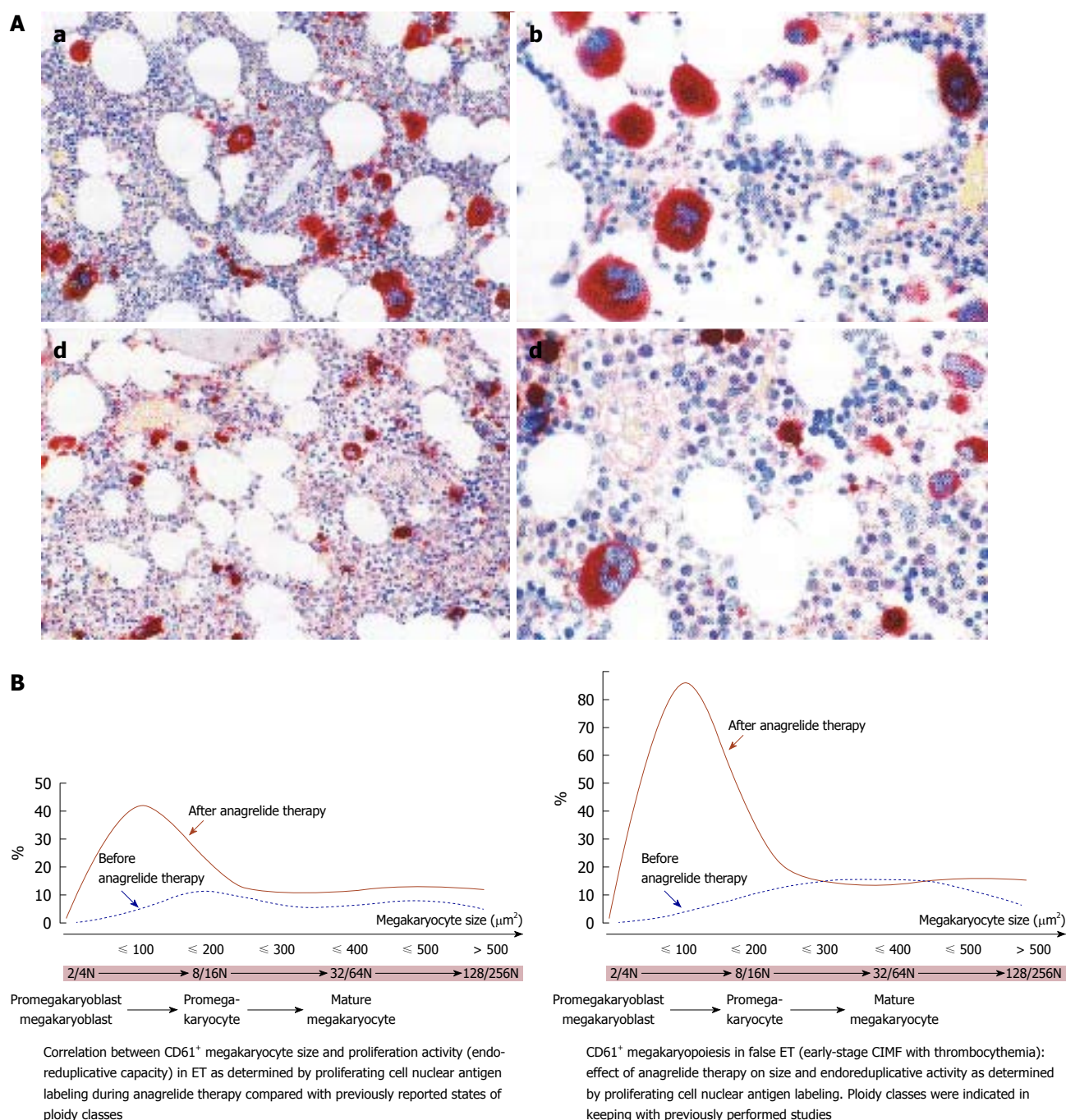


Figure 5 The effect of anagrelide treatment on CD61⁺ stained megakaryocyte size, proliferation and differentiation in bone marrow biopsies. A: Change of megakaryocyte size from large before treatment (a and b) to small after treatment (c and d) with anagrelide, a selective inhibitor of megakaryocyte differentiation; B: The peculiar phenomenon of megakaryocyte change by anagrelide is generated by an arrest of endomitotic activity of the thrombocythemia-specific reversion of large to small megakaryocytes before and after anagrelide treatment in essential thrombocythemia (ET, left) and in hypercellular false ET (early prefibrotic stage chronic idiopathic myelofibrosis, right). Before anagrelide therapy (platelets $9.7 \times 10^{11}/L$), the megakaryocytes in ET are loosely clustered with predominance of large and giant forms (a, b). After 1 year anagrelide monotherapy (platelets $4.5 \times 10^{11}/L$), the megakaryocytes are small (c, d), but the number of small megakaryocytes remained increased (d). Originated from Michiels *et al*^[58].

study were 25 arterial events in 17 ET patients (digital ischemia 7, TIAs 15, stroke 0, myocardial infarction 3), seven venous thrombosis events in 3 (superficial thrombophlebitis = erythromelalgia of the skin, femoral DVT in 1) and unusual localization in 3 (Table 5). It is known to me that digital ischemia, TIA and superficial thrombophlebitis (erythromelalgia of the skin) nicely respond

to aspirin, thereby reducing the incidence of recurrent minor and major thrombosis to less than 2% per 100 patient-years when low dose aspirin would have been prescribed (Table 3)^[61-67]. Therefore, the increased risk of thrombotic incidences in so-called high thrombotic risk ET defined in the Cortelazzo *et al*^[60] study is caused by the increased hypersensitive platelets and its recurrences

Table 6 Low, intermediate and high thrombohemorrhagic risk stratification of essential thrombocythemia patients and a flexible approach towards therapeutic implications with reference to platelet counts including essential thrombocythemia patients with features of early polycythemia vera in blood and bone marrow (prodromal polycythemia vera)

Platelets (400-1500 × 10 ⁹ /L)	Platelets (400-1000 × 10 ⁹ /L)	Platelets (400-1000 × 10 ⁹ /L)	Platelets (> 1500 × 10 ⁹ /L)
Low risk Completely asymptomatic	Low risk Microvascular disturbances only ¹	High risk Major thrombosis, and/or bleeding Vascular risk	High risk > 1000 × 10 ⁹ /L and minor thrombosis/bleeding = high No vascular risk
No vascular risk No bleeding risk Age < 65 yr ² Aspirin uncertain Wait and see?	No vascular risk No bleeding risk Age < 65 yr except ² Low dose aspirin 50 to 100 mg/d Intermediate risk	Age > 65 yr except ³ Platelet reduction to normal or near normal	All ages Platelet reduction to < 1000 × 10 ⁹ /L
Aspirin primary prevention? ET patients and their physician usually prefer the use of low dose aspirin	Microvascular disturbances and platelet count between 1000 and 1500 × 10 ⁹ /L with clear indication aspirin ¹ , →side effects (platelet reduction)	Continue aspirin ¹	When platelets < 1000 × 10 ⁹ /L add aspirin

¹At platelet counts in excess of 1000 × 10⁹/L, aspirin will usually elicit bleeding symptoms, which disappear after reduction of platelet counts to below 1000 × 10⁹/L with continuation of aspirin (Figure 6). If side effects of aspirin (gastritis, gastrointestinal bleeding) occur, reduce platelets to normal and consider indomethacin 25 mg/*bid*; ²Age over 65 plus one of the following: platelets > 1000 × 10⁹/L or the presence of a vascular risk factor, but otherwise asymptomatic while on aspirin seems to be a clear indication to reduce platelet count; ³Age over 65, platelets < 1000 × 10⁹/L, absence of vascular risk and asymptomatic while on low dose aspirin seems to be no clear indication to reduce platelet to normal (Figure 6).

are related to the underlying platelet thrombophilia in ET. This analysis and statement are supported by detailed and re-interpretation of the data in the Bergamo study in view of the real thrombotic risk in PV and ET in the large retrospective Italian cohorts of PV and ET patients (Table 5)^[62]. The real incidence of thrombotic risk of regularly treated (good clinical practice at that time) in the large retrospective study at age over 65 years was not 15% but much lower: 5% for PV and 2.3% for ET (Table 5)^[62]. Consequently, the definition of high thrombotic risk ET by Cortelazzo *et al*^[60] is an overestimation based on statistics mystification derived from one historical cohort of 100 ET patients not on aspirin. The problem of high thrombotic risk as the indication to treat with hydroxyurea has never been evaluated in a prospective outcome study in aspirin treated ET patients. Therefore, this definition of high risk ET does not reflect the reality anymore to which MPD patients nowadays are faced with in the 21st century (Table 3). In our experience, ET at age around and over 65 years without a history of thrombosis and treated with low dose aspirin still remains a low risk ET patient in the absence of vascular risk factors. The presence of one of the risk factors for arterial vascular disease, such as hypertension, hypercholesterolemia, diabetes and smoking, did not contribute in terms of statistical evidence to an additional increased erythromelalgic MIAs in thrombocythemia in three studies^[62-65]. Age was not a risk factor for recurrent cerebral ischemic circulation disturbances (including MIAs) in one retrospective study of 187 ET patients^[63] and also not in our prospective study of 68 ET patients treated with aspirin^[64]. This fits with the observations that increased number of platelets above 350 to 400 × 10⁹/L is the main cause of microvascular events in ET when not on aspirin. Erythromelalgic disturbances and MIAs have never been

reported in age adjusted individuals with reactive thrombocytosis. As vascular risk factors usually present at age over 65 years, it surely will contribute to major atherosclerotic complications in the general population and age-adjusted ET and PV patients; consequently, strict measures to reduce and eliminate them is mandatory (Table 6). ET and PV patients with platelet counts above 1000 × 10⁹/L are at high risk for the paradoxical occurrence of thrombotic and bleeding complications. In that situation, aspirin does prevent platelet-mediated thrombotic events but does increase the bleeding tendency (as well as prolongation of the Ivy bleeding times) and, therefore, are candidates for platelet reductive therapy with continuation of low dose aspirin^[66]. ET and PV patients with platelet counts above 1000 × 10⁹/L are candidates for screening for acquired von Willebrand disease type 2A^[66]. Low dose aspirin surely does increase the risk of bleedings at platelet counts above 1000 × 10⁹/L (Table 6). In our update, low-risk ET patients were younger than 65 years, had no history or manifestations of atherothrombosis or bleeding symptoms and have platelet counts < 1500 × 10⁹/L^[60,64,66-69]. Asymptomatic low risk and symptomatic (microvascular event) low risk ET patients with a clear indication of low dose aspirin do not have an indication to reduce the platelet count because of the complete absence of any vascular risk factor and no history of bleeding and atherothrombosis at a platelet count below 1000 × 10⁹/L (Table 6). Ruggeri *et al*^[63] showed that the risk for atherothrombosis in 65 ET patients under the age of 65 with no history of arterial or venous thrombosis and a platelet count < 1500 × 10⁹/L was not increased compared to match control, but 25 of these patients (38%) needed low dose aspirin to control ET-related erythromelalgic microvascular ischemic symptoms. Those ET patients over the age of

65 with no vascular risk factors, asymptomatic while on low dose aspirin, and platelet count below $1000 \times 10^9/L$ are to be risk stratified as low thrombohemorrhagic risk (Table 6). In our experiences, high thrombohemorrhagic risk ET patients do have a clear indication to reduce platelet counts for various reasons, including ET related major thrombosis or bleeding (and documented AVWS^[66]), aspirin related side effects, platelet count in excess of $1500 \times 10^9/L$, and platelet count in excess of $1000 \times 10^9/L$ when symptomatic while on treatment with aspirin (Table 6, Figure 6). In case of aspirin side effect (gastritis or aspirin allergy), the reversible platelet COX1 inhibitor indomethacine is the alternative. High thrombohemorrhagic risk ET patients do not necessarily have their platelet count corrected to normal ($< 400 \times 10^9/L$) but may remain slightly increased ($400-600 \times 10^9/L$) or even moderately increased ($600-800 \times 10^9/L$). This near to normal platelet count strategy using one of the non-leukemogenic platelet lowering agents, either anagrelide or pegylated interferon (IFN) α -2a is recommended, provided that low dose aspirin (50 to 75 mg/d or 100 mg 4 times a week) will be continued as long as platelet counts are above $350 \times 10^9/L$ (Table 6).

THROMBOHEMORRHAGIC RISK AND THE NEED TO REDUCE PLATELET COUNT PREFERENTIALLY BY NON-LEUKEMOGENIC AGENTS IN ET

The incidence of thrombotic and hemorrhagic complications in 809 patients with ET from 11 retrospective clinical studies is shown in Table 7^[69]. The thrombohemorrhagic complications in 809 ET patients from 11 retrospective studies were recorded in 59%, as thromboembolic complications in 58%, bleeding symptoms in 17%, thromboembolic events without bleeding in 42%, bleeding without thrombosis in 1.4%, and 36% of these 809 ET patients were asymptomatic (Table 7)^[69]. The incidence of deep venous thrombosis, including portal and splenic vein thrombosis, in this review of retrospective studies was as low as 1% and Budd Chiari syndrome was not recorded (Table 7). The arterial thrombotic manifestations of ET in the 809 patients were described as microcirculatory in 41%, involving the extremities in 24% and the cerebral circulation in 17% (Table 7). The frequency of thrombohemorrhagic complications at presentation of ET varied widely in the 11 retrospective studies: 31% to 83% for minor and major arterial complications and 4% to 38% for bleeding complications^[69]. This variation is probably a reflection of the heterogeneity of the patient population studied and differences in the definition of thrombohemorrhagic complications applied. Strikingly, 27% to 70% of the ET patients had developed end-arterial microvascular or functional symptoms reported as acroparesthesias, burning red or blue toes or finger (erythromelalgia), peripheral ischemia,

Table 7 Platelet count and incidences of thrombotic and hemorrhagic complications in 809 patients with essential thrombocythemia from 11 retrospective studies *n* (%)

No. of ET patients	809 (100)
Age (yr), mean (range)	54 (6-90)
Platelet mean ($\times 10^9/L$)	1050
Thromboembolic complications	466 (58)
Microvascular attacks/thrombosis	333 (41)
Peripheral total	197 (24)
Acroparesthesias/erythromelalgia	168 (21)
Acrocyanotic ischemia/gangrene	77 (9.5)
Cerebral total	138 (17.1)
Headache/dizziness	59 (7.3)
Atypical TIAs typical TIAs	46 (5.7)
Visual disturbances	15 (1.9)
Not specified	18 (2.2)
Major arterial thrombosis	164 (20)
Lower extremity	61 (7.5)
Carotic/cerebral	52 (6.4)
Cardiac	44 (5.4)
Others	15 (1.9)
Venous thrombosis	33 (4)
Leg/pelvis vein thrombosis	27 (3.3)
Portal/splenic vein thrombosis	8 (1)
Budd Chiari syndrome	0 (0)
Hemorrhages total	134 (17)
Minor: Bruises, ecchymoses, epistaxis	
Gum bleeding, bleeding after trauma	105 (13)
Major: gastrointestinal tract bleeding, large hematomas	
Hemarthrosis and others	29 (3.6)

ET: Essential thrombocythemia; TIAs: Transient ischemic attacks. Originated from^[69].

poorly localized neurological symptoms, blurred vision or headache (MIAs, Table 7).

The second Bergamo study is a prospective randomized clinical trial (RCT) of 114 ET patients comparing hydroxyurea *vs* placebo^[70]. The results show that 2 of 56 high-risk ET patients on hydroxyurea had major thrombotic events (one stroke, one myocardial infarction) and that 14 of 58 high-risk ET patients in the placebo group had thrombotic complications: minor microcirculatory disturbances in 12, major thrombosis in 1, and deep vein thrombosis in 1. Platelet counts were normal or near normal in the hydroxyurea arm, whereas the ET patients in the placebo arm had a mean platelet count of about $1000 \times 10^9/L$. In this study, 69% of the placebo group and 70% of the HU-treated ET patients received antiplatelet drugs, aspirin (effective) or ticlopedine (not effective). In this second Bergamo study, 10 of the 14 symptomatic patients in the placebo arm manifested mainly microvascular disturbances and were not on treatment with aspirin. Consequently, this RCT demonstrates that hydroxyurea indeed did reduce the occurrence of microvascular thrombotic events, in particular when not on aspirin^[70]. The recommendation from this unbalanced RTC (HU *vs* placebo) to use hydroxyurea in high thrombotic risk ET as first line treatment for ET seems to us significant over-treatment and potentially leukemogenic. A direct comparison of HU *vs* low dose aspirin alone in

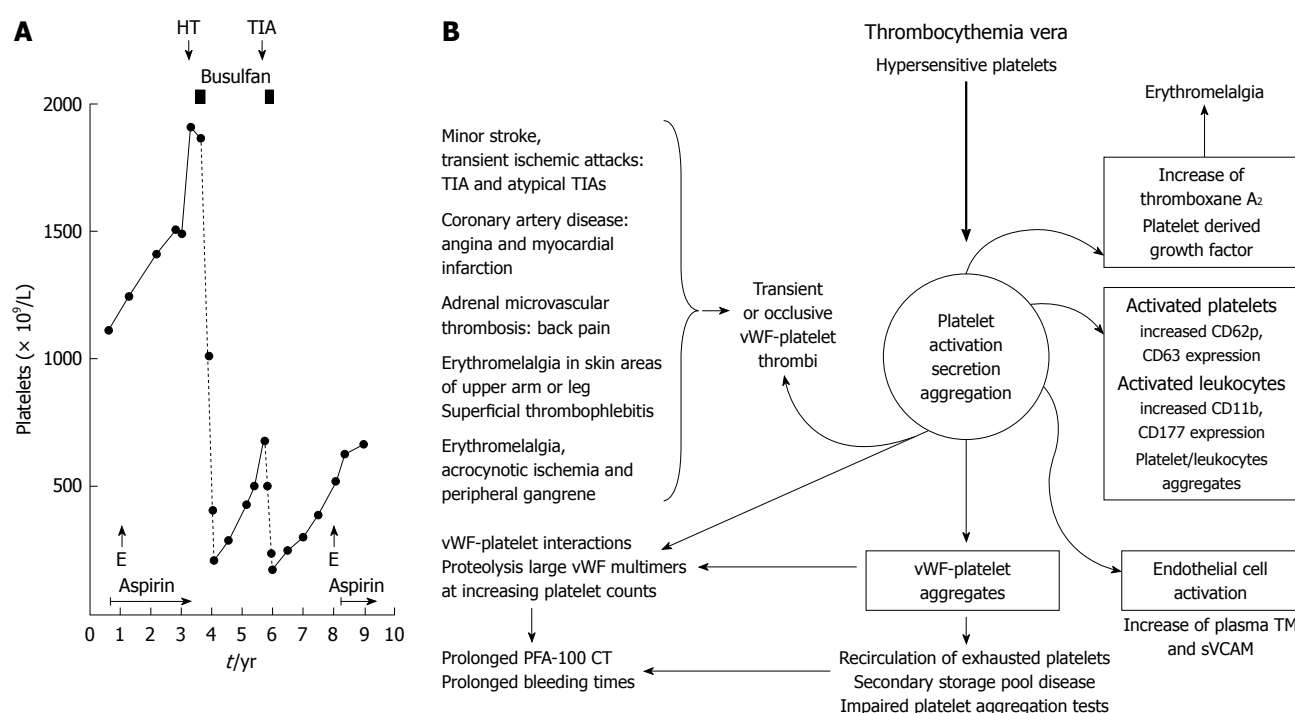


Figure 6 JAK2^{V617F} or MPL⁵¹⁵ constitutively activated (hypersensitive) platelets in thrombocythemia spontaneously aggregate and secrete products (platelet derived growth factor et) at high shear stress in the end-arterial cerebral, ocular, coronary and peripheral circulation. This is associated with a broad spectrum of microvascular ischemic circulation disturbances (B) caused by transient von Willebrand factor (vWF)-rich platelet aggregates or occlusive vWF-rich platelet thrombi on histological investigation (Figure 1). Circulating activated platelets express increase of platelet membrane surface markers, e.g., CD62p, and circulating platelet aggregates induce endothelial cell activation with increased thrombomodulin (TM) and soluble vascular adhesion molecule (sVCAM) plasma levels (right). Increase of platelet counts from below to far above $1000 \times 10^9/L$ is associated with platelet-induced proteolysis of large vWF multimers with clinical features of hemorrhagic thrombocythemia (HT, A) due to an acquired von Willebrand disease type 2A [prolonged PFA-110 closure times (CT) and bleeding times]. TIA: Transient cerebral ischemic attack; E: Erythromelalgia.

ET patients but with platelet counts below $1000 \times 10^9/L$ is predicted to be equally effective for the prevention of microvascular circulation disturbances in ET, but the aspirin arm will be associated with increased bleeding events at platelet counts above $1000 \times 10^9/L$ (Table 3). This has been documented in the prospective 1975-1996 Rotterdam ET study by Van Genderen *et al*^[61] and van Genderen *et al*^[71] of 68 consecutive ET patients who were followed for a total of 419 patient years (Table 3). The 14 ET asymptomatic ET patients in the watch and wait arm suffered from 27 thrombotic and two bleeding episodes which occurred during a follow-up of 127 patient-years. The platelet counts at the time of the thrombotic event were between 410 and 831, mean $610 \times 10^9/L$. Only four of the initially 14 asymptomatic ET patients in the watch and wait arm remained asymptomatic after at least 30 person-years follow-up. In the treatment group of low dose aspirin, 5 thrombotic events during a follow-up of 179 patient-years (2.8%). Ten bleeding episodes occurred in ET patients receiving low dose aspirin monotherapy during a follow-up of 139 patients-years (7.2%) at platelet counts between 661 and 3460 (mean $1737 \times 10^9/L$), indicating the need for platelet reductive therapy. In patients receiving platelet lowering agents, either busulfan or hydroxyurea and no aspirin, 10 thrombotic complications occurred during follow-up of 40 patient-years (25%) at platelet counts of $624 \pm 255 \times 10^9/L$, indicating inadequate control of platelet-mediated throm-

botic events (platelet thrombophilia) in thrombocythemia (ET, PV). In this study, age over 65 years was not a risk factor for thrombotic recurrences when on low dose aspirin. Personal risk assessment for the proper treatment of each individual ET and PV patient is warranted to improve the quality of life with straight forward treatment recommendations that have no or the lowest risk on side effects with no or the least leukemogenic potential in pre-fibrotic MPN disease.

THE PARADOXICAL OCCURRENCE OF THROMBOSIS AND HEMORRHAGES IN THROMBOCYTHEMIA IS RELATED TO INCREASED VON WILIEBRAND FACTOR PROTEOLYSIS

The implications from the prospective Rotterdam ET study of 68 patients (Table 3) are completely in line with the concept that microcirculatory thrombotic complications (platelet thrombophilia) in ET and in PV in remission by venesection already occur at platelet counts in excess of $400 \times 10^9/L$, which are relieved by reduction of platelet counts to normal ($< 359 \times 10^9/L$) or by control of platelet function with low dose aspirin (50 to 75 mg/d or 100 mg 4 times a week) (Figures 3 and 6)^[61,71]. Thrombocythemia of various MPNs with platelet counts

between 1000 up to $2500 \times 10^9/L$ is frequently associated with the paradoxical occurrence of ETT and HT. At platelet counts between 1000 to $2000 \times 10^9/L$, low dose aspirin will prevent ETT but aggravates the hemorrhagic tendency of HT, indicating the need to continue aspirin but to reduce platelet count to below $1000 \times 10^9/L$, preferentially with anagrelide or pegylated IFN and continuation of aspirin (Table 3)^[61,71]. An association between pronounced thrombocythemia in various MPD and an acquired von Willebrand disease type 2A has been increasingly be recognized by and Budde *et al.*^[72,73] and van Genderen *et al.*^[66,74], implicating that thrombocythemic platelets are also involved in the etiology of the bleeding symptoms (Figure 6). However, the mechanism and pathophysiology of the induction of AVWS type 2A by the increased platelet count is related to increased proteolysis of large vWF multimers. van Genderen *et al.*^[74] proposed the hypothesis that the vWF very likely is the link to explain the paradoxical occurrences of both ETT and HT at the same time in one and the same patient. According to this hypothesis, interactive activation of the hypersensitive platelet/ADAMTS13/vWF complex as soon as JAK2^{V617F} or MPL^{S15} mutated hypersensitive platelets enter the circulation will spontaneously aggregate at a high shear stress rate during their first run in the microcirculation. This must occur to understand why both the formation of vWF-rich platelet thrombi formation, as well as increased proteolysis of the vWF at the same time in one and the same patient, does occur at the time of the paradoxical occurrences of both thrombosis and bleeding (Figure 6). It is a matter of the degree of platelet count increase whether erythromelalgic thrombotic, bleeding or both do occur. In addition to the acquired vWF defect (AVWS type 2A), there is a third component of a peculiar bleeding, related to “increased platelet-mediated blood clot retraction” disturbance with erythrocyte fall out, that causes painful subcutaneous hematomas with a central swelling (clot) after a blow, trauma and/or minor or major surgery. This type of bleeding is caused by rapid clot formation of firm clots pulling out the erythrocytes, which in the old literature is known as erythrocyte fall out. This phenomenon is related to increased platelet count around and above $800 \times 10^9/L$ (Michiels 1981-2012, 16 unpublished cases).

THE ROLE OF HEMATOCRIT AND PLATELETS IN THROMBOSIS RISK ASSESSMENT IN PV: REVERSAL BY PHLEBOTOMY AND LOW DOSE ASPIRIN

In the past, the factors productive of vascular complications in PV were not fully understood. Osler^[75] already mentioned that the red painful “neuralgias” of the extremities in cases of PV may simulate erythromelalgia. Oppenheim first recognized that erythromelalgia without evidence of clinically demonstrable vascular occlusion of the extremities may antedate the hypervolemic symptoms

of PV^[76]. The circulatory disturbances of the extremities in a large series of polycythemic patients were classified as burning painful and ulcerating toes, features of erythromelalgia, acrocyanosis of burning type, ischemic attacks of digital arteries and peripheral gangrene with palpable arterial pulsations^[77-81]. This type of vascular complications is completely consistent with the broad spectrum of burning painful ischemic syndrome of thrombocythemia presenting as erythromelalgia and its ischemic complications of painful blue discoloration in the digits leading to gangrene if left untreated. A Swiss study of 86 PV patients (40 men and 46 women, mean age 59 years, observed between 1966 and 1987) nicely demonstrated that migraine-like transient ischemic microvascular disturbances of the cerebral circulation (MIAs, preceded or followed by erythromelalgia) were the main presenting features of PV^[82]. The clinical history at time of diagnosis of PV included headache and dizziness in 49%, painful extremities suggestive for erythromelalgia in 42%, AP in the absence of atherosclerotic disease in 9%, bleeding in 11%, pruritus in 16% and asymptomatic in 16%. The clinical findings at time of diagnosis of PV were peripheral microvascular ischemia in 29%, coronary heart disease in 10%, cerebral ischemia in 5% and venous thrombosis in 8%. The presenting thrombotic events in 200 PV patients in the elegant study of Barabas *et al.*^[79] published in 1973 were microvascular events, including sudden erythromelalgic ischemia of a toe or finger in 15 with amputation of one or more gangrenous digits in 10 (thrombo-angiitis obliterans), attacks of transient blindness (amaurosis fugax) in 4, MIAs, facial weakness or aphasia in 26, MIA followed by stroke in 6, stroke in 7, femoral artery occlusion in 17, coronary artery disease in 10, superficial thrombophlebitis in 30 (15%), deep vein thrombosis in 26 (13%), splanchnic vein thrombosis in 5 (2.5%) and pulmonary embolism in 4. The clinical features and distribution of arterial thrombotic complications in PV differ from an age-matched population with atherosclerosis without PV. In the atherosclerosis group, males predominate but there is equal gender distribution in PV. Minor and major cerebral artery complications are far more common than coronary artery complications in PV compared with non-polycythemic atherosclerosis patients. The intrinsic blood changes of increase platelets, hematocrit and blood viscosity are responsible for this altered distribution of vascular complications in PV.

We studied the effects of bloodletting, coumarin (vitamin K antagonist) and aspirin in great detail in five patients with thrombocythemia associated with classical PV and painful blue and black toe or finger syndrome^[83]. Arteriographic studies in 3 cases with erythromelalgic acrocyanosis or peripheral gangrene showed abrupt and complete occlusions of digital arteries (Figure 7). Maintaining the hematocrit below 0.45 by bloodletting alone in this case appeared to be ineffective, but aspirin completely abolished the painful ischemic swelling of the extremities, whereas the digital arteries remained occluded on the arteriogram 1 year later. PV case 4 suffered from a painful big toe, which progressed to acrocyanosis and subsequent black necrosis of the top together with pain-

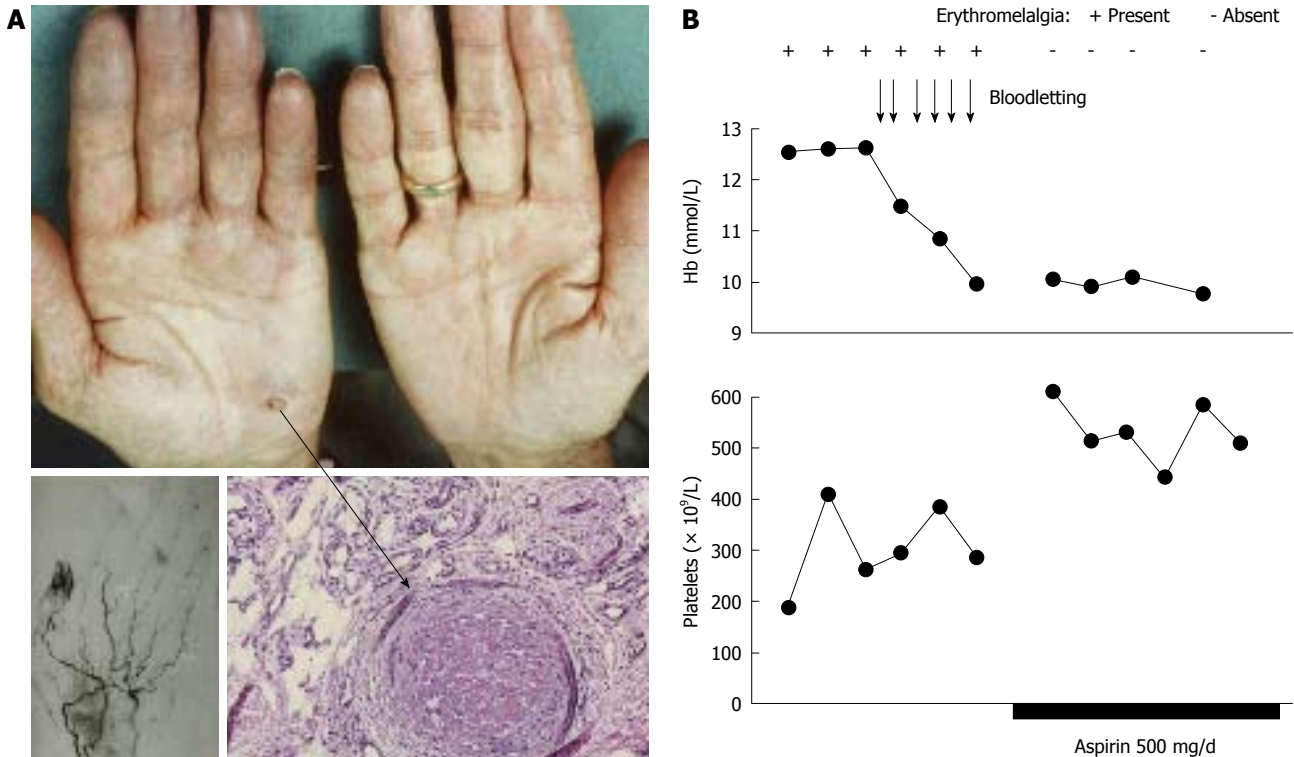


Figure 7 Acrocyanotic ischemic progression in a case of polycythemia vera 6 mo after presentation of erythromelalgia in the fingers of the right hand caused by endothrombotic occlusions of the superficial arterial arcade of the digital arteries. A: A skin biopsy taken from the blue ischemic spot of the handpalm (arrow) showed an occluded arteriole consistent with thrombo-angiitis obliterans; B: The erythromelalgic acrocyanotic congested fingers persisted after treatment of polycythemia vera with phlebotomy for 1 mo (4 wk), but treatment with aspirin during a subsequent second month (4 wk) induced complete relief of the painful blue swelling, which was associated with a significant increase of circulating platelets counts from below to above $400 \times 10^9/L$. Four weeks of phlebotomy treatment (horizontal axis) had no effect on acrocyanotic erythromelalgia of the fingers, and subsequent treatment with aspirin one dose daily for several weeks was associated with the disappearance of painful acrocyanotic erythromelalgic sign and symptoms and a significant rise in circulating platelet counts of about $200 \times 10^9/L$. Originated from Michiels *et al*^[83].

ful bluish discoloration and skin peeling of the second and third toe. Analgesics (paracetamol and pentazocine) and vitamin K antagonists were ineffective. The affected black big toe and red congested second and third toes in this PV patient completely recovered on treatment with aspirin 500 mg per day, which the patient experienced as a miracle. As the patient thought he was cured, he discontinued aspirin and full blown erythromelalgia of the big, 2nd and 3rd toe recurred. With low dose aspirin and control of the PV by phlebotomy he remained asymptomatic for more than 10 years. In case 5 with PV, the erythromelalgic ischemic acrocyanosis was complicated by severe black ischemia of the forefoot despite long-term oral anticoagulation with coumarin. Such a disastrous progression of platelet-mediated erythromelalgic acrocyanotic ischemia of the toes into a black gangrenous forefoot (thromboangiitis obliterans, Figure 7) in the surgical setting resulted in amputation of big and second toes. After adequate control of the PV by aspirin and phlebotomy keeping the hematocrit below 0.45, the patient remained asymptomatic for 9 years and died from lung cancer.

The London PV Study Group chaired by Weitherley-Mein^[84-86] demonstrated that on top of the microvascular disease of thrombocythemia, the incidence of major vascular episodes in PV correlated positively with increased hematocrit levels. Messinezy *et al*^[87] analyzed retrospectively 65 PV patients, who were followed from 1962 to 1983.

Table 8 Vascular complications, hematocrit and platelet counts in 65 patients with classical polycythemia vera at time of presentation and during follow-up treatment: results from a retrospective observational study 1985

	At presentation	After treatment
No. of PV patients	65	65
Follow-up	-	225 patient-years
Hematocrit	0.60 (0.49-0.74)	< 0.48 before 1975 < 0.45 since 1975
Platelet count ($\times 10^9/L$) (mean)	512	390
Major vascular occlusive events	49%	35%
Microvascular/large vessels	13/19 (not specified)	Specified
Cerebrovascular events mainly TIA	-	15
Superficial thrombophlebitis or venous thrombosis	-	20
Microvascular disturbances	-	5
Myocardial infarction	-	1
Mesenteric vein thrombosis	-	1

Study performed from 1962-1983. Phlebotomy to correct hematocrit and busulfan for platelet reduction and not treated with aspirin. Correction of hematocrit in PV to normal by bloodletting plus reduction of platelet count without the use of aspirin does not prevent vascular events. PV: Polycythemia vera; TIA: Transient ischemic attack. Originated from^[87].

A mean hematocrit of 0.60 and a mean platelet count of $512 \times 10^9/L$ at time of diagnosis of PV were associated

Table 9 Fatal and non-fatal major thrombosis in 1630 polycythemia vera patients enrolled in the prospective 2004 European Collaboration on Low Dose Aspirin PV studies^[90,91]

ECLAP study patient populations	n (%)
Observational study ^[91] , n = 1112 of which 66% on aspirin	
RCT low dose aspirin <i>vs</i> placebo ^[73] , n = 518 of which 50% on aspirin	
Total number of PV patients ^[90,91]	1630 (100)
On aspirin observational study plus trial	990 (61)
Previous thrombosis	636 (39)
Overall results, follow-up 2.7 yr	
Fatal thrombosis	67 (4.1)
Cardiovascular disease	35 (2.9)
Stroke	13 (0.8)
Pulmonary embolism	6 (0.4)
Non-fatal thrombosis	187 (11.4)
Arterial	90 (5.5)
Transient ischemic attacks	33 (2.0)
Stroke	23 (1.4)
Peripheral arterial thrombosis	20 (1.2)
Myocardial infarction	14 (0.9)
Venous	97 (5.9)
Superficial thrombophlebitis	46 (2.8)
Deep vein thrombosis	38 (2.3)
Pulmonary embolism	13 (0.8)
Total fatal and non-fatal thrombosis	254 (15.5)

Data of treated polycythemia vera (PV) patients on aspirin versus not on aspirin are only available for the European Collaboration on Low Dose Aspirin in PV (ECLAP) randomized clinical trial on low dose aspirin *vs* placebo. PV treatment modalities: 28% phlebotomy only, 26% chemotherapy only, and 35% phlebotomy plus chemotherapy, no phlebotomy and chemotherapy 11% and hydroxyurea was the cytoreductive drug in 79%.

with microvascular ischemic events and major thrombotic complications in 49% (Table 8). On top of phlebotomy aiming at a hematocrit below 0.48 before 1975 and below 0.45 after 1975, additional low dose busulphan resulted in a mean platelet count around $400 \times 10^9/L$ and no aspirin was given (Table 8). The follow-up of such 65 treated PV patients for a total of 225 patient-years was associated with the occurrence of 42 vascular events in 23 patients (35%) (Table 8). Vascular thrombotic episodes occurred in 7 at hematocrits below 0.44 and 18 at hematocrits above 0.45. The risk of major vascular episodes at was highest, about 35% to 50% at hematocrits above 0.50, when not on low dose aspirin^[86-88]. In personal letters to Weitherley-Mein in 1988 discussing our common experiences of thrombosis and bleeding in ET and PV, The London PV Study Group of Weitherley-Mein^[84-86] introduced the Dutch recommendation of low dose aspirin (Michiels *et al*^[5]) for thrombosis prevention in ET and PV^[71,88,89].

The European Collaboration on Low Dose Aspirin in PV (ECLAP) study was designed between 1994 and 1997 by Landolfi, Michiels and Patrono within the context of the European Working Group on Myeloproliferative Disorders, a scientific working group of the European Hematology Association founded and chaired by Michiels (1994-2004)^[88]. The Rotterdam ET study (van Genderen *et al*^[71]) was used for the design of the ECLAP study in 1997^[90,91]. Because nearly all PV patients in the United Kingdom and The Netherlands were on low dose aspirin, Pearson and

Michiels could not contribute PV patients to the randomized ECLAP study design. The ECLAP consisted of an observational prospective study of treated PV patients already using aspirin and a randomized prospective clinical trial comparing blindly low dose aspirin *vs* placebo in PV not yet on aspirin to address the question whether low dose aspirin indeed is safe and superior to placebo in preventing erythromelalgia, TIAs, non-fatal myocardial infarctions and stroke and vascular death in treated PV patients, aiming at a hematocrit of less than 0.45 (BIOMED 2 program funded by the European Community)^[90,91]. Overall, 1630 PV patients (57% males, median age at recruitment 65 years) were enrolled in the ECLAP study (Table 9). In the observational arm of 1112 of 1630 (68%) PV patients entered into an ongoing, observational prospective cohort study, two third of them had a clear indication for and did use low dose aspirin^[91]. Five hundred and eighteen of 1630 (32%) patients in whom the indication of aspirin was uncertain were randomly allocated to aspirin 100 mg/d or placebo in a double blind controlled trial^[90]. At the time of data collection, the overall cumulative incidence of cardiovascular events in all 1630 PV patients (follow-up 2.7 years, range 0-5.3 years) was 15.5% for fatal and non-fatal thrombotic events (Table 9) was consistent with 5.5 events/100 persons per year. Age over 65 years and a positive history of thrombosis were the two most important predictors of cardiovascular events. Smoking, hypertension and congestive heart failure were the other significant risk factors for major thrombosis^[90,91]. Aspirin therapy was the only variable associated with a lower risk of minor and major thrombosis. Platelet counts and the use of myelo-suppressive drugs compared to phlebotomy were not associated with the risk of major cardiovascular events^[91].

Only 518 out of 1630 PV patients in the ECLAP study were allocated to the randomization aspirin 100 mg *vs* placebo^[90]. Treatment modalities at time of randomization 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 are no differences of vascular risk factors (like hypertension, diabetes, hyperlipidemia, previous thrombosis, *etc.*) in the aspirin and the placebo group^[90]. Mean values in randomized PV patients were 0.45 for hematocrit and $330 \times 10^9/L$ for platelet count. Treatment with low dose aspirin 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. Absolute risk reduction was 8.4% and the number needed to treat to prevent one thrombotic event is 12^[90,91]. The rates and relative risk reduction according to primary end points for major thrombosis and for a secondary end point of major thrombosis are shown in Figures 8 and 9. These significant risk reductions in major thrombosis were seen very soon after randomization. Major total and gastro-intestinal bleeding were slightly increased without reaching statistical significance^[90]. These ECLAP data indeed confirmed the long-term experiences of the Rotterdam MPD Study Group (1981-1998) that low dose aspirin should be included on top of phlebotomy

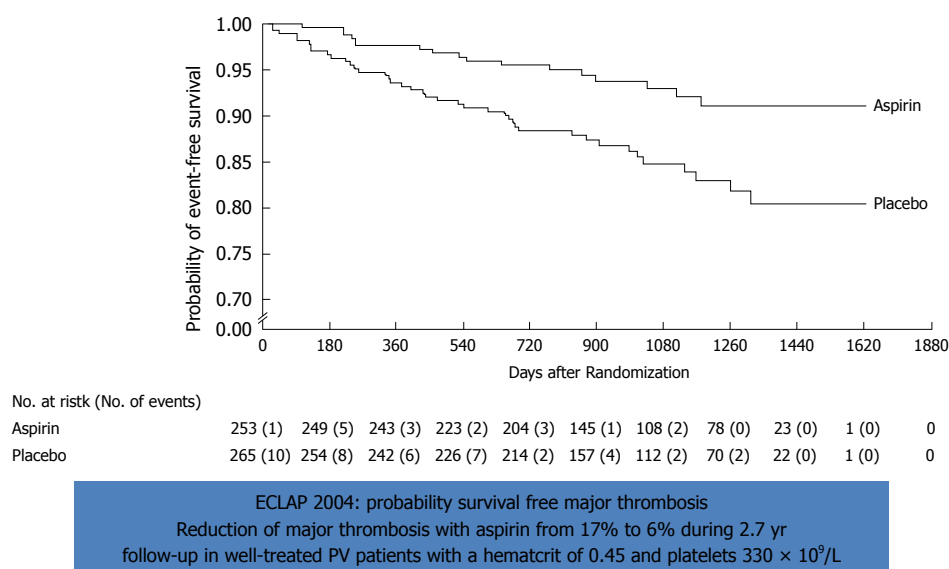


Figure 8 Effect of low dose aspirin on major thrombosis in treated polycythemia vera with bloodletting (40%) or hydra (60%).

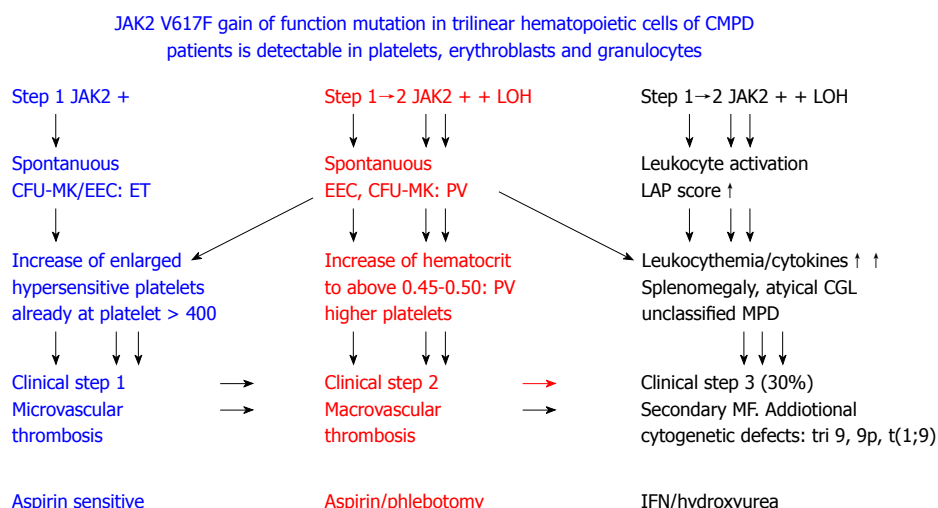


Figure 9 2005 ECLAP concept on the molecular etiology of essential thrombocythemia, prodromal and overt polycythemia vera, leukocytosis, platelet-mediated microvascular thrombosis, increased blood volume and secondary myelofibrosis in JAK2 mutated classical myeloproliferative disorders. ET: Essential thrombocythemia; PV: Polycythemia vera; LAP: Leukocyte alkaline phosphatase; MPD: Myeloproliferative disorders; IFN: Interferon. Originated from Michiels *et al.*^[106,107].

my alone in early stage PV, but also on top of treatment with hydroxyurea in high risk PV patients, even when in remission at a mean hematocrit of 0.45 and mean platelet count of around 300 to $350 \times 10^9/L$ ^[5,64,89].

INDUCTION OF COMPLETE HEMATOLOGICAL REMISSIONS BY IFN IN JAK2^{V617F} MUTATED PREFIBROTIC PV AND ET.MGM

From the results of a few prospective randomized studies in PV (PVSG 01, *etc.*), international PVSG experts concluded in 2000 that new clinical trials in previously untreated PV patients should focus on comparing IFN- α , a non-leukemogenic approach, *vs* a potential leukemogenic

myelosuppressive treatment modality^[92]. Compared to P32 and pipobroman, hydroxyurea is the least leukemogenic myelosuppressive agent in long-term prospective clinical PV-studies extending observation periods of more than 10 years^[93,94]. The rationale for using IFN- α as the first-line treatment option in newly diagnosed PV-patients include its effectiveness to abate constitutional symptoms and to induce a complete remission, thereby avoiding phlebotomy, iron deficiency and macrocytosis associated with hydroxyurea^[95-99]. IFN- α may prevent myelofibrosis if used early in the early prefibrotic stage of prodromal and overt PV disease^[92]. Clinicians will be reluctant to postpone the use of hydroxyurea in early stage PV as long as it is a conservative approach using phlebotomy aiming at a hematocrit below 0.45, on top of low dose aspirin for the control platelet function, and if indicated, anagrelide for

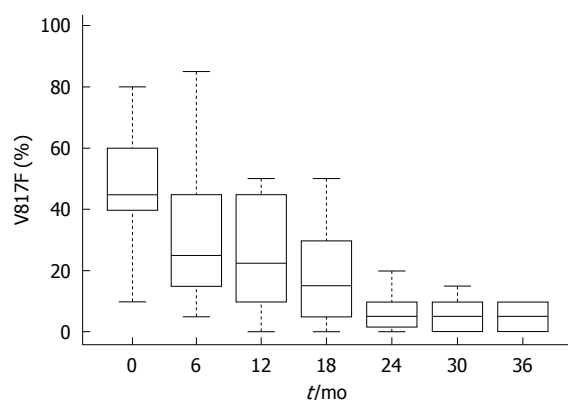


Figure 10 JAK2^{V617F} molecular responses in 38 patients with polycythemia vera during the first three years of treatment with interferon α -2a (Pegasys R) Courtesy of Jean Jacques Kiladjian, France.

the control platelet number is used to keep the PV patient healthy as long as possible. Silver^[95] did the pioneer clinical studies and never gave up. Kiladjian *et al.*^[96] and Quintás-Cardama *et al.*^[97] did the critical studies showing that complete hematological responses (CHR) did occur within one year, and that major molecular responses (MMR) were reached after a follow-up of 2 to 3 years in PV and ET patients (Figure 10). The cumulative incidence of MMR was 14% at 2 years and 30% at 4 years follow-up in the first study^[96]. Pegylated IFN α -2a reduced the median JAK2-allele burden from 45% to 5% in 37 PV patients in Kiladjian's study^[96] (Figure 10), and from 64% to 12% in 79 PV and ET patients in the study of Quintás-Cardama *et al.*^[97]. Larsen *et al.*^[98,99] confirmed the effectiveness of pegylated IFN α -2a in these two studies by the demonstration that a complete molecular response (CMR) may be reached, which was associated with normalization of bone marrow histology (Figure 11). Preliminary animal studies indicate that both hydroxyurea and JAK2 inhibitors are not able to eliminate the JAK2V617F clone in the bone marrow of JAK2^{V617F} mutated animals, whereas pegylated IFN in principle is able to reduce or even eliminate it^[100]. However, MPN patients and their physicians should be cautious and attentive not to become too enthusiastic since the use of pegylated IFN α -2a is associated with significant side effects in about one third of PV patients. We do know that only a proportion (30% to 50%) but not all early and intermediate stage PV patients are responsive to IFN. The misconception in the past was to start with too high dosages of IFN. A recent retrospective study of 118 MPN patients with various degrees of MPN disease burden, ranging from PVSG defined ET ($n = 46$), PV ($n = 55$) and PMF ($n = 17$), were comparable with regards to age and peripheral blood features, the JAK2^{V617F} mutation was present in PV 91%, ET 37% and MF 53%, and the spleen palpable (splenomegaly) in PV 25%, ET 13% and MF 47%^[101]. Data on bone marrow histopathology, circulating CD34 cells, lactate dehydrogenase and mutation load are lacking in this retrospective study^[101]. The complete response rate according to ELN criteria was 54% for PV and 63% for ET, but whether they reached complete re-

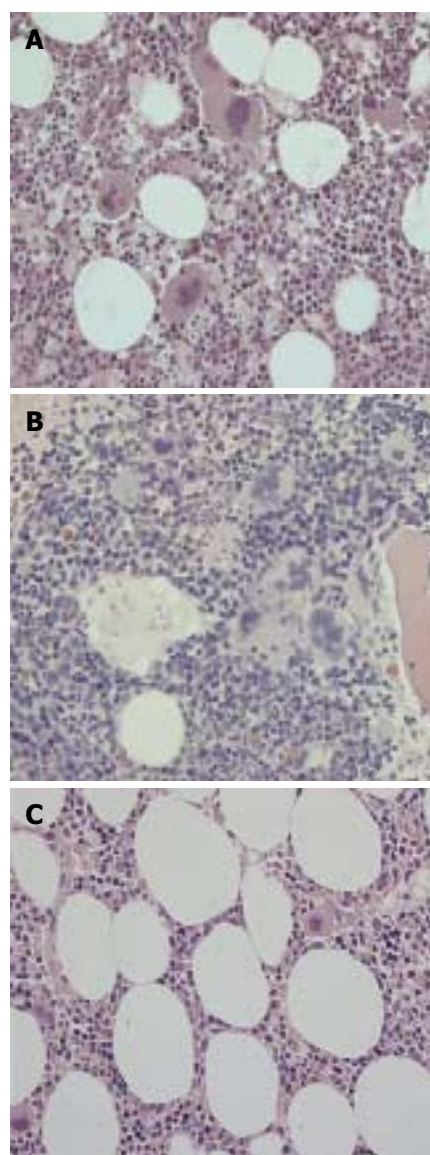


Figure 11 Bone marrow histomorphology from a patient with early prefibrotic polycythemia vera at diagnosis in 1996 (A) (hemoglobin 19.7 g/dL, hematocrit 0.60, leukocytes normal, platelets $750 \times 10^9/L$, modest splenomegaly, JAKV617F > 54%) just prior to interferon α -2b treatment (B) showing typical hypercellular polycythemia vera pictures with clustered large pleiomorphic megakaryocytes and normal cellular bone marrow with megakaryocytes of normal size and morphology (JAK2V617F < 1%) after 8 years of treatment with interferon α -2b treatment (C). Courtesy of Hans Hasselbalch, Denmark. A and B: Pleiomorphic (normal sized and enlarged) loosely clustered megakaryocytes with hyperploid nuclei and increased cellularity due to increased erythropoiesis in a case of polycythemia vera (PV) before start of interferon α -2b treatment in 1996; C: Megakaryocytes of normal size and morphology in a normocellular bone marrow in the case of PV (A, B) in complete hematological and molecular remission. Originated from Larsen *et al.*^[98].

sponses at the bone marrow and molecular level remained elusive. It is known to hematologists that IFN may be rather effective in prefibrotic primary myelofibrosis (PM-FMF 0/1) but much less and usually ineffective in advanced WHO defined PMF grade (MF/2/3). It is clearly shown that complete hematological and even significant molecular responses indeed do occur and only are reached 1 to 3 years after initiation of INF (Figure 10)^[96]. Conse-

quently, a loading dose of IFN seems to us to be useless and starting with low dose pegylated IFN has advances in prefibrotic JAK2^{V617F} mutated prodromal and early stage PV has two advantages: firstly, less side effects and secondly, it offers the unique opportunity to assess the sensitivity of IFN to reduce JAK2^{V617F} mutated cells and MPN disease load in that particular MPN patient. If responsive to low dose pegylated IFN (PegasysR) 45 ucg/wk, and having overcome the eventual initial hurdle of (minor) side effects during the first 6 mo to a year, it is recommended to slowly increase the dose in order to give confidence to the MPN patient that IFN really works better than hydroxyurea in terms of hematological and molecular responses. I have followed this strategy since 2000 in a few early stage PV patients with a previous 10-year history of JAK2^{V617F} mutated ET (manuscript in press). The JAK2^{V617F} mutated prodromal PV and early stage PV, as well as JAK2^{V617F} mutated ET patients with a hypercellular megakaryocytic-granulocytic myeloproliferation (MGM) of the bone marrow (ET.MGM) or prefibrotic PMF as defined by Thiele, are among the candidates for low dose pegylated IFN to postpone the use of hydroxyurea as long as possible. If IFN is not responsive or has been shown to elicit serious side effects, hydroxyurea becomes the second line treatment of choice to treat PV patients, with the aim to improve quality of life by control or reduction of MPN disease burden. In this situation, we should address the question of whether the JAK2 inhibitors as a non-leukemogenic approach is equal or superior to hydroxyurea in overt and advanced PV with a hypercellular bone marrow (80%-100%) at the stage just before the change of reversible benign reticulin fibrosis (RF 0/1 or MF 1) into irreversible reticulin/collagen fibrosis (RCF or MF 2) had occurred.

MOLECULAR ETIOLOGY OF PLATELET-MEDIATED ARTERIOLAR THROMBOSIS IN ET AND PV

The discovery of JAK2^{V617F} gain of function mutation by James *et al.*^[102] and his team in June 2004, published with significant delay in Nature in 2005, has become a real evolutionary event for a better understanding towards an unifying concept on the molecular etiology for ET and also for the clinical manifestations of platelet-mediated thrombosis, for the increased red cell mass complicated by major and secondary myelofibrosis. JAK2 plays an essential role in hematopoiesis by mediating signals from several hematopoietic cytokines, including EPO, TPO IL-3 G-CSF and GM-CSF. The JAK2^{V617F} mutation makes the mutated hematopoietic progenitor cells hypersensitive for TPO, EPO, IL3, G-CSF and GM-CSF, thereby leading to growth advantage of the mutated above the normal trilinear hematopoietic cells in the bone marrow. The discovery of the JAK2^{V617F} mutation by James *et al.*^[102] was rapidly confirmed by three groups of multidisciplinary investigators^[103-105]. Half of the ET patients and

the majority of PV patients have the mutated the JAK2 allele. The JAK2^{V617F} mutation affects the trilinear hematopoietic bone marrow cells and is detectable in platelets, erythroblasts and granulocytes. The gain of function mutation is in line with the concept that all “stops” to blood production in the bone marrow seem to have been pulled out by one factor JAK2^{V617F} causing, due to hypersensitivity of hematopoietic progenitor cells to growth factors, trilinear myeloproliferation. The 2005 concept of Vainchenker, Green and Michiels (France, United Kingdom and The Netherlands) (Figure 9)^[106,107] is that heterozygous JAK2V617F mutated hematopoietic progenitor cells may be enough for megakaryocyte proliferation with increase of hypersensitive platelets (ET mimicking PV), with no or slight increase of erythropoiesis (prodromal PV), and that heterozygous/homozygous or homozygous JAK2^{V617F} mutated hematopoietic progenitors cells will produce pronounced trilinear megakaryocyte, erythroid or even granulocytic proliferation with the clinical pictures of PV, granulocytic leukemia, unclassifiable MPD with secondary myelofibrosis. The sequential occurrence of heterozygous and homozygous JAK2^{V617F} mutation can readily explain the spontaneous megakaryocyte and erythroid colony formation (EEC), and the granulocyte precursors growth advantage with the production of increased hypersensitive platelets as a first step in ET (Figure 9) and increased hematocrit as a second step, aggravating the microvascular disturbances of thrombocythemia into the macrovascular complications of PV (Figure 9). Similarly, the sequential occurrence of heterozygous and homozygous JAK2^{V617F} mutation load can readily explain the dual granulocytic megakaryocytic proliferation in prefibrotic and early fibrotic ET with a hypercellular megakaryocytic/granulocytic (ET.MGM) bone marrow associated with constitutively activated leukocytes (increased LAP-score) and granulocythemia followed by secondary myelofibrosis without features of PV because of significant splenomegaly (Figure 9). ET.MGM is clearly in between ET and post-ET myelofibrosis. The association between JAK2^{V617F} mutation load with severity of both MPN disease burden as the cause of trilinear MPN (James *et al.*^[102]) and platelet-mediated thrombotic risk (Michiels *et al.*^[4,5])^[108,109] was already recognized by the bright clinician Dameshek^[1,110] as platelet thrombophilia with multiple small peripheral vascular thromboses in patients with trilinear PV.

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Another look at the life of a neutrophil

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Core tip: The lifespan of neutrophils is very dependent on the method used to determine it. Neutrophils are stored in pools and traveling from one location to another dependent on the occurrence of inflammation or not. It appears that isolating neutrophils and labeling them shortens their lifespan considerably. Their longer lifespan enables new functions assigned to them recently.

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Abstract

Neutrophils are considered as the privates of the innate immune system. They are born in the bone marrow, migrate to the tissues where they kill putative intruders. After their job they are quickly removed from the battlefield by macrophages. This view of a predetermined pathway fitted nicely in their short lifespan of 5 h. However, recent studies indicated that their lifespan was in the order of several days. Recently, it became clear that neutrophils have functions beyond killing of pathogens. The reported half-life of 5 h is hardly compatible with those functions. Moreover, the organism actively invests in rescuing primed neutrophils from clearance by the body. It appears that their half-life is highly dependent on the method used to measure their life span. Here, we discuss the literature and show that neutrophils compartmentalize which could explain partially the differences reported for their lifespan. Moreover, the methodology to label neutrophils *ex-vivo* could have similar deteriorating effects on their lifespan as found for transfused red blood cells.

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Key words: Neutrophils; Granulopoiesis; Homeostasis; Inflammation; Circulation; Radioactive labeling

INTRODUCTION

Neutrophils are polymorphonuclear leukocytes (PMNs), the main cell type of white blood cells in humans and are known for their specific segmented nucleus and their granules. They are the human body's main cellular components of the innate immune system, having an anti-infectious and a pro-inflammatory function. Upon infection, neutrophils are the first responders of the innate immune system to migrate towards the site of inflammation. They can ingest and kill invading microorganisms intracellularly by phagocytosis and the subsequent fusion of the phagosome with lysosomes containing antimicrobial peptides, enzymes and reactive oxygen intermediates (ROI). Neutrophils can also kill microbes extracellularly by the release of antimicrobial peptides and enzymes, stored in their granules^[1]. Besides its antimicrobial function, the neutrophil is able to express genes encoding inflammatory mediators such as growth factors, chemokines and cytokines^[2]. The presence of fully functional neutrophils in tissues is critical for the defense against microbial infections. This importance is seen in patients with leukocyte adhesion deficiency, which has neutrophil adhesion defects, resulting in poor crossing of the neutrophil across the endothelium covering the blood vessel into the diseased tissue area. These patients suffer from several bacterial infections,

which are life-threatening, due to the inability to destroy the pathogens by neutrophil phagocytosis^[3]. The importance of reactive oxygen species (ROS) formation is seen in patients with chronic granulomatous disease, who have defective oxidase function and are susceptible to recurrent bacterial and fungal infections^[4].

During activation not only extracellular pathogens are affected, the surrounding cells and tissues of the host are also damaged^[5]. To reduce the damage on host cells, neutrophils are quickly and efficiently removed from an inflammatory site by macrophage phagocytosis after functioning^[6,7]. However, in neonates^[8] and in some clinical settings such as sepsis^[9,10], COPD and acute coronary syndromes^[11-13], neutrophils are deactivated or apoptosis is reduced.

Every day, 10^{11} neutrophils are produced in the bone marrow making them the most abundant white blood cells^[14]. Neutrophils are thought to live only a couple of hours outside of the bone marrow after which they are phagocytosed and cleared, in the same rate as the production rate^[15]. In order to produce this amount of cells, and producing them at such a high rate, the bone marrow harbors a large constantly active granulopoiesis compartment. When during infection more neutrophils are needed, the bone marrow has reserve capacity to scale up the production.

Recently, neutrophils were found to have a blood lifespan of 5.4 d^[16], which is more than twenty times longer than found before^[17]. Although there are some concerns expressed about these recent findings^[18], this new lifespan also changes the paradigm of the neutrophil as a short living cell, produced in huge quantities only to kill microbes. Interestingly, with this significantly longer life span, new functions of a neutrophil can be foreseen. Indeed the first papers appear that describe a role for neutrophils in the shaping T-cell independent antibody responses^[19,20], but also functions such as antigen presentation and interactions with T cells are reported^[21-23]. Such functions demand a longer life span than the reported 5 h and could explain some recently identified roles of neutrophils in inflammatory diseases^[24-28].

Here, we review the current knowledge about neutrophil production, function and clearance. We address the question if the neutrophil is just a microbe killer with a unidirectional short life span or whether the neutrophil can reverse its unidirectional fate and by doing so prolong its life span.

First a general description of the exciting life of a neutrophil from birth to death is given. Different parts of the neutrophil life cycle are discussed as well as the kinetics. Several functions of neutrophils and the consequences of these functions to their life span will be discussed. Subsequently, the clearance of neutrophils is discussed in light of recent calculations of neutrophil populations and methods used, focusing on how the calculations are performed and which assumptions are made.

GRANULOPOIESIS

Neutrophils are produced in the bone marrow, where the

blood-forming process called hematopoiesis takes place^[29]. A hematopoietic stem cell (HSC) can proliferate and differentiate into a wide range of white and red blood cells (Figure 1A). Approximately two-thirds of the hematopoiesis is devoted to myelopoiesis: the formation of monocytes, megakaryocytes, red blood cells, dendritic cells and granulocytes^[1]. Each day, approximately 10^{11} neutrophils, the largest group of the granulocytes, are produced from HSCs under normal conditions (Figure 1B) but the rate of neutrophil production is highly dynamic. Factors influencing the rate of production are the rate of neutrophil apoptosis and immunological stress conditions. In immunologically stressed conditions, granulopoiesis and thereby the formation of neutrophils, is induced due to the production of several cytokines. For example, T-helper 17 cells have been shown to secrete interleukin (IL)-17 and other cytokines during inflammation that promote granulopoiesis, neutrophil proliferation and accumulation^[30]. On the other hand, during inflammation, neutrophils produce Pre-B cell colony-enhancing factor, thereby inhibiting neutrophil apoptosis and subsequently granulopoiesis^[31].

The granulopoietic compartment in the bone marrow can be divided into three pools: the stem cell pool (HSCs), the mitotic pool and the post-mitotic pool. The mitotic pool is the group of progenitor cells that are massively proliferating and differentiating. The bone marrow also comprises a reserve pool of mature neutrophils, approximately 20 times the number of neutrophils in circulation^[14]. The fully differentiated mature neutrophils define the post-mitotic pool, a pool ready for on demand release. Several stages of maturation of neutrophils can be discerned (Figure 1B). As differentiation and maturation progress, cells lose their ability to proliferate^[1]. In the terminally differentiated mature neutrophil state, cells can only progress unto death^[32].

For maintaining homeostatic levels of peripheral neutrophils and other blood cells, proliferation and differentiation of progenitor cells is tightly regulated and controlled by several intrinsic and extrinsic factors. For example, in bone marrow niches, HSCs retain in the niches through interaction of β -integrins on their membrane with osteoblasts and with the extracellular matrix (Figure 1B). An interaction essential for homing of HSCs and mature neutrophils is the interaction of chemokine receptor (CXCR4) with the bone marrow stromal cell derived factor 1 (SDF-1)^[33]. The interaction of Notch on HSCs with Jagged1 on osteoblasts is known to inhibit differentiation of HSCs in the bone marrow^[34]. Soluble factors known to maintain HSCs in the bone marrow are for example IL-1, -6, and -10 and thrombopoietin^[34].

One of the main regulating factors essential for tuning the production of neutrophils, is granulocyte colony stimulating factor (G-CSF)^[35]. G-CSF affects hematopoietic cells, through commitment of progenitor cells to the granulocyte lineage, massive proliferation of granulocytic precursors (*e.g.*, promyelocytes and myelocytes) and release of mature cells from the bone marrow^[36]. It induces effects *via* the G-CSF receptor, thereby activating an intracellular signaling cascade *via* signal transducer and activa-

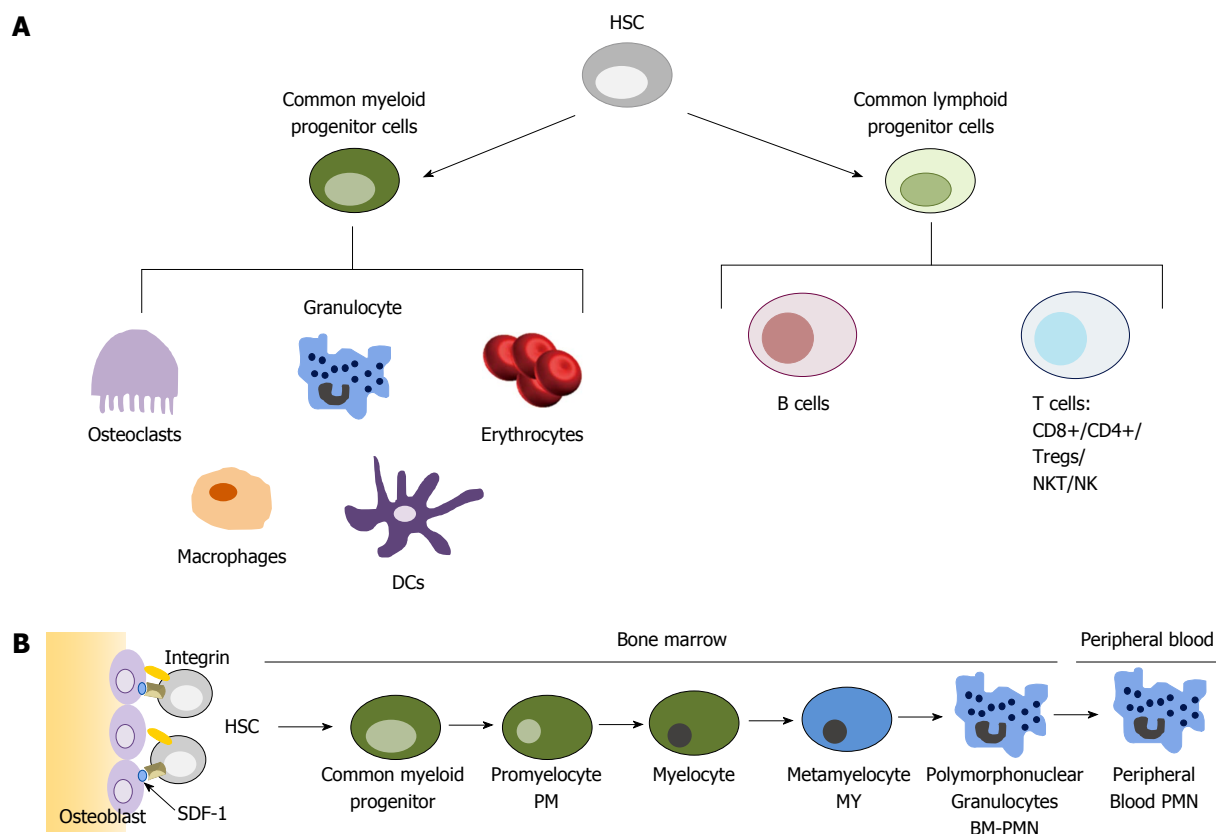


Figure 1 Granulopoiesis process. A: The production of blood cells from a hematopoietic stem cell. Modified from^[131]; B: Neutrophil maturation from the hematopoietic stem cell to mature neutrophils. Modified from^[1]. HSC: Hematopoietic stem cell; NK: Natural killer; DCs: Dendritic cells; PMN: Polymorphonuclear leukocyte.

tor of transcription 3 (STAT3). Where loss of the G-CSF receptor decreases the number of circulating neutrophils, injection of G-CSF increases neutrophil numbers in circulation^[37,38]. Furthermore, its production is up regulated with neutrophil apoptosis in the bone marrow and down-regulated when the number of neutrophils increases. In addition, during inflammation, different cytokines induce the production of G-CSF^[39] or act in synergy, like IL-1 β ^[40,41]. Other factors regulating neutrophil maintenance are IL-3, granulocyte-macrophage CSF (GM-CSF) and lymphoid enhancer-binding factor-1, targeting genes like survivin, cyclin D1, CEBP- α and c-myc^[42].

NEUTROPHIL RELEASE FROM THE BONE MARROW

After maturation in the bone marrow, neutrophils are stored, awaiting release into the circulation. To exit the bone marrow, the neutrophils have to migrate across the bone marrow endothelium that separates the marrow from the circulation. Stimulation to leave the bone marrow occurs during inflammation or infection by the presence of chemoattractant factors as leukotriene LTB₄, complement factor C5a, CXCL8 and intrinsic regulation factors like G-CSF, but recent findings describe that also circadian rhythms can contribute to neutrophil recruitment from the bone marrow^[43]. Under homeostatic conditions, G-CSF is the main regulator release of neutrophils.

During maturation, G-CSF receptors maintain highly expressed on the surface of neutrophils^[44], as well as on bone marrow stromal cells. G-CSF inhibits stromal cell production of SDF-1, thereby inhibiting the interaction with its receptor CXCR4 on neutrophils^[45]. G-CSF also functions in another bone marrow interaction with neutrophils. Bone marrow endothelial cells express vascular cell adhesion molecule 1 (VCAM-1), which interact with the integrin very late antigen-4 on neutrophils. G-CSF administration results in a loss of VCAM-1 on endothelial cells. G-CSF stimulates granule release of neutrophils, which contain proteases able to cleave VCAM-1^[46]. A third effect of G-CSF is exerted on the cytokine receptor CXCR2, which is essential for neutrophil release. G-CSF stimulates the expression of CXCR2 ligands on bone marrow endothelial cells, facilitating neutrophil release^[47]. In summary, G-CSF stimulates bone marrow endothelial cells in several ways to down regulate their neutrophil homing receptors and increase the expression of ligands inducing neutrophil release. After release, neutrophils can follow the gradient of chemoattractants into the tissues.

LEAVING THE CIRCULATION: HOMEOSTATIC VS INFLAMMATORY CONDITIONS

Upon infection and inflammation, several pro-inflam-

matory signals, like fMLP, LTB₄, CXCL8, C5a, CXCL1 and CXCL5, activate the vascular endothelium causing it to present adhesion molecules and chemotactic factors on the surface^[48-50]. P-selectins and E-selectins induced on endothelial cells will interact with PSGL-1, L-selectin and CD44 on neutrophils, mediating rolling and activation of the neutrophil integrins at the site of maximal chemokine concentration. These integrins then interact with ICAM-1 molecules on the endothelial cells, causing neutrophil arrest. Adhesion strengthening occurs with subsequent spreading of the neutrophil, resulting in intravascular crawling. The chemotactic process and the chemoattractant gradient both lead to a cytoskeletal rearrangement, necessary for the spreading and transmigration^[51]. The leukocyte adhesion cascade is described in detail elsewhere and this information can be found in^[49].

Once in the tissues, neutrophils are more prone to phagocytosis than blood neutrophils. As transmigration is partly mediated by fusion of secretory vesicles with the neutrophil membrane, several surface membrane receptors are added to the membrane as well as other functional proteins like chemoattractant and phagocytosis receptors. Upon stimulation by microbial moieties, G-CSF or GM-CSF, tumor necrosis factor- α (TNF- α) or Type I and II interferons in the inflamed tissue, neutrophils are functionally activated and start to transcribe and produce other chemokines, for example CXCL8^[2,52].

Priming

Activation of neutrophils is a two-step process, starting with priming by an initial exposure to mediators such as cytokines, which don't activate the neutrophils directly, but leave them in a "primed" state. These cytokines can be early-phase cytokines like TNF- α , IL-1 α and pathogen associated molecular patterns like endotoxin, as well as the earlier mentioned late-phase chemoattractants as IL-8, LTB₄ and GM-CSF. Priming can be described as a resting state of a neutrophil but with a functional response (*e.g.*, chemotaxis, ROS production) to be amplified upon another stimulus. Without priming, no maximal degranulation and activation of the NADPH oxidase can occur^[53]. Priming affects the neutrophil cytoskeletal organization to reduce deformability in order to retain in capillary beds^[54]. *In vitro*, priming (and subsequent shape change) has been shown to be reversible^[55], but there is limited data on the effects of priming on neutrophil kinetics *in vivo*. It is suggested that as 15% of the cardiac output can pass through an inflamed site each minute, all neutrophils are exposed to the priming stimulus within min. However, *in vivo* studies show a maximum 60% of primed circulating neutrophils, suggestive for de-priming *in vivo*^[56]. De-priming should protect the systemic circulation from the potentially damaging effects of primed cells, for example because of the produced H₂O₂, a marker of primed or activated neutrophils. Mixed venous blood (blood before the pulmonary circulation) has higher H₂O₂ compared with arterial blood (blood after the pulmonary circulation), suggesting the lung to be the de-priming compart-

ment^[57]. De-priming may have effects on the life span of the neutrophil, as priming can lead to neutrophil-mediated tissue damage and therefore these neutrophils are phagocytosed by macrophages early in the inflammatory response^[58]. Depriming may thus give an alternative way of clearance of harmful neutrophils in inflammatory responses^[55].

Functioning of activated neutrophils

Once activated in the tissues, transcriptional activity of neutrophils is up regulated, in part mediated by local G-CSF production, resulting in the production of cytokines^[2]. Also, the neutrophil will start phagocytosing microorganisms, degranulate, activate the oxidative metabolism intracellularly and will finally undergo apoptosis.

Degranulation is one of the first steps of neutrophil activation and is initiated during transmigration. The components of the different granules are well known and are described elsewhere^[59,60]. Not only anti-microbial proteins are stored in these compartments, but also proteases, components of the respiratory burst oxidase (described below) and a wide range of receptors, extracellular matrix proteins and soluble mediators of inflammation^[61]. Soluble inflammatory factors are for example chemotactic proteins^[62,63], inducers of vascular permeability changes^[64] and antigen presenting cell-activators^[65]. Degranulation transforms the neutrophil from passively circulating to being an effector cell of the innate immune system^[60].

Upon activation of the neutrophil, also the oxidative metabolism of the cell is activated. Neutrophils are very effective at the generation of ROS, a process called the oxygen metabolism or the respiratory burst. ROS or ROI are generated by the NADPH oxidase complex on the membrane of the cell. Some components are stored in storage sites, like secondary granules, which associate with the oxidase complex after fusion of these storage sites with the membrane or with phagosomes^[59]. These ROS serve as highly effective antimicrobial agents but are also highly damaging the host as the produced components are highly reactive. ROS producing neutrophils are rapidly cleared by macrophages.

An extend in the antimicrobial activity of the neutrophil is the formation of neutrophil extracellular traps (NETs)^[66]. The formation of NETs is a result of nuclear swelling and dissolved chromatin. Along with the nuclear swelling, granules are also disintegrated and as a result, large strands of unpacked DNA are extruded from the cell, carrying along proteins from granules and from the cytosol. At this time, already 24 different neutrophil proteins are associated with NETs, which are primarily proteins from primary granules (such as MPO and elastase), secondary granules (*e.g.*, lactoferrin and pentraxin 3) and tertiary granules (*e.g.*, MMP9)^[66,67]. NETs have been shown to trap microorganisms and promote interaction with the granule proteins, resulting in microbial recognition, antimicrobial activity and tissue remodeling. NET formation is a cell-death dependent process, also influencing the life span of neutrophils^[66,68].

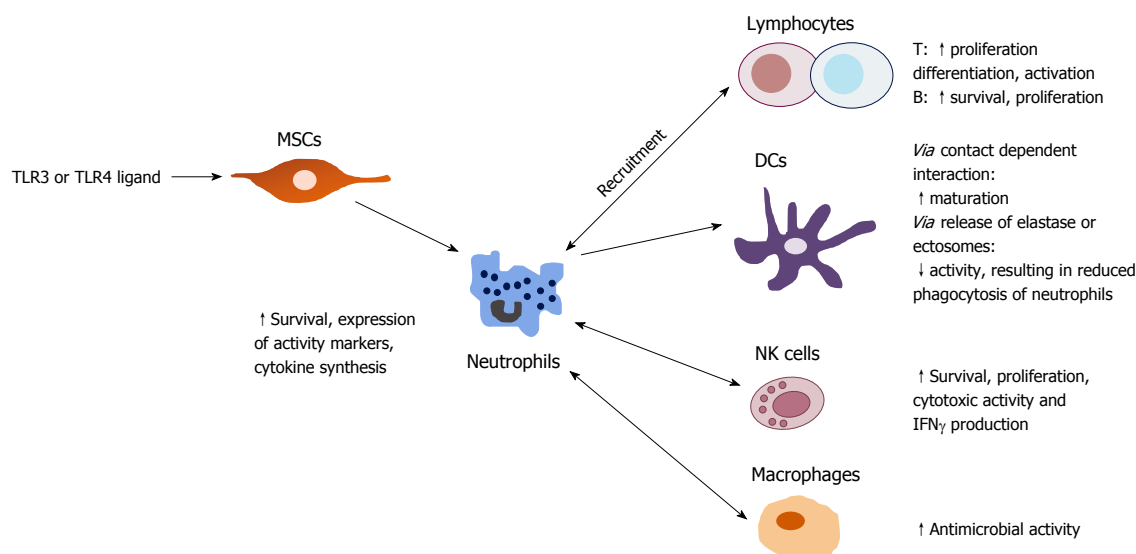


Figure 2 Cellular crosstalk of neutrophils in the tissues and in the lymph nodes. Modified from^[78]. TLR: Toll-like receptor; NK: Natural killer; DCs: Dendritic cells; MSCs: Mesenchymal stem cells; IFN: Interferon.

Deactivation of neutrophils

In addition to being harmful for microbes, the proteins that neutrophils secrete also damage the host tissue. Therefore it is important to control the influx of neutrophils to prevent excessive tissue damage. Neutrophil influx is controlled by several negative feedback loops at different stages of the inflammatory response. For example during the chemotactic process, it has been described that after a first encounter with CXCL8, neutrophils are desensitized to additional chemotactic signals^[69,70]. Intracellularly, there are proteins recruiting phosphotyrosine phosphatases, which deactivate receptors on the surface. For example suppressor of cytokine signaling 3 down regulates G-CSF receptor signaling by blocking the phosphotyrosine on the activated receptor thereby preventing the interaction with STAT3. Extracellularly, neutrophils and macrophages partner in the termination of inflammation^[71]. Neutrophils for example will express “eat-me signals” due to phospholipid asymmetry, triggering macrophages to phagocytose neutrophils^[72].

The receptor Chem R23 on macrophages, DCs and endothelial cells mediates activation of macrophages that enhances the phagocytic capacity of macrophages for uptake of apoptotic neutrophils. Neutrophil apoptosis itself is a specific process with different signals triggering apoptosis *via* different pathways. This process is described in detail elsewhere^[73,74]. Importantly, phagocytosis by macrophages reduces the risk of necrotic neutrophil death and down regulates the local G-CSF production to limit neutrophil activation^[1].

Other deactivating processes are granule-proteins like LL-37 and cathepsin G that stimulate rolling monocytes to migrate into the inflamed tissue. Neutrophil-derived proteins then stimulate the extravasated monocytes to mature into macrophages and subsequently phagocytose apoptotic neutrophils. The macrophages in turn release anti-inflammatory mediators such as IL-10, fur-

ther limiting the damage neutrophils do to host tissues^[71]. Importance of these deactivating signals is seen in clinical settings such as cystic fibrosis, in which neutrophils are insensitive to signals as IL-10 and corticoids^[75,76].

Additional known functioning of activated neutrophils

For a long time, neutrophils were thought to only be recruited to the inflamed tissue, act as phagocytic cells, release lytic enzymes and produce ROS, after which they were cleared. However, additional functions of neutrophils in inflammatory sites have recently been described. First of all, neutrophils were shown to express genes encoding inflammatory mediators^[2]. Secondly, neutrophils were found to produce anti-inflammatory molecules and factors promoting the resolution of inflammation, as described above and elsewhere^[56,77] and thirdly, neutrophils were shown to engage in interactions with different cells of the immune system^[20]. These new insights are very important for our understanding of inflammatory diseases, their resolution and possibility of neutrophils as targets to modulate immunity.

In vitro interactions with neutrophils have been shown for monocytes^[78], macrophages, DCs, natural killer (NK) cells, lymphocytes and mesenchymal stem cells in the tissues and were reviewed by^[79] (Figure 2). Also, crosstalk with platelets^[80] and regulatory T cells are described^[81]. First, neutrophils can induce the maturation of DCs *in vitro* through contact-dependent interactions involving CD18 and CEACAM1 on neutrophils and DC-SIGN on DCs. Subsequently, mature DCs induce T cell proliferation and polarization towards a Th1 response. However, neutrophils can also deactivate DCs *via* the production of elastase or ectosomes, containing transforming growth factor (TGF)- β 1^[79,82]. Deactivated DCs showed a reduced phagocytic activity, thereby preventing the phagocytosis of neutrophils.

Second, an interaction was unraveled between neutro-

phils and NK cells. Neutrophils are required both in the bone marrow as well as in the peripheral development of NK cells^[83]. They can modulate the survival, proliferation, cytotoxic activity and interferon γ (IFN γ) production of NK cells *via* the generation of ROS and/or the release of granules. NK cells can in turn promote neutrophil survival, expression of activation markers, priming of ROS production and cytokine synthesis^[84].

Direct cell-cell contact between neutrophils, NK cells and DCs has been shown *in vitro* as well, resulting in the increased release of IL-12 by DCs and an up regulated IFN γ expression by NK cells. IFN γ in turn stimulates neutrophil survival, expression of activation markers and cytokine synthesis^[85]. These effects have only been described for neutrophils *in vitro*, so further *in vivo* investigation is needed, but it gives new insights in the expanding functions of inflammatory site neutrophils. The importance of these additional functions is still elusive.

A third interaction is reported for neutrophils and lymphocytes. Neutrophils and lymphocytes can modulate each other's recruitment to the site of infection *via* the release of several released chemokines. Activated CD4⁺ and CD8⁺ T cells produce cytokines modulating neutrophil survival and expression of activation markers *in vitro*^[86]. In a similar fashion, $\gamma\delta$ T cells strongly promote neutrophil survival and activation by up regulation of CD64 and HLA-DR expression^[79]. Neutrophils also play an important role in B-cell help where they can even induce class switching of B-cells, a property solely assigned to T-cells^[20].

The next interaction described is the crosstalk with platelets. In transfusion-related acute lung injury, the leading cause of death after transfusion therapy, activated platelets were described to induce the formation of NETs^[80]. In another study, platelet were suggested to bind to neutrophils in the lungs, with subsequent activation of neutrophils by platelet toll-like receptor (TLR)4^[87].

In the interaction with monocytes, apoptotic neutrophils trigger the monocyte elicit an anti-inflammatory cytokine response through IL-10 and TGF- β , and to down-regulate the production of pro-inflammatory cytokines TNF- α and IL-1 β . In order to induce this response, cell-cell contact between the apoptotic neutrophil and monocytes was required^[78].

THE MARGINATED POOL

After leaving the bone marrow, the neutrophil becomes part of one of the two compartments found in blood: the circulating pool and the marginated pool. The circulating pool consists of neutrophils flowing freely through vascular spaces and the marginated pool consists of neutrophils adhered to the endothelium of capillaries and post capillary venules, often in the lung, liver and spleen^[15]. Already in 1867, Cohnheim observed cells in a marginal position along venule walls. Almost 50% of labeled granulocytes injected into healthy volunteers disappear rapidly from the circulation^[17]. This gave rise to the hypothesis that a marginated pool should exist. Next,

it was found that leukocytes circulate freely in the blood, then adhere to the vascular endothelium, especially in sites where the blood flow is slow and then re-enter the circulation in a continuously exchanging process^[88]. The relative size of the marginated and circulating pool however, can be affected during exercise or induced by adrenaline or drugs (Figure 3). It has been suggested that during infection the marginated pool is minimized, while the freely circulating pool becomes larger^[89]. The marginated pool consists of neutrophils adhered to the endothelium of capillaries and postcapillary venules, often in the lung, liver and spleen. The bone marrow has also been suggested as a margination site^[90]. Margination means a prolonged transit through these specific organs, resulting in an intravascular neutrophil pool. The lung has been a controversial margination site. Some data suggest that the lung is the predominant site of margination^[91], but this has been called into question by others^[92]. Interestingly, different neutrophil types localized in different organs^[93]. Suratt *et al*^[93] showed that mature peripheral blood neutrophils localize to the liver, bone marrow and to a lesser extent to the spleen. Younger marrow-derived neutrophils prefer to home back to the bone marrow, a process that will be described below, and inflammatory peritoneal neutrophils prefer the liver and the lungs. The biodistribution of inflammatory neutrophils might be non-comparable with homeostatic conditions as these neutrophils are different in surface expression of receptors and in functioning.

HOMING

Apoptotic neutrophils are not detected in normal circulation, so the need for an efficient removal system is evident, as 10^{11} neutrophils are believed to be produced and removed every day.

Surface receptor expression is highly dynamic upon infection, but receptor expression also changes upon aging. As neutrophils become senescent, expression of a receptor for chemotaxis, CXCR2, decreases, while the expression of a chemokine receptor, CXCR4, increases^[77,94]. Interestingly, the responsiveness to SDF-1 α , the ligand of CXCR4, increases in coincidence, resulting in homing of senescent neutrophils to the bone marrow. CXCR4 thus is not only a signal to retain neutrophils in the bone marrow, but is also acting on homing senescent cells to the marrow for destruction.

CXCR4 expression is up regulated just before apoptosis and after homing to the bone marrow, the neutrophils will undergo apoptosis and are subsequently phagocytosed by stromal macrophages, which are present in the hematopoietic cords^[73,95]. Furze *et al*^[96] showed that in mice, about one third of ^{111}In -labeled neutrophils were cleared *via* bone marrow stromal macrophages. Before, stromal macrophages were only known for the removal of cellular debris and non-productive B cells^[97]. Interestingly, if the labeled neutrophils were pretreated with pertussis toxin that inhibits the chemokine receptors,

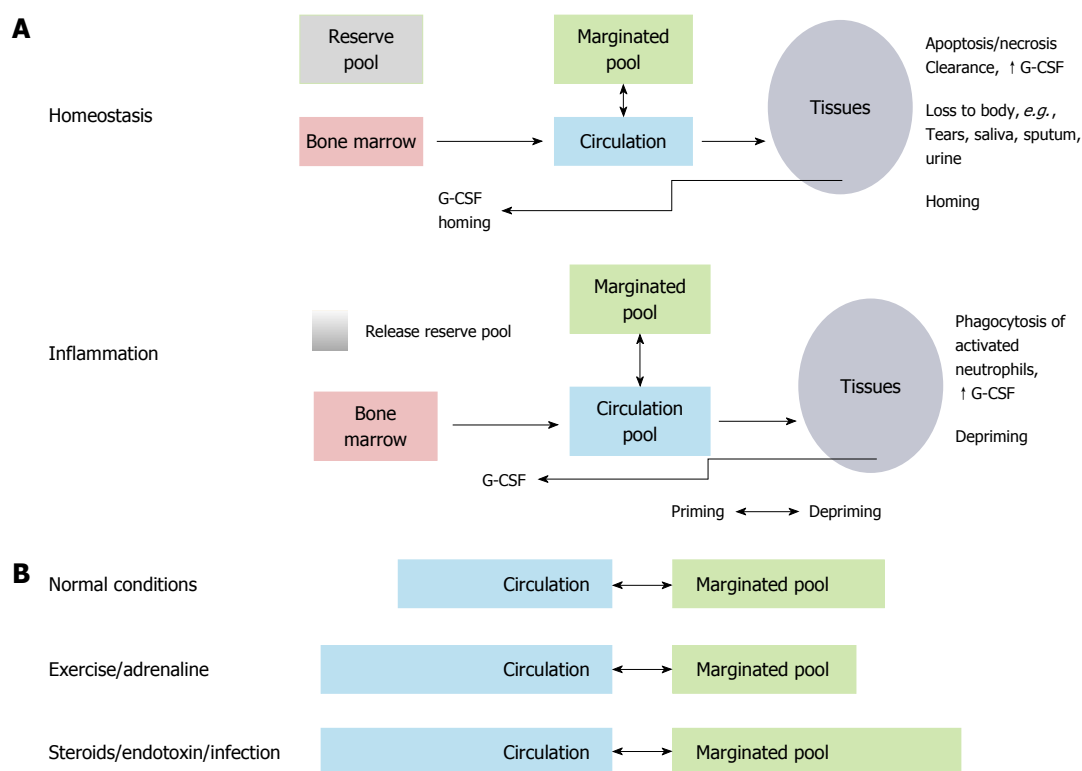


Figure 3 The margined pool. A: Neutrophil pools under homeostatic and inflammatory conditions. All pools remain present, but the pool sizes change significantly; B: The size of the margined and circulating granulocyte pool can be affected due to exercise or endotoxins or steroids. Modified from^[88]. G-CSF: Granulocyte colony stimulating factor.

neutrophil clearance *via* the bone marrow was inhibited for 75%, which is consistent with a role for chemokines, as clearance by the liver was unaffected by pertussis toxin treatment^[96].

Homing neutrophils must actively migrate through the bone marrow endothelium, a process that is not possible for apoptotic neutrophils. Neutrophils also home back to the bone marrow while the liver and spleen also remove circulating neutrophils. Furze *et al*^[96] showed that phagocytosis of neutrophils in the bone marrow stimulates G-CSF production which in turn induces neutrophil production in the bone marrow. Interestingly, when apoptotic neutrophils are phagocytosed by reticular endothelial macrophages in the spleen and liver or by macrophages on a site of infection, the production of G-CSF is suppressed to limit the inflammation^[98]. This way, *via* the up regulation of G-CSF production directly in the bone marrow, the production of new neutrophils can be tightly regulated. So if neutrophils are already apoptotic in circulation, the spleen and liver will clear them. On the other hand, senescent neutrophils can migrate back into the bone marrow and will be cleared there, as a positive feedback loop for neutrophil production.

To determine whether homing neutrophils can return to circulation, isolated neutrophils from the bone marrow and peripheral blood of mice were labeled and injected back into the mice^[92]. About 20 percent of labeled mature bone marrow neutrophils remobilized during an inflammatory response. However, homed bone mar-

row peripheral neutrophils could not be remobilized in response to inflammation. Therefore, the bone marrow could be seen as a site for clearance. In addition, this study also showed that infused marrow neutrophils may be remobilized. Other experiments indicated that 10% of labeled injected HSCs could leave the bone marrow, enter the blood, re-enter the bone marrow and still mature into granulocytes^[99]. It would be very interesting to further investigate the recirculating potential of mature neutrophils, as this can greatly influence our understanding of neutrophil kinetics.

KINETICS

The kinetics of neutrophil production, the amount of cells that are produced each day, is measured as a rate of turnover of neutrophils in the blood. Blood neutrophil turnover has been determined by labeling neutrophils with [³²P] DFP (di-isopropyl fluorophosphate) and has been described to be about 1.5×10^9 cells/kg per day^[100,101].

Marrow neutrophil production has been determined from the number of neutrophils in the post mitotic pool, divided by their transit time (the appearance in circulating neutrophils of injected ³H-thymidine) (Figure 4). The post mitotic pool consists of about 5.5×10^9 neutrophils/kg body weight and the transit time was about 6.6 d. The marrow neutrophil production has therefore been calculated to be 0.85×10^9 cells/kg per day. This amount corresponds to the calculated neutrophil turnover in blood.

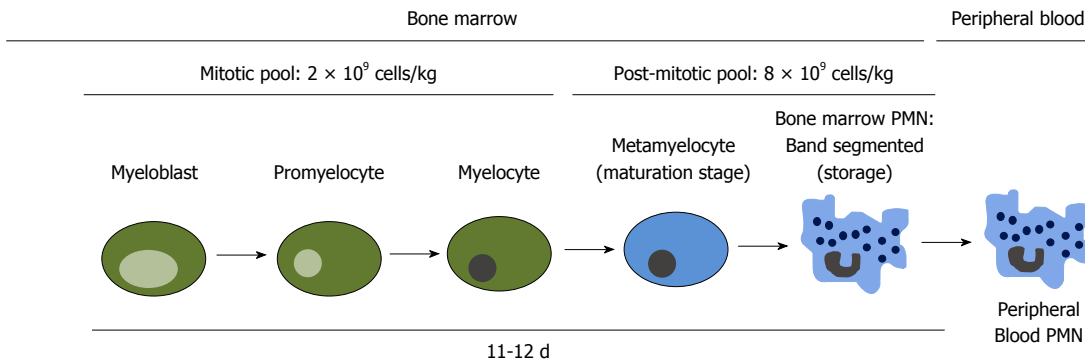


Figure 4 Neutrophil kinetics of the differentiation towards neutrophils in the bone marrow. Modified from^[106]. PMN: Polymorphonuclear leukocyte.

However, when cells were labeled with di-isopropylfluorophosphate-³²P, a larger turnover of neutrophils was found. Care should thus be taken with calculations and amounts, as they depend on the method to label cells^[14].

The different maturation stages all have different kinetics, which are studied *in vivo* and *in vitro* using radioisotopic labeling. These studies indicate that between the myeloblast and the myelocyte stages, approximately five cell divisions occur^[102,103]. Myelocytes probably undergo about three cell divisions, indicating the major expansion of the neutrophil pool to be at the myelocyte stage. The mitotic pool of neutrophils contains about 2 × 10⁹ cells/kg^[14], whereas the post mitotic pool contains about four times as much. These radionuclide studies suggest that the transit time from myeloblast to myelocyte takes about 135 h, divided over the different myelocyte stages (Figure 2). The transition from myelocyte to blood neutrophil takes about 131-158 h, indicating a total time of approximately 12 d from precursor to mature neutrophil^[102]. During infection, transition time from myelocyte to blood neutrophil can be shortened to 48 h.

Following production, mature post mitotic neutrophils (approximately 10¹¹ cells) will remain in the bone marrow for 4-6 d^[14,104]. In response to infection, the storage pool in the bone marrow will be used as source of neutrophils for blood neutrophilia^[105]. In conclusion, before a neutrophil leaves the bone marrow, it takes 17 d to be produced and matured^[106].

The kinetics of neutrophils leaving the vascular compartment and their take-over by new neutrophils can easily be measured by labeling neutrophils and measure the transit time through the vascular compartment. When healthy individuals are injected with neutrophils, they leave the vascular compartment with a 7 h half life time^[17,107]. Using radiolabelled neutrophils and other analytical techniques, the neutrophil intravascular transit time has been measured for the liver, spleen and bone marrow, being respectively 2 and 10 min. The intravascular transit time can be seen as the mean time taken for neutrophils to pass through the capillary bed of a specific organ. The influence of the marginated pool, homing back to the bone marrow and the kinetics in the spleen and liver on this transit time is unknown.

As the regulation of neutrophil production and clear-

ance is an important homeostatic mechanism and also involved in the development of systemic inflammatory states, it is of great importance that the kinetics of circulation and clearance are clear. Now we know that not only the liver and spleen, but also the bone marrow clears neutrophils, and that the different organs clear different types of neutrophils^[92]. But the function of neutrophils leaving the vascular compartment is largely unknown.

As described before, inflammatory neutrophils were found to have many more functions than only clearance of microbes. Possibly, neutrophils in marginated sites outside the vascular compartment, also have additional functions. There is growing evidence that to a certain extent neutrophils influence the adaptive immune response, either through pathogen shuttling to the lymph nodes^[108], through antigen presentation^[109], and through modulation of T helper responses^[110]. However, these described functions have still not been shown *in vivo* and also, are they neutrophil specific or do they occur as side effects of the functioning as a microbe-killer?

Methodology used for obtaining kinetic data: the effects of radioactive labeling

Without signs of infection, neutrophils do not get activated and have no need to go into the tissues. They also do not exocytose their granules, meaning that they are not as harmful for the host as activated neutrophils. The fate of these unactivated neutrophils is hard to investigate. Labeling neutrophils has revealed some of their fate, but labeling can also cause changes in the neutrophil (*e.g.*, prime or activate), which makes it a non-optimal technique for measuring unprimed circulating neutrophils. However, the studies which labeled neutrophils and followed their route through the human body are still very useful in this context.

In studies measuring neutrophil kinetics, different types of radioactive labeling have been used. ³²P-diisopropylfluorophosphate (DFP³²) is a potent and irreversible esterase inhibitor, which binds to granulocytes without modifying the viability of the cells and without being re-used after degradation of neutrophils. Furthermore, the label is only slightly or not at all attached to lymphocytes or monocytes^[111]. Other radioactive labels are In-111 oxine, Tc-99m sulfur colloid, Ga-67 labeling and Na⁵¹CrO₄

or SnCl₂-reduced ^{99m}TcO₄⁻. The effects of these radioactive labels on neutrophils have been studied by several authors, for example the effects on chemotactic responsiveness^[112]. Some labels are no longer in use, for example Na₂⁵¹CrO₄ and SnCl₂-reduced ^{99m}TcO₄⁻, which showed less optimal results in the chemotactic responsiveness studies. Other labels are still used, for example ³²DFP or ³H-thymidine.

The ideal radioactive agent should have the following properties: only label cells *in vivo*, only label neutrophils, do not elute from cells after labeling or being reused after degradation of the neutrophil, cause no radiation damage to the cells, emit γ radiation suitable for external detection and have a long enough half-life for studies without radioactive decay but short enough to limit patient-suffering^[113]. For a long time, only *in vitro* labeling was possible, where neutrophils were isolated from a blood sample, which could easily stimulate the neutrophils. Upon stimulation, neutrophils release their granules and are altered in surface receptor expression, and although the labeling experiments have been improved hardly any research was done to assess the activation of neutrophils or the change in surface receptor expression due to labeling^[114]. Some authors claim that there is no difference in neutrophil activation, without showing the data. But as neutrophils are quick responders to differences in their homeostatic environment, *in vitro*, *in vivo* or *in situ* labeling can have tremendous effects on the cell, affecting the outcome of a study as well. In mice, neutrophils were shown to have a half-life of 8 to 10 h when labeled *in vivo*^[115]. But when neutrophils were labeled *ex vivo*, 90% were cleared after 4 h, resulting in a half-life of only 1.5 h^[92]. This shows that the methods for labeling can have a devastating effect on the outcome of the study. But unfortunately, extrapolation of mice experiments is often very difficult. In mice, neutrophils are not the main circulating white blood cell-type, they do not express the same receptors as human neutrophils (for example there is a lack CXCR1) and also chemoattractant CXCL-8 does not exist in mice. Therefore, care should be taken when mice are used for calculating neutrophil life spans.

Most experiments done with *in vitro* labeling have not been repeated with *in vivo* labeling, meaning that some knowledge needs to be adjusted. Recently, Pillay *et al*^[116] used ²H₂O, a new labeling method for labeling neutrophil pools *in vivo*, to calculate the rate of division of the mitotic pool in the bone marrow, the transit time of new neutrophils through the post mitotic pool and the delay in mobilization of neutrophils from the post mitotic pool to the blood. They recalculated the life-span of neutrophils and found an average circulatory neutrophil lifespan of 5.4 d, which is 10 times longer than previously reported^[14]. However, there are also doubts concerning this report, as the previously used radioactive labels (*e.g.*, ³²DFP, H³-Th, Cr-51, In-111 and Tc-99m) all showed a lifespan of approximately 10 h. The new model is thought to lack the right temporal resolution to make these conclusions, as the mean value of the total life span of a neutrophil

is in line with the previously described total life span^[102]. Also, the authors did not show that the deuterium was not reutilized in newly dividing neutrophil precursor, thereby possibly influencing the results^[118]. Furthermore, if a concentration of 3×10^6 neutrophils/mL blood is maintained, the disappearance from the blood should be 5 h, considering the production rate of 1×10^9 cells/kg body weight^[88]. Either one of these two numbers should be reconsidered.

Interestingly, different maturation states of neutrophils are labeled by different radioactive labels. Warner and Athens compared the three most common radioactive labels *in vitro* until 1964, ³H-thymidine, ³²P-labeled sodium phosphate and ³²DFP, in their kinetics regarding the blood granulocyte radioactivity curves measured after administration^[103]. ³H-thymidine, a compound built in the DNA of newly formed neutrophils, showed a labeling of myelocytes and more immature forms, but neither PMN neutrophils in the blood nor PMNs and metamyelocytes in the bone marrow were labeled. ³²P-labeled phosphate was found in the same subsets of neutrophils, as it is also incorporated in DNA^[103]. ³²DFP labels granulocytes intracellularly and therefore, PMNs are directly labeled in the blood. The component(s) in the granulocytes to which DFP binds is unknown as DFP binds many different esterases and proteolytic enzymes^[111]. When the blood kinetics of all three populations is compared, they are all three totally different: ³²DFP levels start high, where after the labeled neutrophils disappear in marginated pools and the level of ³²DFP declines. ³H-thymidine labeled neutrophils appear later in the blood, after proliferation and differentiation and then the level declines (Figure 5).

In our opinion *in vivo* labeling is the better method. Isolating blood cells, processing and inject them again in the recipient can have dramatic effects on their life span. When leukemia patients are transfused with donated red blood cells after bone marrow transplantation, the half life on the donated red blood cells is dramatically reduced, leading to massive clearance of red blood cells. The released iron due to this enhanced turnover is a well known complication of red cell transfusion^[116]. This indicates that even careful isolation of blood cells without any labeling has an impressive effect on their life span.

A proper understanding of the lifespan and distribution of the neutrophil is very important, as the neutrophil can vary in phenotype and function with a longer lifespan, and the lifespan determines the need for influencing the neutrophil function in inflammatory diseases. Further investigation of these different labeling techniques, their influence on neutrophil life span and the actual life span of a neutrophil are needed.

BEHAVIOR OF TISSUE NEUTROPHILS IN COMPARISON TO BLOOD NEUTROPHILS

Besides the effects of labeling neutrophils, the behavior of blood neutrophils compared to tissue neutrophils should also be taken into account. During *in vitro* culture,

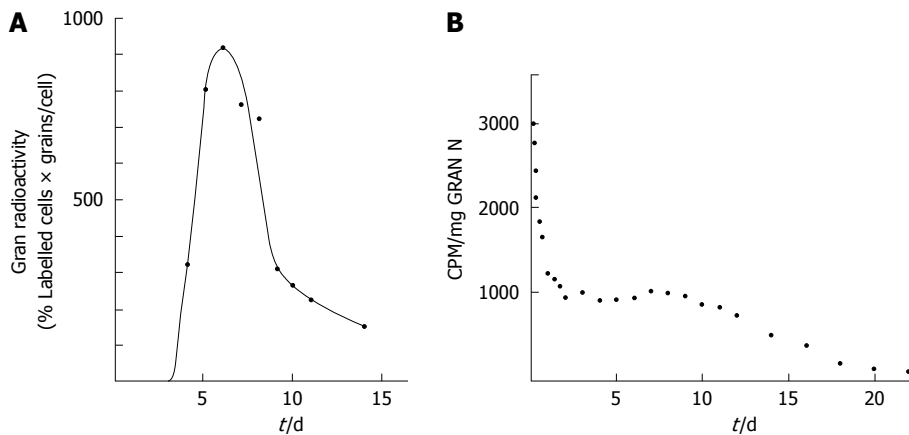


Figure 5 The kinetics of neutrophil production, the amount of cells that are produced each day, is measured as a rate of turnover of neutrophils in the blood. A: Decay of radioactivity of blood granulocytes after intravenous injection of ^3H -thymidine; B: Decay of radioactivity of blood granulocytes after intravenous injection of ^{32}Di -Isopropylfluorophosphate. Modified from^[103].

neutrophils are able to spontaneously enter apoptosis, a process which initiation can be accelerated or delayed by several factors. Danger signals such as TLR ligands are potentially anti-apoptotic^[117] while pro-inflammatory cytokine GM-CSF^[118] and signaling from death receptor Fas can induce cell death^[119]. Furthermore, culturing neutrophils in hypoxia reduces apoptosis, which improves the lifespan *in vitro*^[120]. Interestingly, neutrophil apoptosis and the regulation of death processes are almost all studied in blood neutrophils, while the bulk of neutrophil apoptosis takes place in the tissues, as well as the clearance. This results in a lack in information about tissue neutrophil apoptosis. Tissue neutrophils can be obtained *in vitro* by experiments with so-called aseptic skin chamber techniques^[121]. These transmigrated neutrophils have different gene transcription and behave differently than peripheral blood neutrophils^[122], as transmigration induces mobilization of certain intracellular granules^[123]. Interestingly, these transmigrated neutrophils also differ in responsiveness to stimulating agents. A wide variety of anti-apoptotic factors (the earlier mentioned TLR ligands and GM-CSF) were unable to delay apoptosis in transmigrated neutrophils^[124]. This way, Christenson *et al.*^[124] showed functional differences in transmigrated tissue neutrophils compared to blood neutrophils. As tissue neutrophils differ from blood neutrophils in surface receptor expression and respond differently to certain stimulation, maybe earlier made conclusions regarding apoptosis pathways and life spans based on studies on blood neutrophils are only particularly true for tissue neutrophils, and this has to be further investigated.

TRANSMIGRATION OF TISSUE NEUTROPHILS

Neutrophils are thought to have little functional plasticity after differentiation, in comparison to monocytes and macrophages^[125]. After recruitment into the tissue, they fulfill their immune function and die by apoptosis and phagocytosis by macrophages. Studies with rats have

suggested that neutrophils can emigrate out of inflamed tissue and return to the circulation^[126]. Recently, a study in humans showed neutrophils that emigrate out of the tissues *in vitro* via the lymphatics^[127], in a manner similar to that described for monocytes^[128]. These reverse transmigrated neutrophils are phenotypically and functionally different from circulating neutrophils and are found *in vivo* in the blood of healthy persons. Interestingly, these neutrophils are also found at significantly higher levels in patients with chronic active inflammatory disease, suggesting a role for these neutrophils in the persistence of inflammations in humans. This new perspective on the possibility of neutrophils to reverse transmigrate gives new insight in chronic inflammation, but also in the kinetics of neutrophil clearance in the tissues. The clearance of neutrophils in tissues is no longer only subscribed to apoptosis and phagocytosis, but also to reverse transmigration back into the circulation. The *in vivo* kinetics of transmigration is therefore a much needed future study.

CONTAMINATION

When using sensitive quantitative studies such as RT-PCR or measuring cytokine production, there is a big risk of contamination by monocytes and lymphocytes. Depending on the cytokine, neutrophils possess 10-20 fold lower RNA levels per cell than monocytes or lymphocytes. This means that neutrophils synthesize 10-300 times less cytokines than monocytes individually and causes a 1%-2% monocyte contamination to influence the RNA yield with 20%-30%^[12]. It is thus important when measuring cytokine levels, to keep the level of contamination with monocytes and lymphocytes very low (< 0.5%). The neutrophil-specific surface marker CD66b could be used to determine purity of samples^[129].

As earlier mentioned the exclusion of prestimulation of neutrophils is important. Every reagent, solution or lab ware with small levels of endotoxin can stimulate neutrophils. Inappropriate methods of erythrocyte lysis can lead to stimulation as well. To exclude stimulated

neutrophils, the cells should be checked for CD62L, a membrane bound antigen, rapidly released upon neutrophil stimulation^[130].

With this in mind, it will be interesting to investigate some of the current papers about neutrophil kinetics. For example in the paper of Suratt *et al.*^[92], the neutrophils are only tested for viability using Trypan blue dye exclusion. No control experiment for CD62L expression and thus activation was performed. The authors did obtain neutrophils with a modified method, to ensure depletion of contaminating monocytes.

Other studies compared tissue neutrophils with blood neutrophils, but no investigation into the functionality of neutrophils after collecting them was done^[124]. Care should be taken when drawing conclusions from papers without proper controls for monocyte contamination or neutrophil activation. Also the type of labeling is important and the type of neutrophil used for different studies, as tissue neutrophils differ from blood neutrophils.

CONCLUDING REMARKS

The neutrophil has more functionality than just killing microbes, it also has a role in signaling to both the innate and adaptive immunity, the resolution of inflammation and cellular signaling with DCs and T cells. The mechanism of clearance of neutrophils and homing to the bone marrow is of great importance to the balance of cellular homeostasis. Clearance in the bone marrow leads to new neutrophil production; clearance in the spleen, liver and tissues reduces damage. This way, our body is able to continue the cellular homeostasis and the levels of circulating neutrophils. The neutrophil is important, but its clearance too. Because neutrophils are readily activated in experiments, a proper *in vivo* experiment is difficult to set up. More investigation is needed to elucidate the role of the different types of neutrophils in immunity. Tissue neutrophils differ from blood neutrophils, as well as the marginated pool differs from the circulating pool.

There are numerous studies done to the kinetics and life span of the neutrophil. The calculated blood circulation time varies from 10 h to over 5 d, each life span having tremendous effects on the functions of neutrophils. Further investigation to the lifespan and production rate is necessary, as current calculations are all based on different labeling techniques with different disadvantages and no clear conclusions can be drawn. In analogy to red blood cells, it is to be expected that after collection of cells, the life span decreases tremendously. *In vivo* labeling of neutrophil can prevent such effects on life time of a neutrophil. Also, the different pools that are present have to be taken into account when assessing the lifetime of neutrophils. Many tools for investigating the function of neutrophils in mice *in vivo* are now available. Although understanding the role of the neutrophil *in vivo* in man is much more difficult, it is of great importance for the potential role of neutrophils as targets in inflammatory diseases.

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Autologous lymphocytes infusion

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our case, suggesting that this treatment modality might benefit lymphoma patients.

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Abstract

The graft *vs* tumor effect produced by the infusion of allo-reactive lymphocytes is considered to be the main mechanism of action in the eradication of tumor cells only reported in allogeneic stem cell transplantation. We present a case of a lymphoma patient infused with his collected bystander lymphocytes from is stem cell autograft after failing to collect enough stem cells to proceed with autologous stem cells transplantation, resulting in tumor response with no treatment related toxicity. This case illustrates the concept of autologous lymphocyte infusion, suggesting the possibility of an autograft *vs* tumor effect, as an effort to parallel donor lymphocyte infusion in allogeneic stem cell transplantation to create a graft *vs* tumor effect by increasing donor lymphocytes in the patient.

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Key words: Autologous lymphocytes infusion; Autograft *vs* tumor effect; Natural killer cells; Tumor regression; Survival

Core tip: This case report illustrated the possibility of an autograft *vs* tumor effect by infusion autologous lymphocytes collected during stem cell collection without the side effect graft *vs* host disease. The infusion of autologous lymphocytes produced tumor regression in

INTRODUCTION

The clinical evidence of graft *vs* tumor effect reported in the allogeneic stem cell transplantation setting has been recognized based on indirect observations: (1) anecdotal reports showing the sudden withdrawal of immunosuppression in relapsed patients post-allogeneic stem cell transplantation can re-established complete remission; (2) increased relapse rates in patients receiving syngeneic marrow graft compared with recipients of allogeneic grafts; (3) decreased relapse rates post-allogeneic stem cell transplantation in association with graft-*vs*-host disease; (4) T-cell depletion of an allogeneic graft increases the risk of relapse; and (5) donor lymphocyte infusion induces complete remission after relapse following allogeneic stem cell transplantation^[1]. In contrast to allogeneic stem cell transplantation, the high-dose chemotherapy used in autologous stem cell transplantation is considered to be the sole mechanism of action to eradicate resistant tumor clones that escaped standard chemotherapy because of the presumed lack of an autologous graft (autograft) *vs* tumor effect that parallels the graft *vs* tumor effect reported in allogeneic stem cell transplantation^[1]. However, our published studies reporting that the recovery of absolute lymphocyte count post-autologous stem cell transplantation is associated with superior clinical outcomes without the detrimental effects of graft *vs* host disease suggests the possibility of an autograft *vs* tumor effect^[2-4]. Furthermore, the discovery that early absolute lymphocyte recovery post-autologous stem cell transplantation, as surrogate marker of host immunity in autologous stem cell transplantation, is directly dependent on the absolute numbers of infused

7/05

Autologous lymphocyte infusion = infused 0.48×10^9 lymphocytes/kg

	6/05	8/05	1/06	5/08	Normal range
Hgb	11.8	11.3	10.6	8.7	(13.5-17.5)
WBC	2.4	2.3	2.2	1.6	(3.5-10.0)
ANC	1.59	1.42	1.18	0.4	(1.7-7.0)
Plts	60	61	65	19	(150-450)
ALC	0.24	0.44	0.60	0.5	(0.9-2.9)
NK	53	105	128	89	(80-597)
CD4	121	149	190	42	(401-1532)
CD8	88	85	132	108	(352-838)

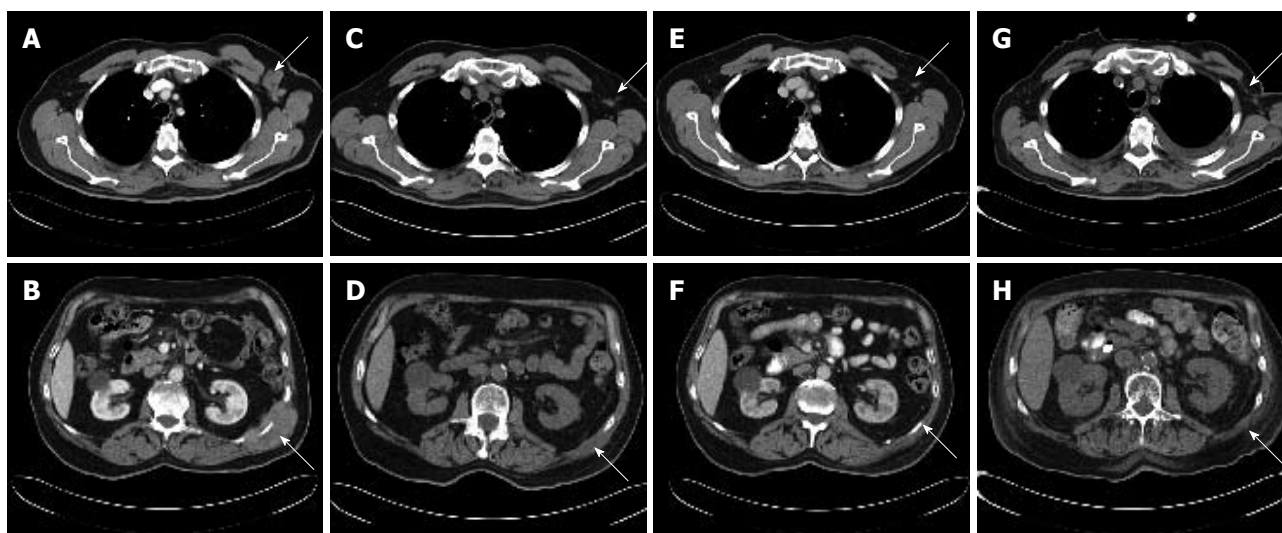


Figure 1 Computer tomography scans before and after autologous lymphocyte infusion (arrow). A, B: In June 2005 he developed new flank pain and a computer tomography scan; C, D: Follow-up computer tomography scan in August 2005 showed improvement of the left axillary adenopathy (1.4 cm $\times$ 0.5 cm) and the left flank mass (4.3 cm $\times$ 1.8 cm); E-H: Subsequent scan on January 2006 and May 2008. The units for absolute lymphocyte count (ALC), absolute neutrophil count (ANC), platelets (Plts), and white blood cell (WBC) count are $\times 10^9/L$, the units for the lymphocyte subsets are in cells/ μL , and the unit for hemoglobin (Hgb) is in g/dL. NK: Natural killer cells.

bystander lymphocytes (immune effector cells) harvested during CD34⁺ stem cells collection argues that the manipulation of the immunocompetent effector cells in the autograft can affect not only immune recovery but also clinical outcomes in the post-autologous stem cell transplantation^[3]. Thus, the infusion of collected “bystander” lymphocytes from a stem cell autograft correlates with lymphocyte recovery and clinical outcomes in lymphoma patients undergoing autologous stem cell transplantation. Herein, we report a case of a lymphoma patient that failed to collect enough stem cell to proceed to autologous stem cell transplantation, but received his collected bystander autologous lymphoma resulting in tumor regression.

CASE REPORT

In November 2000, a 70-year-old man presented with worsening abdominal pain, distension, heartburn and early satiety. Patient was evaluated by local physician who identified an abdominal mass measuring 6.0 cm by 5.0 cm. Computer tomography scan demonstrated an abdominal mass measuring 11.0 cm by 7.0 cm arising from the pancreas into the mesenteric. Needle biopsy of the mesenteric mass revealed diffuse large B-cell lymphoma (DLBCL) is Eastern Cooperative Oncology Group performance

status was 1, bone marrow biopsy was negative, lactate dehydrogenase of 297 U/L, and his stage was IIE. He achieved a complete remission after six cycles of cyclophosphamide, adriamycin, vincristine, and prednisone chemotherapy in February 2001. In August 2001, the patient presented with hypercalcemia (12.8 mg/dL), and left axillary adenopathy. A bone marrow biopsy demonstrated 80% of DLBCL lymphoma involvement. Salvage chemotherapy with ifosfamide, etoposide and carboplatin was initiated resolving the lymphadenopathy, hypercalcemia and repeated bone marrow biopsy demonstrated 5% lymphoma involvement. In February 2002, patient preceded to peripheral blood stem cell collection. The patient only collected $1.03 \times 10^6/kg$ stem cells through is peripheral blood; 2.0×10^6 cells/kg is the minimum required for autologous stem cell transplantation. The patient did not proceed to autologous stem cell transplantation due to his low stem cell numbers. The decision was to observe the patient and he did well. In June 2005 he developed new flank pain and a computer tomography scan (Figure 1A and B) demonstrated new masses: left axillary mass of 3.5 cm $\times$ 2.6 cm and left flank mass of 8.4 cm $\times$ 5.4 cm. Biopsy of the left flank mass revealed DLBCL. The patient refused any further cytotoxic chemotherapy but was willing to consider non-cytotoxic approaches. We discussed

with him the option of infusing his autograft lymphocytes that were collected at the same time of his stem cell collection, based on our previous publications in autologous stem cell transplantation that recovery of lymphocytes improves clinical outcomes in lymphomas^[2-4]. In July 2005 patient received 0.48×10^9 lymphocytes/kg. The infusion of autologous lymphocytes was approved by our Institutional Review Board and patient consented also for the procedure. The patient did not experience any side effects or toxicities from the autologous lymphocytes infusion. His absolute lymphocyte count prior to the lymphocyte infusion was $0.24 \times 10^9/L$. After the infusion, the absolute lymphocyte count improved to $0.44 \times 10^9/L$ (Figure 1). His peripheral blood lymphocyte subsets showed normalization of natural killer (NK) cells (Figure 1). Patient received interleukin-2 million units three times a week for 6 wk. Follow-up computer tomography scan in August 2005 showed improvement of the left axillary adenopathy (1.4 cm $\times$ 0.5 cm) and the left flank mass (4.3 cm $\times$ 1.8 cm) (Figure 1C and D) and subsequent scan on January 2006 (Figure 1E and F) and May 2008 (Figure 1G and H) continued to showed resolution of the masses. In addition, patient's NK cells count remained in the normal range after the autologous lymphocyte infusion. In May 2008, patient showed pancytopenia and bone marrow biopsy revealed therapy-related myelodysplastic syndrome with refractory cytopenia with multilineage dysplasia and documented monosomy 7 secondary to prior chemotherapy treatment with cyclophosphamide, carboplatin, and etoposide. The bone marrow biopsy was negative for involvement for lymphoma. In September 5, 2008 the patient succumbed to complications from myelodysplastic syndrome with no recurrence of his DLBCL.

DISCUSSION

To our knowledge; the infusion of autologous bystander lymphocytes from the stem cell autograft in a non-transplant setting has never been reported. We present a case of a lymphoma patient who failed to collect sufficient stem cells for autologous stem cell transplantation. The stored autograft was infused in an attempt to halt lymphoma progression by increasing lymphocyte counts, as an effort to parallel a "donor lymphocyte infusion" of allograft recipients to create "graft-*vs*-tumor effect" by increasing donor lymphocyte counts in the patient. After the autologous lymphocyte infusion, our patient showed increment of his absolute lymphocyte count with normalization of his NK cells. NK cells have been associated as the key lymphocytes affecting clinical outcomes in lymphoma patients undergoing autologous stem cell transplantation^[5]. Patient remained in remission in concordance with normal peripheral blood NK cells counts (Figure 1). A limitation of this case report was the lack of reporting

the absolute lymphocyte subsets infused to the patient. From our previous publication the infusion of 0.5×10^6 lymphocytes/kg was associated with faster lymphocyte recovery post-autologous stem cell transplantation. Our patient received 0.48×10^6 lymphocyte/kg resulting in the normalization of the peripheral blood NK cells observed just 1 mo after her infusion of his autologous lymphocytes. Patient did not develop any side effects from the autologous lymphocyte infusion and he did not develop any signs or symptoms of autologous graft versus host disease. Due to the immunosuppressive effects of the tumor, higher number of non-cytotoxic NK cells and overexpression of inhibitory NK cells receptors have been observed in conjunction low numbers of cytotoxic NK cells and reduced expression of activating receptors ailing tumor progression. In our case, the NK cells collected in the autograft where collected when the patient was in complete remission. Thus, it is reasonable to assume that infused NK cells have not been tampered by the immunosuppressive effects of the lymphoma leading to the recognition and eradication of the lymphoma in our case^[6]. Our intervention resulted in a dramatic and sustained treatment response with no treatment related toxicity, suggesting the concept of autograft *vs* tumor effect.

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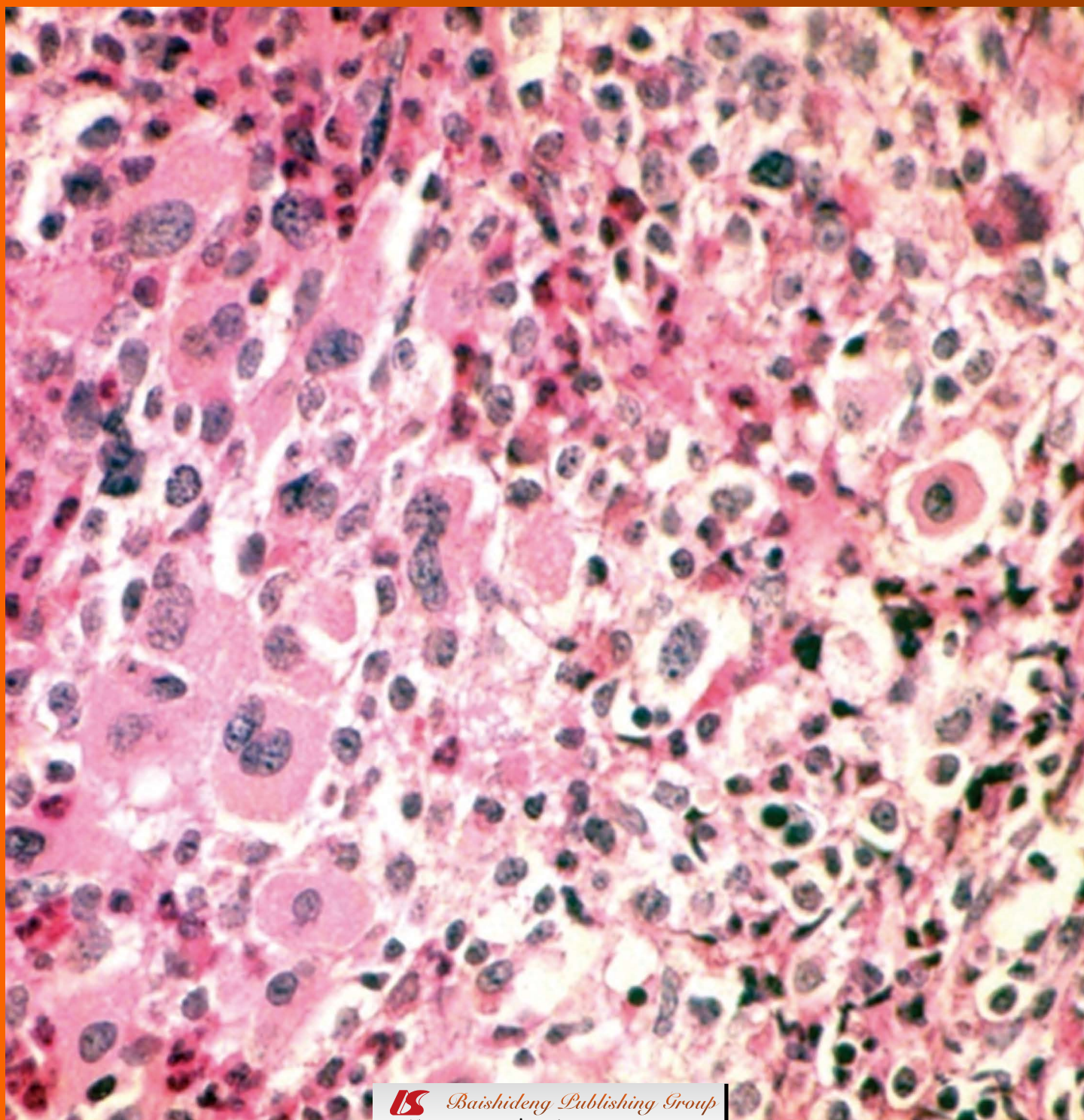
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First contributors in the history of leukemia

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Core tip: Towards the end of the nineteenth century, a clearer definition of the classification of leukemia had been established leading to different subtypes. These well-defined subtypes of leukemia were used for the development of effective chemotherapy, which has represented the most important advance in leukemia research during the past half century.

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Abstract

While the modern era of leukemia chemotherapy began recently, the recognition of leukemias has been mainly recorded in the second part of the nineteenth century. This brief historic review reports the first descriptions of the disease and the major advances in its history from its roots to the beginning of the twentieth century. Although most treatments for leukemia were ineffective until the middle of the twentieth century, it seemed of interest to review some pertinent examples of the evolution in the knowledge of this disease (relied upon chronology as an organizing framework, while stressing the importance of themes), since our current knowledge about leukemia is still mainly based on the first accounts of scientific and medical discovery. Early in the nineteenth century, a small number of cases of patients with uncommon or peculiar alterations of the blood were published. Of the cases, four might suggest symptoms of chronic leukemia. The first published case was the detailed report prepared by John Hughes Bennett in the "*Edinburgh Medical and Surgical Journal*" October 1845. Leukemia gradually became accepted as a distinct disease and published case reports grew in number. Concomitantly, clinical and pathological description of the disease became more detailed.

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INTRODUCTION

The oldest description of cancer was discovered in Egypt and dates back to approximately 1600 BC. The origin of the word "cancer" is credited to the Greek physician Hippocrates (460-370 BC). Hippocrates noticed that blood vessels around a malignant tumor looked like the claws of crab^[1]. He named the disease karkinos (the Greek name for crab) to describe tumors that may or may not progress to ulceration. The credit for the discovery of leukemia goes to the ancient Greeks, who recognized this blood disease way back in the 4th or 5th century BC. However, the literature until AD 500 revealed no evidence of blood malignancies. Accounts and traces of leukemia in the literature are of relatively recent origin. It is obvious that modern knowledge about leukemia owes a debt to numerous European physicians.

In this brief historical review, we examined the major initial advances in the history of leukemia and the main characters involved in the first descriptions. Although "discovery" could not be attributed to one unique observation and that accurate knowledge and effective treatment of leukemia were not available until the middle of the twentieth century, it seemed of interest to review some pertinent examples of the first descriptions and

advances in this disease. Targeted more specifically at the young hematologist and the general educated reader, this review aims to communicate accounts of scientific and medical discovery and initial practice in this disease. It relies upon chronology as an organizing framework, while stressing the importance of themes.

FIRST STUDIES OF BLOOD

Naked-eye inspection of blood by phlebotomy was practiced far back to ancient times. The ancients readily recognized the importance of blood as a life-giving substance, believing it to hold the body's vital force. Hebrews back to the patriarchal age maintained that blood was the seat of the soul and demanded through the Mosaic laws that it be drained before an animal was prepared as food. The Romans drank the blood of their enemies, thinking it would confer on them the courage of their vanquished foes. The scientific study of blood had to wait for the invention of the microscope. While magnifying lenses were known to the monastic scholar and natural historian Roger Bacon (1214-1294), lenses of sufficient quality for scientific use were not available for another three centuries^[2]. Invention of the compound microscope around 1590 by Hans and Zacharias Janssen made possible the examination of the content of the blood^[3]. In 1658, the Dutch naturalist, Jan Swammerdam (1637-1680) was the first person to observe red blood cells under the microscope^[4]. Another Dutch microscopist Anton van Leeuwenhoek (1632-1723) who developed a number of primitive compound microscopes, described the size and shape of "red corpuscles" and rendered the first illustration of human red blood cells in 1695^[5]. The white blood cells ("*the globuli albicante?*") were first noted by Joseph Lieutaud (1703-1780)^[6], some 20 years before William Hewson (1739-1774) first described the lymphocyte^[7]. The earliest reports of leukemia were therefore made by pathologists who recognized early in the nineteenth century, first by gross observation and later through microscopy, that the blood was composed predominantly of white cells, but the evidence provided in the publications was insufficient to support a definite diagnosis of leukemia^[8-12]. This is only in the 19th century that several European physicians noticed that quite a few of their patients suffered from abnormally high levels of white blood cells.

FIRST DESCRIPTIONS OF LEUKEMIA

Similar to most discoveries in medicine, it is not clear who first actually discovered leukemia. Early in the nineteenth century, a small number of cases with uncommon alterations of the blood were published^[13]. The earliest report of the illness is generally considered to have been made by Velpeau^[12] (Figure 1). Velpeau^[12] reported a case of a 63-year-old woman who had fever, a swollen stomach, as well as being generally weak. At autopsy, she was found to have an enormous spleen (twenty times larger than normal) and whose blood was "thick like gruel such that one might have asked if it were not rather laudable pus, than blood". However, at the time no official name was

assigned to the condition and the scant evidence provided in the publication was insufficient to support a definite diagnosis of leukemia. The disease might well have been first observed by Alfred Donné of Paris, the founder of French clinical microscopy (Figure 2). In 1839, Dr. Barth and Dr. Chomel requested his consultative services for a woman patient aged 44 suffering from a painless left-sided abdominal tumor filling the left side of the abdomen. After requesting a specimen of blood for examination, Donné indicated that the large excess of white blood cells which might resemble purulent matter, was not in fact such and concluded that the patient presented with a new disease^[14]. Donné wrote Barth as follows: "The blood you sent me shows a remarkable and most conspicuous change. More than half of the cells were mucous globules. You know that normal blood contains three types of cells: (1) red cells, the essential cells of the blood; (2) white cells or mucous cells; and (3) the small globules. It is the second variety which dominates so much, that, one wonders, knowing nothing about the clinical course, whether this blood does not contain pus. As you know the pus cells cannot yet be differentiated with accuracy from mucous cells"^[15]. In a later case, he reported far more accurately on leukemia and wrote the following text for a chapter entitled "De l'altération des globules blancs" which appeared in his book "Cours de microscopie complémentaire des études médicales": "There are conditions in which white cells seem to be in excess in the blood. I found this fact so many times, it is so evident in certain patients, that I cannot conceive the slightest doubt in this regard... I had an opportunity of seeing these cells in a patient under Dr. Rayer at the La Charité Hospital. This man was affected by arteritis especially in his leg vessels. Both legs showed ecchymoses and gangrenous blisters. The blood of this patient showed such a number of white cells that I thought his blood was mixed with pus, but in the end, I was able to observe a clear-cut difference between these cells and the white cells... In fact, I believe that the excess of white blood cells is due to an arrest of maturation of blood... From my theory on the origin of blood cells, the overabundance of white blood cells should be the result of an arrest of development of intermediate cells"^[16]. Here we find leukemia linked with abnormal blood pathology for the first time in medical history. He made this description of the disease in 1844, calling it an unknown disease whose findings were not published until 1855^[17]. Among other published cases, two might suggest symptoms of chronic leukemia and the blood examined after death showed that the purulent matter and lymph has been mixed with the blood and had been circulated^[10,18]. John Hughes Bennett (Figure 3), pathologist at the Royal Infirmary Edinburgh, gave leukemia its first published recognition as a clinical entity and as a blood-related disease. He was then often referred to as the person who first discovered leukemia because his description was more complete and scientific in nature. Bennett became interested in the disorder when his mentor, Dr. David Craigie, observed two patients admitted to the Royal Infirmary in Edinburgh with unusual blood consistency and a splenic

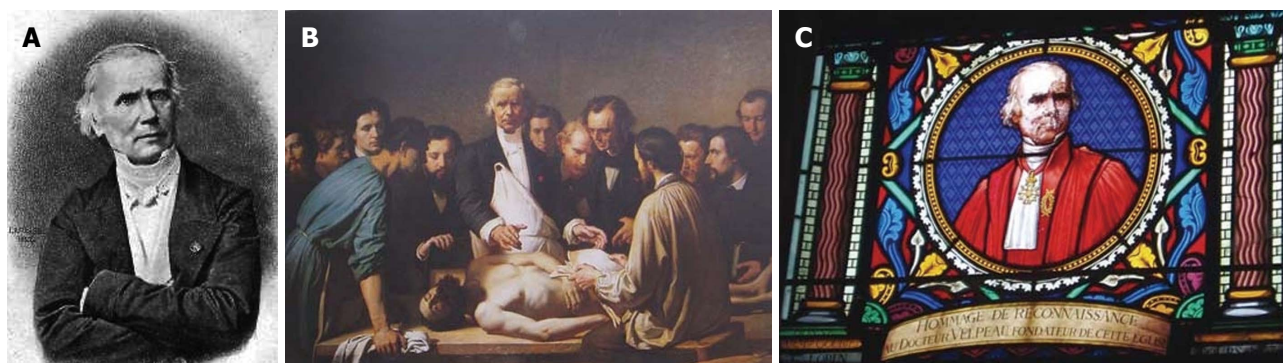


Figure 1 Alfred Velpeau (1795-1867). A: Alfred Armand Louis Marie Velpeau was born on 18 May 1795 in the Touraine village of Brèches in France, where his father was a farrier. He was expected to follow his father's footsteps, but a chance event changed his life. Interested in medicine, in an attempt to dispel the sadness of a depressed young girl, he poisoned her with hellebore. The local physician called for help was so impressed by his knowledge and intelligence that he introduced him to Vincent Gourand, surgeon at the hospital of Tours, who in turn passed him to Pierre-Fidèle Bretonneau in 1816; B: Velpeau was 21 years old^[49,50]. Bretonneau was one of the outstanding physicians of his day in France. He quickly recognized the exceptional talent of his young assistant, treated him like a son, and trained him in clinical medicine and pathology. By 1819, Velpeau was "officier de santé" at the hospital. In 1820, Bretonneau sent him to Paris and obtained for him a position in the Saint-Louis hospital. There Velpeau gained both the anatomy and physiology prizes, while also teaching junior medical students. In 1823, Velpeau qualified and was appointed "agrégé de médecine" with honors, writing his thesis in Latin under the direction of Laennec on intermittent and chronic fevers, based on studies made with Bretonneau in Tours. At the age of 29 years, Velpeau came to be appointed to the junior surgical staff of various hospitals: Saint-Antoine, La Pitié, and La Charité. In 1828, he passed the "Chirurgicale", a higher degree in surgery, and was appointed surgeon to La Pitié. Five years later, he took the university chair of clinical surgery, a position he then held for the next 33 years. Throughout his life, his work was enormous. His published works included 340 titles. There were texts on surgical anatomy, obstetrics, operative medicine, embryology, and diseases of the uterus and breast. Velpeau's "hernia", "canal", "deformity", and a "pressure bandage" for the treatment of phlebitis and burns are among the items linked with his name that have come down to us. Velpeau was elected to the Academy of Medicine in 1832 and to the prestigious Academy of Science in 1843. In 1860 honoured and famous, he visited Brèches, where he had been born. He gave a substantial sum of money to renovate the village church; C: His generosity is still remembered in a stained glass window there, in which he is represented in his professional dress with the inscription "Hommage de reconnaissance au Docteur Velpeau, fondateur de cette église". In 1867, Velpeau caught flu. He died on 24th of August, few days after performing an amputation. His funeral at Saint-Thomas d'Aquin and at the cemetery of Montparnasse was magnificent. It was a fitting end to the life of a man who, from humble origins, had by his own endeavours risen to the front rank of his profession as one of the leading surgeons of the century.

tumor. The first patient was observed in 1841 but was dismissed as unusual until 1844 when a 28-year-old man presented with similar symptoms. John Bennett was given permission to perform an autopsy and study the pathology of this second case. Bennett had attended the lectures on clinical microscopy given by Alfred Donné in Paris and supported Donné's ideas to use the microscope as a clinical instrument. His report entitled "Case of hypertrophy of the spleen and liver in which death took place from suppuration of the blood" was published in the "*Edinburgh Medical and Surgical Journal*" in October 1845^[19]. The symptoms described together with the extensive post mortem report would today be diagnosed as chronic granulocytic leukemia. His drawings were the first illustrations of the blood cells of a patient with leukemia^[20]. The second case of leukemia, published 6 wk later, was reported by Virchow^[18] (Figure 4), a demonstrator in pathological anatomy at the Charité Hospital in Berlin, who described a similar case with enlargement of the spleen specifying, however, that the excess of cells was not purulent matter but instead originated in the blood. A 50-year-old woman was admitted to the Charité Hospital in Berlin complaining of fatigue, nosebleeds, swelling of the legs and abdomen, and died within 4 mo. Virchow noted the enlarged spleen and liver, but also described blood vessels filled with material resembling pus. Virchow described the disparity between white and red blood cells as "weisses blut" (white blood). Few years later, Henry Fuller, a physician at St George's Hospital in London described the first case of childhood leukemia^[21]. In 1852, Bennett published

the first data collection of 35 cases of leukemia or cases which might suggest leukemia. These first series demonstrated a wider geographical distribution of the disease.

CONTROVERSY ABOUT THE PRIORITY OF THE DISCOVERY

Controversy ensued from 1854 on the naming of the new disease and on the priority of its discovery: Bennett arguing precedence on the basis of the publication date and Virchow^[22] suggesting that Bennett's claim was false because of his incorrect determination of purulent matter. In 1847, Virchow^[22] reported a second case and for the first time used the name "leukämie" (leukemia) (the name leukemia is a combination of the Greek words "leukos" and "heima" which means "white blood") to describe this newly observed disease. This refers to the abundance of white blood cells in the body. In 1852, Bennett recommended the term "leucocythaemia", meaning increased white blood cells, which was better accepted^[23]. In 1854, Kölliker^[24] wrote the history of the discovery as it was understood in Germany. Bennett^[23] replied in the same journal and defended his position. However, there was mutual respect between the two men. Virchow wrote testimonials in support of Bennett's candidature for two chairs in Edinburgh in 1848 and then in 1855: "I hereby testify, that having for a long time assiduously followed the very valuable researches of Dr. J Hughes Bennett of Edinburgh, I entertain the highest esteem for his scientific contributions, and consider them as among the most important of



Figure 2 Alfred François Donné (1801-1878). Alfred François Donné was born on 13 September 1801 at Noyon (France). At the age of 20 years, he moved to Paris with his family. Although he had a dislike of law as a career, he embarked on this discipline at his parents' insistence. He qualified as a lawyer but did not practice, and then became a late starter as a medical student, when he entered the Paris Faculty at the Sorbonne at 25 years of age. A few years later he married Marie Antoinette Joantho, and through his wife, became linked with a well known medical family, the des Essarts. Donné graduated in 1831 at the age of 30 years. His clinical work was concentrated at the Charité hospital under the very experienced clinician Bouillaud. His qualities were recognized by the University Council and they appointed him to the honorary post of sub-librarian to the Faculty of Medicine^[51]. In 1836, he made one of his greatest contributions to medicine by discovering the protozoon, *Trichomonas vaginalis*, in vaginal secretion of Parisien prostitutes^[52] and recorded it in a publication addressed to the Academy of Sciences. During this period of intensive research and clinical work, Donné had realized that microscopes were invaluable for the proper illustration and understanding of his lectures. His pediatric successes with feeding difficulties of premature infants led to his election as a Chevalier of the Legion of Honor and his nomination as Inspector General of Medicine. His successful researches into hematology have not received the publicity and fame they deserve. In 1842, he announced his discovery of blood platelets to the Academy of Sciences and these were incorporated in his "*Atlas de microscopie médicale*"^[53]. After losing his office of Inspector General following the 1848 revolution, he was installed in 1853 as the new Rector of the University of Strasbourg, then became in 1855 Rector of the University of Montpellier. During his stay of almost 20 years at Montpellier, he became interested in theories and studies on spontaneous generation. On retiring from office in 1875, he returned to Paris where he died of a cerebral vascular accident on 7 March 1878^[51]. He remains virtually unknown outside of France, never obtained the title of professor and was a practical man fond of microscope and laboratory work, but his contribution to medical and scientific progress is inestimable.

those which have enriched the Pathology now struggling for a physiological basis. It appears to me, therefore, that it would be only a well-merited reward, if the Chair of Physiology and Pathology should be given to him, who is able to fill it so worthily". The question of priority was resolved publicly by Virchow in a lecture he delivered in 1858, in which he stated that Bennett observed a case of indubitable leukemia few months before he saw his first case: "It is moreover the same conclusion which Bennett came to in the much discussed matter of priority between us when he observed a case of individual some months before I saw my first case".

IDENTIFICATION OF LEUKEMIA AS A SEPARATE DISEASE

Pus and inflammation continued to dominate hematological thoughts until the middle of the nineteenth century. In a third publication (1856), Virchow^[25] was credited with concluding that the disorder was not the result of an in-



Figure 3 John Hughes Bennett (1812-1875). Born in London on 31 August 1812, Bennett (Figure 3) was educated at Exeter (England) and being destined for the medical profession, he entered an apprenticeship with a surgeon in Maidstone (Kent). In 1833, he began his studies in Edinburgh. He published his first article in "*London Medical Gazette*" in 1836. He graduated in 1837 with the highest honors and gold medal, with a dissertation entitled "The physiology and pathology of the brain". During the next 4 years, he studied in Paris, where he founded the English-speaking Medical Society, and then in Germany. On his return to Edinburgh in 1841, he published a "Treatise on cod-liver oil as a therapeutic agent" and became physician at the Royal Public Dispensary of Edinburgh. He began to lecture as an extra-academic teacher on histology, drawing attention to the importance of the microscope in the investigation of diseases^[54]. In 1843, he was appointed professor of the Institute of Medicine in Edinburgh. Opposed bloodletting and the indiscriminate use of drugs, he was an important influence in changing British therapeutic practices during the second half of the nineteenth century. In 1845, he published a paper entitled "Case of hypertrophy of the spleen and liver in which death took place from suppuration of the blood" in the "*Edinburgh Medical and Surgical Journal*". In 1846, he became editor and later proprietor of the *Monthly Journal of Medical Science*. In 1851, Bennett founded and became the first president of the Physiological Society of Edinburgh. His publications were very numerous including "*Lectures on clinical medicine*" (1850-1856), "*Clinical lectures on the principles and practice of medicine*", "*Leucocythaemia*" (1852), "*Outlines of physiology*" (1858), "*Pathology and treatment of pulmonary tuberculosis*" (1853), "*Textbook of physiology*" (1871-1872). In 1869, he supported the admission of women medical students in Edinburgh. In 1873, he was elected a member of the French Academy of Medicine and granted recognition by the French government to practice medicine in France. In 1875, after his participation at the meeting of the British Medical Association, he was compelled to have the operation of lithotomy performed. He sank rapidly and died on September 25 at Norwich. In 1901, the University of Edinburgh inaugurated the John Hughes Bennett Laboratory of Experimental Pathology. A second laboratory with his name was opened in 1998, in a joint venture between Britain's Leukaemia Research Fund, the University of Edinburgh and the Western General Hospital Trust.

fectious process but rather was caused by the tissue that produced the white blood cells. The debate centred upon whether leukemia was in fact a separate disease was then conducted by some of the leading physicians in France. Among the minority that supported the autonomy of leukemia, Gabriel Andral, Professor at the University of Paris (Figure 5), proposed the study of the blood as a clinical discipline and defended the use of the microscope in clinical medicine. He was the founder of the science of hematology and is credited with the integration of that science into clinical and investigative medicine. Leukemia gradually became accepted as a distinct disease and clinical and pathological description became more detailed. However, the definition of leukemia was far from precise until a first classification was introduced in 1857 by Nikolaus Friedreich, a pathologist in Würzburg (Figure 6).



Figure 4 Rudolf Virchow (1821-1902). Born in Germany in 1821, he studied medicine and chemistry in Berlin at the Prussian Military Academy from 1839 to 1843. After graduation in 1843, he went to serve as assistant at the Charité Hospital. In 1847, he qualified as a lecturer at the University of Berlin and participated in founding the "Archiv für pathologische anatomie und physiologie und für klinische medizin". Virchow is credited with multiple important discoveries. Besides his role in recognizing leukemic cells, he was one of the first to accept the work of Robert Remak who showed that the origin of cells was the division of preexisting cells. He also described that an enlarged left supra-clavicular node is one of the earliest signs of gastrointestinal malignancy. He elucidated the mechanism of pulmonary thromboembolism and founded the medical fields of cellular pathology and comparative pathology. He also developed a standard method of autopsy procedure. In 1861, he was elected a foreign member of the Royal Swedish Academy of Sciences. In 1862, he was awarded the Copley Medal. In 1869 Virchow founded the Society of anthropology, ethnology and prehistory which was very influential in coordinating German archaeological research^[55,56]. More than a laboratory physician, Virchow was an impassioned advocate for social and political reform. He made himself known as a pronounced democrat in the year of revolution, 1848, and his political activity caused the government to remove him from his position. In 1859, he became a member of the Municipal Council of Berlin, and began his career as a civic reformer. Elected to the Prussian Diet in 1862, he became leader of the Radical or Progressive party, and from 1880 to 1893 he was a member of the Reichstag. He is widely regarded as a pioneer of "social medicine", focusing on the fact that disease is never purely biological, but often socially derived or spread^[57]. Virchow died of heart failure in 1902.



Figure 5 Gabriel Andral (1797-1876). Gabriel Andral was born in Paris, the son of a well-known physician who was a member of the academy and personal physician to the French revolutionary leader Jean-Paul Marat. Andral received his doctorate in 1821 with a thesis on expectoration. He was habilitated in 1824 and became "agrégé". The faculty appointed him professor of hygiene in 1828 on the death of René-Joseph-Hyacinthe Bertin. When baron René-Nicolas-Dufrièche Desgenettes retired, he became professor of internal pathology, and in 1839 he succeeded François-Joseph-Victor Broussais in the chair of general pathology and therapy, holding this tenure for 27 years. Besides he was physician at the Charité. In 1823, Andral became a member of the Academy of Medicine. In 1843 he became member of the Institute, and in 1858 was made a commander of the Legion of Honor. In 1866 he abandoned his chair and retired, but still he took part in the advance of science and participated in the transactions of learned societies. He died of a heart condition on February 13, 1873. His main oeuvre "*Clinique médicale*", a five-volume work, comprises almost every aspect of medicine. This treatise on general medicine may be considered a summary of French medicine as it had developed in the first decades of the 19th century. He was the founder of the science of hematology. He is said to be the originator of the word "anemia" and was the first physician to see the potential of chemical analysis of the blood.



N. Friedreich

Figure 6 Nikolaus Friedreich (1825-1882). Born in Würzburg in 1825, Nikolaus Friedreich received medical training in this city where his father and grandfather had been professor of medicine. He received his doctorate in 1850. He became Assistant at the clinic of clinician Karl Friedrich von Marcus and in 1853 was habilitated as Privatdocent of special pathology and therapy. When Virchow came to Würzburg, Friedreich became an ardent student of this great pathologist and considered abandoning clinical medicine for pathology. In 1857, he was appointed professor of pathological anatomy at Würzburg, and in 1858 moved to the tenure of professor ordinarius of pathology and therapy at Heidelberg, a post which he held for the remainder of his career. He was also director of medical clinic. He took an interest in all branches of medicine, especially neurology. He has left 8 major and 51 larger and smaller treatises, among them a number of monographs. These include works on leukemia. He died in 1882 from a ruptured aortic aneurysm.

THE MORPHOLOGICAL ERA OF HEMATOLOGY

During his period of intensive research and clinical work, Donné had realized that microscopes were invaluable for the proper illustration and understanding of his lectures. In spite of hostility, he organized a microscopy course (the first microscopic workshop in medicine) that attracted French as well as foreign students. One of Donné's foreign students was John Hughes Bennett. After Louis Daguerre, one of the inventors of photography presented his discovery to the Academy of Sciences, Donné became most interested in these photographic reproductions and resolved to incorporate them in his lectures. In 1844 he described his procedure in a book entitled "*Cours de microscopie complémentaire des études médicales*" which demonstrated the scope of the instrument^[16]. The first reported use of the microscope to diagnose leukemia in a living patient was performed by Fuller^[26]. The neoplastic nature of leukemia and the origin of the disease in organs of blood formation having been established, the clinical and biological science of hematology were given a tremen-

dous boost in the period between 1878 and 1888, when it became possible to examine the microscopic details of

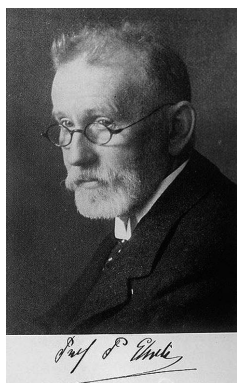


Figure 7 Paul Ehrlich (1854-1915). Paul Ehrlich was born on March 14, 1854 at Strehlen in Silesia (Germany). Educated at the Gymnasium at Breslau and subsequently at the universities of Breslau, Strassburg, Freiburg-im-Breisgau and Leipzig, he obtained his doctorate of medicine in 1878 by means of a dissertation on the theory and practice of staining animal tissues. In 1882 he published his method of staining the tubercle bacillus that Koch had discovered. This method was the basis of the subsequent modifications introduced by Zhiel and Neelson and of the Gram method of staining bacteria. In 1882 Ehrlich became Titular Professor and in 1887 he qualified as a Privatdozent in the faculty of medicine in the University of Berlin. Later he became an Associate Professor and Senior House Physician to the Charité Hospital in Berlin. After becoming Robert Koch's assistant in 1890, he began immunological studies. He worked out the details of preparing an antitoxin for diphtheria, which represented the first use of immunotherapy to specifically treat an infection. In 1896 he was appointed Director of the Institute for the control of therapeutic sera at Steglitz in Berlin, and formulates his side-chain theory of immunity. In 1897 Ehrlich was appointed Public Health Officer at Frankfurt-am-Main and in 1899 became director at the Royal Institute of Experimental Therapy. He then began another phase in his varied researches and devoted to chemotherapy with the aim to find chemical substances which have special affinities for pathogenic organisms and would be "magic bullets" which would go straight to the organisms at which they were aimed. He produced trypan red and established the correct structural formula of atoxyl effective against trypanosomes. This opened the way of obtaining new organic compounds with trivalent arsenic. One arsenic drug was found very effective against syphilis. Ehrlich announced it under the name of "Salvarsan". Another arsenical substance named "Neosalvarsan" became more easily administered. During the later years of his life, Ehrlich was concerned with experimental work on tumors and on his view that sarcoma may develop from carcinoma, also on his theory of atreptic immunity of cancer. In 1908 he shared with Metchnikoff the highest scientific distinction, the Nobel price. On August 20, 1915 a stroke ended his life.

blood cells. The event that provided this opportunity was biological stains. At this point of medical history, despite the fact that iodine, saffron, and ammonia carmine were available for staining tissues and cells, practically no advances were made in morphology of blood cells. The state of hematology from the 1850s to the 1870s was delineated by Beale^[27] (1828-1906), a British professor, in a book that went through several editions. Beale^[27] illustrated and described "the various corpuscles met within healthy blood". A major advance in understanding leukemia pathophysiology came in 1877 when Ehrlich^[28] (Figure 7) developed a triacid stain and introduced the names acidophil (later changed to eosinophil), basophil and neutrophil for the three different granulocyte types. His techniques initiated the true morphological era of hematology. In 1891, the triacid stain was replaced by the eosine methylene blue stain invented by DL Romanowsky (1861-1921) of St. Petersburg in Russia. The "Romanowsky stain"

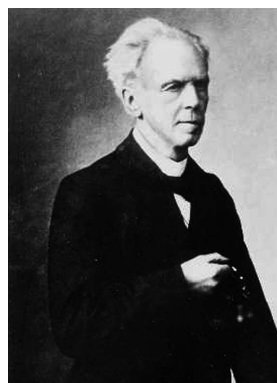


Figure 8 Ernst Neumann (1834-1918). Ernst Neumann was born in 1834 at Königsberg, capital of eastern Prussia, as great son of Karl Goofried Hagen (professor of chemistry and pharmacy) and son of Franz Ernst Neumann (a pioneer in the mathematical physics). In 1850, he enrolled at the famous university of Königsberg (Albertina). In 1855, he took his doctor's advice degree. After studies in Prague, Berlin (under Rudolf Virchow) and Königsberg, he became a lecturer in medicine in 1859. He got interested in the rising field of pathological anatomy and was appointed professor of pathology at Königsberg in 1866. He described the presence of nucleated red blood cells in bone marrow sap of humans and rabbits obtained by squeezing bones. He was the first to conclude that during postembryonic life, erythropoiesis is taking place in bone marrow. Further studies pointed to the fact that leukocytes are also formed in the bone marrow. He postulated a common stem cell for all hematopoietic cells. He later was made Geheimer Medicinalrath - privy medical councillor, and received honorary doctorate from the universities of Tübingen and Geneva, in 1898 and 1915. He died in 1918.

was further modified by Richard May of Munich in 1902, Gustav Giemsa (1867-1948) of Hamburg in 1905, and JH Wright of Boston in 1906. However, all of these modifications were direct descendants of Ehrlich's original ideas.

THE ORIGIN OF LEUKEMIA

A vital discovery came in 1868 when Neumann^[29] (Figure 8), professor of Pathological Anatomy at Königsberg, reported changes in the bone marrow in leukemia and established the link between the source of blood and the bone marrow. One year later, he published an extensive description of cells in the marrow, and, 1 year later again, that the circulating red cells were derived from an ancestral cell^[30]. Around the same time, Bizzozero^[31,32] (1846-1901) of Pavia confirmed the observation that non-nucleated red blood cells were formed from nucleated red cells in the bone marrow and that the blood formation function of the bone marrow also included white blood cells. He also identified the platelet^[33]. Neumann^[34] stated in 1872 that leukemia was a disease of the marrow. Shortly thereafter, Mosler^[35] introduced bone marrow puncture as a means of antemortem diagnosis of leukemia. For some years, the mystery remained of how the cells were able to travel through the bone to the circulatory blood system.

THE CONCEPT OF THE "STEM CELLS"

The concept of "stem cells" was first proposed by German zoologists and medical scientists^[36]. Adopting the term "*stammzelle*" from Ernst Haeckel (1834-1919), a con-

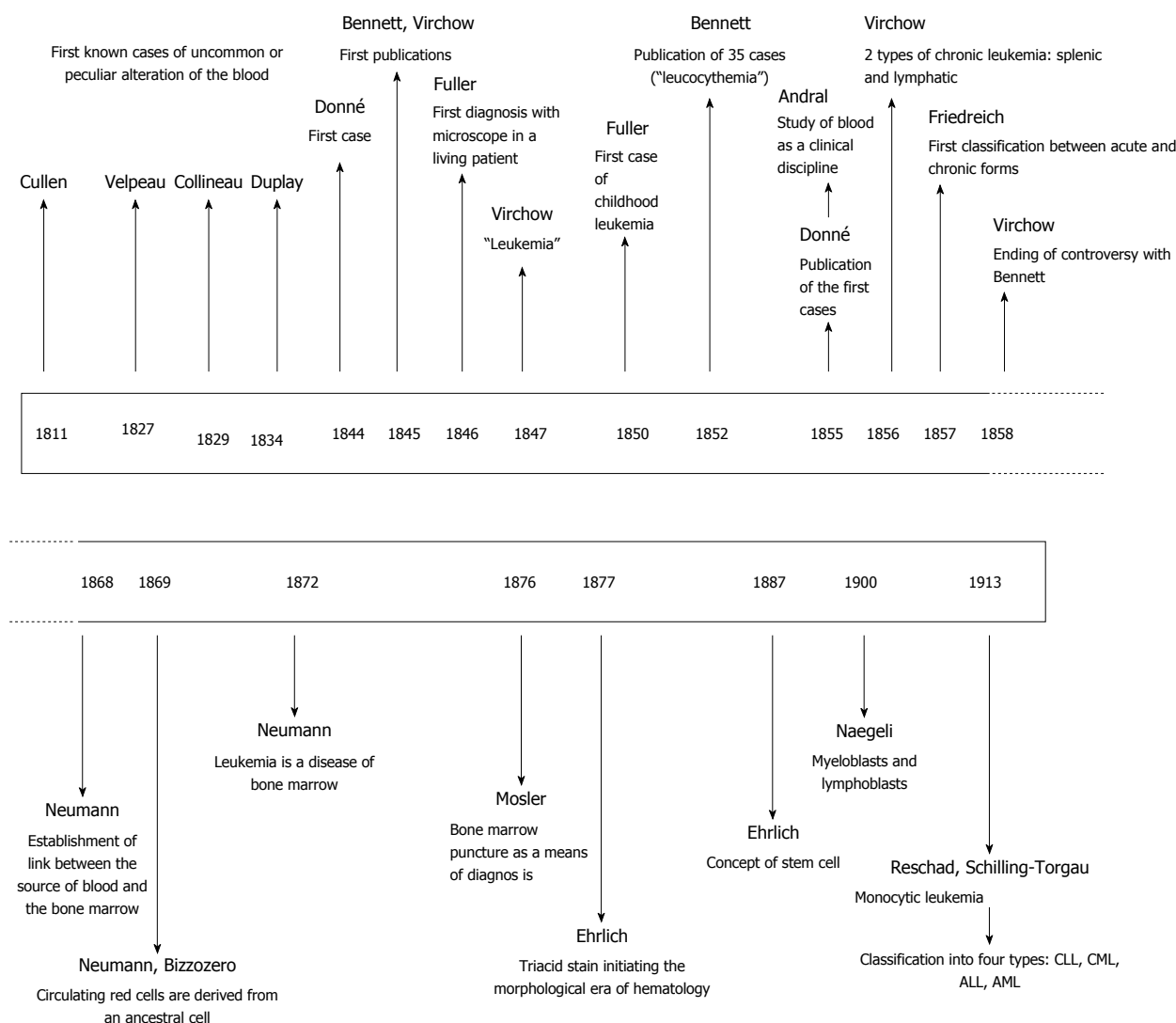


Figure 9 First contributors in the history of leukemia. CLL: Chronic lymphocytic leukemia; CML: Chronic myeloid leukemia; ALL: Acute lymphocytic leukemia; AML: Acute myeloid leukemia.

troverfial Darwinist and professor of zoology at Jena^[37], Theodor Boveri (1862-1915) was influential in making these cells known as carriers of the so-called "germ plasm" and as the starting points in embryological development of differentiated body cells as well as germ cells^[36]. The essential characteristic of a "stem cell" was a capacity for self-renewal as well as for differentiation into specific types of somatic cells or germ cells. Valentin Haecker (1864-1915), who was made director of the Zoological Institute at the University of Stuttgart and subsequently at the University of Halle, propagated the notion of "pluripotency", which he ascribed to the "germ plasm" of an organism^[38]. In 1896, Artur Pappenheim (1870-1916), who worked at Virchow's Pathological Institute in Berlin on the formation of red blood cells, developed the conception that "stem cells" or "mother cells" ("mutterzellen") (as he called them) were embryonic cells that had the potential to differentiate into different cell lines and to form the basis of different types of blood cells^[39]. Pappenheim also argued that myelocytes and lymphocytes originated from the same "multipotent stem cell"^[40]. Julius Cohnheim's theory

was also widely discussed at the end of the nineteenth-century. He defined tumors as "atypical neoplasms of tissue based on an embryonic rudiment"^[41]. The lymphocyte was described as the "common, indifferent stem cell" for erythrocytes and granulated granocytes by Dantschakoff^[42] or even as the "common stem cell" of all types of blood cells by Maximow^[43]. These authors were committed to the "Unitarian" view of the different types of blood cells. In contrast, Ehrlich^[44] was the first scholar to propose the "dualist doctrine", which assumed that the lymphocytes and leucocytes originated from morphologically different precursor cells in different organs: lymph nodes and spleen for the first ones and bone marrow for the second ones. In 1900, the Swiss hematologist Naegeli described a new cell in the myeloid cell line, which he named the myeloblast, as an ancestor of granulocyte cells^[45]. He also showed the lymphoblast as an ancestor of lymphocytes. The presence of myeloblasts or lymphoblasts in the circulating blood formed a classic diagnosis of acute leukemia. Monocytic leukemia was first described by Reschad *et al*^[46], and leukemic cells recognized as unusual forms of myeloblasts.

CLASSIFICATION OF LEUKEMIAS

In 1856, Virchow^[25] categorized two types of the disease - the splenic and the lymphatic forms - by the starting site of the disease, which we now know as leukemia and lymphoma, respectively. A first classification was introduced in 1857 by Nikolaus Friedreich. A distinction between acute and chronic forms of leukemia was appreciated. He reported at length on a case which he described as acute leukemia, the first time the term was used^[47]. Few years later, Mosler added the classification myelogenous leukemia to the splenic and lymphatic types of the disease^[48]. The development of a triacid stain by Paul Ehrlich in 1877 simplified the classification of leukemia into the myeloid group and the lymphoid group. Towards the end of the nineteenth century, a clearer definition of the classification of the subtypes of leukemia had been established, but there was still a lack of any form of effective therapy. This is only in 1913 that leukemia was classified into four types: chronic lymphocytic leukemia, chronic myelogenous leukemia, acute lymphocytic leukemia, and acute myelogenous leukemia.

CONCLUSION

While the modern era of leukemia chemotherapy began recently, the recognition of leukemias has been mainly recorded in the second part of the nineteenth century. This brief historic review reports the first descriptions of the disease and the major advances in its history from its roots to the beginning of the twentieth century. Although most treatments for leukemia were ineffective until the middle of the twentieth century, it seemed of interest to review some pertinent examples of the evolution in the knowledge of this disease (relied upon chronology as an organizing framework, while stressing the importance of themes), since our current knowledge about leukemia is still mainly based on the first accounts of scientific and medical discovery. Towards the end of the nineteenth century, a clearer definition of the classification of leukemia had been established leading to different subtypes. These well-defined subtypes of leukemia were used for the development of effective chemotherapy, which has represented the most important advance in leukemia research during the past half century (Figure 9).

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PVSG and WHO vs European Clinical, Molecular and Pathological Criteria for prefibrotic myeloproliferative neoplasms

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Abstract

The Polycythemia Vera Study Group (PVSG), World Health Organization (WHO) and European Clinical, Molecular and Pathological (ECMP) classifications agree upon the diagnostic criteria for polycythemia vera (PV) and advanced primary myelofibrosis (MF). Essential thrombocythemia (ET) according to PVSG and 2007/2008 WHO criteria comprises three variants of JAK2^{V617F} mutated ET when the ECMP criteria are applied. These include normocellular ET, hypercellular ET with features of early PV (prodromal PV), and hypercellular ET due to megakaryocytic, granulocytic myeloprolifera-

tion (ET.MGM). Evolution of prodromal PV into overt PV is common. Development of MF is rare in normocellular ET (WHO-ET) but rather common in hypercellular ET.MGM. The JAK2^{V617F} mutation burden in heterozygous mutated normocellular ET and in heterozygous/homozygous or homozygous mutated PV and ET.MGM is of major prognostic significance. JAK2/MPL wild type ET associated with prefibrotic primary megakaryocytic and granulocytic myeloproliferation (PMGM) is characterized by densely clustered immature dysmorphic megakaryocytes with bulky (bulbous) hyperchromatic nuclei, which are never seen in JAK2^{V617F} mutated ET, and PV and also not in MPL^{S515} mutated normocellular ET (WHO-ET). JAK2^{V617F} mutation burden, spleen size, LDH, circulating CD34⁺ cells, and pre-treatment bone marrow histopathology are mandatory to stage the myeloproliferative neoplasms ET, PV, PMGM for proper prognosis assessment and therapeutic implications. MF itself is not a disease because reticulin fibrosis and reticulin/collagen fibrosis are secondary responses of activated polyclonal fibroblasts to cytokines released from the clonal myeloproliferative granulocytic and megakaryocytic progenitor cells in ET.MGM, PV and PMGM.

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Key words: Myeloproliferative neoplasms; Essential thrombocythemia; Prodromal polycythemia vera; Polycythemia vera; Myelofibrosis; JAK2^{V617F} mutation; JAK2 wild type myeloproliferative neoplasm; Bone marrow pathology

Core tip: The integrated World Health Organization (WHO) and European Clinical, Molecular and Pathological classification of the myeloproliferative neoplasms include JAK2^{V617F} mutated normocellular essential thrombocythemia (WHO-ET), prodromal polycythemia vera (PV), classical PV, and hypercellular ET due to megakaryocytic, granulocytic myeloproliferation. Evolution of prodromal PV into overt PV is common. JAK2/MPL wild hypercellular ET associated with prefibrotic primary megakaryocytic

and granulocytic myeloproliferation is characterized by densely clustered immature dysmorphic megakaryocytes with bulky (bulbous) hyperchromatic nuclei, which are never seen in JAK2^{V617F} mutated ET and PV, and also not in JAK2 wild type normocellular ET (WHO-ET) carrying the MPL⁵¹⁵ mutation.

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INTRODUCTION

Vaquez^[1] and Osler^[2] first described polycythemia vera (PV) as a distinct disease entity. In 1950, Dameshek^[3] described PV as a trilinear myeloproliferation of the bone marrow with various degrees excessive production of red blood cells, granulocytes and platelets. Dameshek^[3] proposed two highly speculative possibilities for the etiology of trilinear PV: first, the presence of excessive bone marrow stimulation by an unknown factor, second, a lack or a diminution in the normal inhibitory factor. This hypothesis is confirmed by the discovery of the JAK2^{V617F} mutation by James *et al*^[4] in 2005 demonstrating that the JAK2^{V617F} mutation induces a loss of inhibitory activity of the JH2 pseudokinase part on the JH1 kinase part of JAK2, leading to enhanced activity of the normal JH1 kinase activity of JAK2. The JAK2^{V617F} mutation makes the mutated hematopoietic stem cells hypersensitive to hematopoietic growth factors TPO, EPO, IGF1, SCF and GCSF, resulting in trilinear myeloproliferation with clinical manifestations of essential thrombocythemia (ET), PV and myelofibrosis (MF) (Figure 1)^[4,5].

The Polycythemia Vera Study Group (PVSG) followed the 1951 recommendations of Dameshek to define PV by increased red cell mass (RCM) as a major criterion and proposed criteria for the clinical diagnosis of Ph-negative ET, PV and agnogenic myeloid metaplasia (AMM) with MF^[6-10]. The unifying concept of the lumping of the chronic myeloproliferative disorders (MPD) ET, PV, AMM, chronic myeloid leukemia (CML) by Dameshek in 1951 has been broken up by the PVSG in 1975 into Ph-positive (Ph⁺) CML and Ph-negative ET, PV and AMM (Figure 1)^[6-11]. The Ph-negative MPDs ET, PV and MF form a benign group of chronic MPD, whereas the Ph⁺ chromosome is the result of the *BRC/ABL* fusion gene and protein. *BCR/ABL*-positive CML appears to be a real neoplasia (leukemia) with an inevitable transition into acute leukemia when strict morphological, biochemical, cytogenetic and molecular criteria are used in routine daily practice^[12-14]. The Thrombocythemia Vera Study Group introduced bone marrow biopsy as a specific, pathognomonic clue for early stage ET and PV^[15,16]. The PVSG^[6-8] and the 2001 World Health Organization (WHO) criteria

for the classification of the MPDs are not refined enough to also take the early prefibrotic stages of thrombocythemia in various MPDs into account^[15-20]. The availability of the current clinical and molecular markers endogenous erythroid colony (EEC) formation, serum EPO levels, the JAK2^{V617F} mutation and bone marrow histology allow the detection of early stage ET and PV. Within the context of the European Working Group on MPD Michiels *et al*^[15-17] contributed significantly to the European consensus criteria for ET, PV and chronic idiopathic MF (CIMF) by including bone marrow histology and subsequently defined between 2002 and 2005 the European Clinical and Pathologic (ECP) criteria (http://www.mpn-stichting.nl/doctors_brochure_2004.pdf)^[17-19]. In the present study, we extend the PVSG, the ECP^[18-20], and the 2007/2008 WHO^[21,22] MPD/myeloproliferative neoplasms (MPN) classifications into simplified and integrated WHO and European Clinical, Molecular and Pathological (WHO-ECMP) criteria by including bone marrow pathology together with a complete set of established laboratory and molecular markers for diagnostic differentiation of each of the latent (masked), early and overt MPNs^[19,20].

DIAGNOSIS OF MPN

ET

In the 1980s, Georgii *et al*^[23-25] and Thiele *et al*^[26-29] defined the pathological features of ET, PV and chronic megakaryocytic granulocytic myeloproliferation (CMGM) or CIMF as derived from bone marrow histopathological morphology (Figure 1)^[30]. ET was defined by Georgii *et al*^[23-25] and Thiele *et al*^[26-29] as persistent increase of platelets in excess of $400 \times 10^9/L$ without the Ph⁺ chromosome together with monolinear proliferation of mature enlarged megakaryocytes in the bone marrow with normal cellularity, normal erythropoiesis and normal granulopoiesis (Figure 1). This bone marrow definition for the diagnosis of normocellular ET has been used by Michiels *et al*^[17,18] in the ECP classification of MPD (http://www.mpn-stichting.nl/doctors_brochure_2004.pdf) and by the 2007/2008 WHO classification (WHO-ET)^[21,22]. Normocellular ET (WHO-ET) only comprises about one third of PVSG defined ET patients^[21,22]. The 1997 PVSG and 2001 WHO classifications used a platelet count in excess of $600 \times 10^9/L$ as the minimum criterion for the diagnosis of ET^[7,14] and therefore did not include the early stages of ET, which consequently were diagnosed as masked or unclassifiable MPD^[18]. This comprises about 30% of early stage or latent ET (Table 1) indicating the need to lower the platelet count cut-off to $400 \times 10^9/L$ (upper limit of normal) for the diagnosis of thrombocythemia in various MPDs (Table 2)^[12-20]. The relatively high incidence of early prefibrotic thrombocythemia with a platelet count between 400 and $600 \times 10^9/L$ strengthen the need for use of specific laboratory and molecular markers to differentiate thrombocythemia from reactive thrombocytosis followed by bone marrow histopathological evaluation in order to correctly diagnose patients with suspected

Table 1 Blood and bone marrow features in one prospective study of Thrombocythemia Vera Study Group-defined essential thrombocythemia and one retrospective study of Polycythemia Vera Study Group defined essential thrombocythemia at platelet counts above the upper limit of normal

Ref.	Michiels <i>et al.</i> ^[11]	Lengfelder <i>et al.</i> ^[30]	
Type of study	Prospective 1975-1985	Retrospective 1975-1995	
Diagnosis ET	TVSG criteria	PVSG criteria	
Inclusion criterion	ET	ET	Tentative diagnosis WHO-ECMP
Platelet count × 10 ⁹ /L	> 400	> 350	
Number of ET patients	30	143	
Platelets × 10 ⁹ /L range	420-1500	< 350-> 000	
Below 600	13%	29%	Early latent ET
Between 600-1000	54%	45%	Fits with ET
Above 1000	33%	26%	Fits with ET
Leukocytes			
Above 12 × 10 ⁹ /L	10%	51%	
Hemoglobin			
Below 16 g/dL	-	80%	
Below 17 g/dL	-	100%	
Above 16 g/dL	-	20%	Fits with PV
Splenomegaly			
No	63%	56%	
Yes	37%	44%	
Spleen size on echogram (cm)			
n < 12/12-15/> 15	2019/8/3	-	
Bone marrow biopsy			
Normal cellularity	17 (57%)	52%	Fits with true ET
Increased cellularity	13 (43%)	60%	
Increased erythropoiesis	13 (43%)	17%	Fits with early PV
Increased granulopoiesis	0	45%	Fits with CMGM
Myelofibrosis	No	No	

Essential thrombocythemia (ET) according to Polycythemia Vera Study Group (PVSG) criteria appears to be a spectrum of normocellular ET, prodromal polycythemia vera (PV) and ET due to megakaryocytic, granulocytic myeloproliferation or ET associated with chronic or primary megakaryocytic granulocytic myeloproliferation (CMGM/PMGM) when diagnostic World Health Organization and European Clinical, Molecular and Pathological (WHO-ECMP) bone marrow features are applied. TVSG: Thrombocythemia Vera Study Group.

slight splenomegaly and borderline erythrocytosis RCM is the gold standard to make the distinction between hypercellular ET due to increased erythropoiesis with normal RCM (prodromal PV) from classical PV with increased RCM. In our experience, cases of prodromal PV, masked PV do show a typical PV picture in the bone marrow histopathology (Figure 3). This controversial topic has been addressed in a separate report (manuscript in press). PV patients do have erythrocytes above $6 \times 10^{12}/L$ even when the hemoglobin and hematocrit are in the upper range of normal due to microcytosis of erythrocytes caused by iron deficiency and/or significant splenomegaly^[3,39]. Consequently, RCM measurement is of debatable additional diagnostic value in classic PV carrying the JAK2^{V617F} or exon 12 mutation, since all patients with 2008 WHO/ECMP defined PV do have erythrocyte counts above $6 \times 10^{12}/L$ and demonstrate a bone marrow histology that is pathognomonic for PV (Table 3).

Nomenclature, clinical and bone marrow diagnosis of primary MF

The terms AMM and IMF are applied to hypercellular advanced fibrotic stages of MPN^[8,23-29]. MF is a reactive feature secondary to progressive disease seen in AMM, CIMF, PV and CML. In 1988, 1996 and 1999 Thiele *et al.*^[26-29] clearly defined the bone marrow features of normocellular true ET (WHO-ET, Table 2, Figure 2), of hypercellular trilinear PV (Table 3, Figure 2), and prefibrotic CIMF with associated thrombocytosis (Figure 2). According to Thiele *et al.*^[26-29], “true” ET clearly differs from ET associated with prefibrotic CIMF labeled as “false” ET. In true ET megakaryocytes display large to giant megakaryocytes showing hyperlobulated staghorn-like nuclei in a normocellular bone marrow (WHO-ET, Table 2, Figure 2). Interestingly, the megakaryocytes in true ET are larger than in PV^[26]. PV is typically featured by small to large (pleomorphic) megakaryocytes with hyperploid nuclei in a hypercellular bone marrow due to increased erythropoiesis or increased erythro-granulocytic myeloproliferation (WHO-PV, Table 3, Figures 2, 3 and 5). In 1980, Georgii *et al.*^[23] described CMGM as a distinct MPD entity apart from ET. In 1990, Georgii *et al.*^[24,25] proposed the Hannover Bone Classification of the myeloproliferative disease and defined CMGM as hypercellular prefibrotic stages preceding AMM or IMF (Figure 2). As prefibrotic CIMF-0 is a contradiction of terms and MF is not idiopathic but secondary seen in various MPDs, Georgii *et al.*^[24] replaced the term CIMF by CMGM as the third entity of prefibrotic MPD different from ET and PV at the bone marrow pathology level (Hannover Bone Marrow Classification of MPD). The prefibrotic stage of CMGM or CIMF precedes the fibrotic stages of AMM (Figure 2) and initially present as primary megakaryocytic and granulocytic myeloproliferation (PMGM) defined by Michiels and Thiele in Table 4. PMGM is characterized by a specific disturbance of a hypercellular bone marrow with striking abnormalities of megakaryocyte maturation (dysmegakaryopoiesis) (Figures 6-10), which consist of variations in size including giant forms and deviations of the nuclear-cytoplasmic ratio accompanied by bulbous and hyperchromatic cloud-like nuclei, which are not seen in ET and PV. Thiele *et al.*^[29] in their 1999 Cologne Clinical and Bone Marrow Classification of the MPDs used the term prefibrotic chronic IMF (CIMF-0) for this third CMGM MPD entity. Prefibrotic CIMF/CMGM is typically featured by hypercellular ET associated with megakaryocytic, granulocytic myeloproliferation (MGM) at the bone marrow level with no or slight increase of reticulin fibrosis (RF) in the Gomori’s silver or Gordon Sweet stain of bone marrow biopsy specimen (Table 4, Figure 2). With the advent of the JAK2^{V617F} mutation, Michiels distinguish in this report two variants of MGM (Figure 2): JAK2^{V617F} mutated ET due to MGM (ET.MGM) (Table 2, Figure 4) and JAK2 wild type ET associated with primary MGM (PMGM, Table 4, Figures 6-8 and 10). ET associated with JAK2 wild type PMGM (Table 4, Figure 2) is not preceded by any variant of JAK2 or MPL mutated ET or PV.

Table 2 2008 World Health Organization and European Clinical, Molecular and Pathological criteria for the diagnosis and classification of JAK2^{V617F} mutated essential thrombocythemia into 3 stages or phenotypes: important to differentiate because natural history differs

Clinical and molecular criteria	WHO bone marrow criteria
ET stage 1 Platelet count of $> 350 \times 10^9/L$ and the presence of large platelets in a blood smear in all stages of ET Presence of JAK2 ^{V617F} mutation	Normocellular ET Predominant proliferation of enlarged megakaryocytes with hyperlobulated nuclei and mature cytoplasm, lacking conspicuous morphological abnormalities. No increase, proliferation or immaturity of granulopoiesis or erythropoiesis No progression to post-ET myelofibrosis
ET stage 2 Platelet count of $\geq 350 \times 10^9/L$ and normal hematocrit: male $< 51\%$, female $< 48\%$ erythrocytes $< 6 \times 10^{12}/L$ Presence of JAK2 ^{V617F} mutation Low serum EPO level and/or increased score for leukocyte alkaline phosphatase Spontaneous EEC	Prodromal PV Increased cellularity with trilineage myeloproliferation (<i>i.e.</i> , panmyelosis). Proliferation and clustering of small to giant (pleomorphic) megakaryocytes No pronounced inflammatory reaction (plasmacytosis, cellular debris). Absence bone marrow features consistent with congenital polycythemia and secondary erythrocytosis Progression to overt PV during follow-up
ET stage 3 Platelet count of $\geq 3500 \times 10^9/L$ and no signs of leuko-erythroblastosis Erythrocytes $< 6 \times 10^{12}/L$ Presence of JAK2 ^{V617F} mutation Slight splenomegaly on ultrasound and no anemia Hb > 12 g/dL No preceding or allied of CML, PV, RARS-T or MDS	ET.MGM Increased cellularity due to MGM and normal or relative reduction of erythroid precursors with various degrees pleiomorphic loosely clustered megakaryocytes containing dysmorphic (not cloud-like) nuclei and maturation defects No or slight RF (RF 0 or 1) Progression to post ET myelofibrosis

Masked myeloproliferative neoplasms: normal platelets, leukocytes and hematocrit, but slight splenomegaly on echogram with the presence of JAK2^{V617F} mutation and/or a World Health Organization (WHO) bone marrow is rare (rather frequent in patients with splanchnic vein thrombosis and/or Budd Chiari syndrome). ET: Essential thrombocythemia; PV: Polycythemia vera; ET.MGM: ET due to megakaryocytic, granulocytic myeloproliferation; EEC: Endogenous erythroid colony; CML: Chronic myeloid leukemia; RARS-T: Thrombocythemia associated with refractory anemia with increased ringed sideroblasts; RF: Reticulin fibrosis.

Table 3 The 2008 World Health Organization and European Clinical, Molecular and Pathological criteria for the diagnosis of polycythemia vera and diagnostic differentiation between polycythemia vera and congenital or acquired erythrocytosis

Clinical and molecular criteria	Pathological criteria (WHO)
Major PV criteria A0. Early PV. Hematocrit in the upper limit of normal: Ht: 0.45 to 0.51 in male and 0.43 to 0.48 in female, Erythrocytes $< 6 \times 10^{12}/L$ A1. Classical WHO defined PV: Hematocrit $> 0.51/> 0.48$ in male/female, Erythrocytes $> 6 \times 10^{12}/L$ A2. Presence of JAK2 ^{V617F} mutation (sensitivity 95%) or exon 12 mutation A3. Low serum EPO level and/or spontaneous endogenous erythroid colony formation	P1. Early PV Increased cellularity of bone marrow predominantly due to increased erythropoiesis and loose clusters of large megakaryocytes with hyperlobulated nuclei. No or slight increase of granulopoiesis and RF P2. Overt PV Hypercellular (75%-100%) bone marrow due to trilinear increase of erythropoiesis, megakaryopoiesis and granulopoiesis and clustering of small to giant (pleomorph) megakaryocytes with hyperlobulated nuclei. Absence of stainable iron
Minor MPD criteria B1. Persistent increase of platelet count: grade I : 400-1500, grade II : > 1500 B2. Granulocytes $> 10 \times 10^9/L$ or Leukocytes $> 12 \times 10^9/L$ and/or raised LAP-score or increased PRV-1 expression in the absence of fever or infection B3. Splenomegaly on palpation or on ultrasound echogram (> 12 cm length in diameter)	P3. Erythrocytosis Selective increase of erythropoiesis, normal granulopoiesis and megakaryocytes of normal size, morphology and no clustering of megakaryocytes in primary or secondary erythrocytosis Grading of RF (RF 0, 1, 2, 3) Grading of reticulin and collagen fibrosis; myelofibrosis MF grade 1, 2 and 3

World Health Organization (WHO) and European Clinical, Molecular and Pathological (ECMP) criteria criteria for early and overt polycythemia vera (PV). A0, A2, B1 and P1 establish prodromal PV (ET stage 2) PV ECMP stage 0, or masked PV; A1, A2, P1 and none of B establish so-called idiopathic erythrocytosis or polycythemic PV ECMP stage 1. A1, A2, P2 and one or more of B establish WHO defined classic and advanced PV ECMP stage 2 and 3. A1 and P3 with normal or increased values of serum EPO is consistent with congenital or secondary erythrocytosis. A3 confirms early and overt PV without the need of red cell mass measurement for clinicians who do not have access to a hematopathologist expert in myeloproliferative neoplasms. MPD: Myeloproliferative disorders; LAP: Leukocyte alkaline phosphatase; MF: Myelofibrosis; RF: Reticulin fibrosis.

The diagnosis of 2008 WHO fibrotic primary MF (PMF) (Figure 2) is identical to fibrotic stages of PMGM

as based on the presence of at least 2 minor criteria and typical bone marrow features including: (1) dense clusters

Bone marrow alone (Georgii 1990)	ET	PV	ET PMGM	PAMM
Thiele 1988-2012 PVSG→WHO	ET RF 0	PV	PMF MF-0,1,2	AMM/IMF/CIMF/PMF
WHO	ET	PV	unclear	2001 CIMF 2008 PMF
PVSG	ET	PV	-----	IMF/PMF
↓ translation				
2008 ECMP	3 stages of ET		Hypercellular ET	
2008 ECMP	ET stage 1	ET stage 2	PV	ET stage 3
				ET.MGM
Bone marrow Michiels	ET picture	ET/PV picture	PV picture	PMGM picture
Cellularity %	< 50	50-80	80-100	50-80
		prodromal PV		80-100
Megakaryocytes	Mature	Pleomorphic	Pleomorphic	ET.MGM
				PMGM
Enlarged/clusters	+ / ↑	+ / ↑	+ / ↑ ↑	+ / ↑ ↑
Erythropoiesis	N/N	↑	↑ ↑	N / ↓
Granulocytosis	N/N	N / ↑	↑ ↑	↑ ↑
Hematocrit	N/N	< 0.51	> 0.51	N
Platelets > 400 × mm ³ /L	+ / +	+	+	+
JAK2 ^{V617F} 2005	+ / -	+	+ / + +	- / + / + +

Figure 2 Bone Marrow diagnosis alone: chronic megakaryocytic granulocytic myeloproliferation by Georgii *et al.*^[24] vs chronic idiopathic myelofibrosis by Thiele *et al.*^[28,75], and comparative World Health Organization and European Clinical, Molecular and Pathological criteria for prefibrotic essential thrombocythemia, polycythemia vera and primary megakaryocytic and granulocytic myeloproliferation or chronic idiopathic myelofibrosis/primary myelofibrosis or agnogenic myeloid metaplasia. Translation of Polycythemia Vera Study Group (PVSG) and 2008 World Health Organization (WHO) defined essential thrombocythemia (ET), polycythemia vera (PV) and chronic idiopathic myelofibrosis (CIMF), chronic megakaryocytic granulocytic myeloproliferation (CMGM) or primary megakaryocytic and granulocytic myeloproliferation (PMGM) according to European Clinical, Molecular and Pathological (ECMP) criteria subdivided in JAK2^{V617F} mutated ET, prodromal PV, overt PV and ET.MGM (red) vs prefibrotic PMGM (blue) and 2 types of normocellular ET (JAK2^{V617F} + left red, MPL^{S15} + black). PMF: Primary myelofibrosis; AMM: Agnogenic myeloid metaplasia; ET.MGM: ET due to megakaryocytic, granulocytic myeloproliferation; MF: Myelofibrosis.

of large dysmorphic megakaryocytes with immature cloud-like nuclei not seen in ET and PV; (2) increased and normal maturation of granulopoiesis; and (3) various degrees of MF, consistent with fibrotic stage of AMM (Figure 2, clinical stage C2 and C3 in Table 4). Fibrotic stage of PMF is also observed in post-ET MF and post-PV MF (Figure 2).

WHO-ECMP CRITERIA TO DIAGNOSE AND CLASSIFY MPD

The 2008 WHO^[21,22] classification of the MPNs ET, PV and PMF is a very important step forward as compared to the PVSG diagnostic MPD criteria for ET, PV and AMM^[7-10], but do not meet the needs in daily practice for four reasons^[18-20]. First, 2008 WHO criteria for ET only define normocellular ET (WHO-ET), and the diagnosis of ET type 2 with features of early PV (prodromal PV) in blood and bone marrow but normal RCM and erythrocytes ($< 6 \times 10^{12}/L$) is suggested by the 2008 WHO but not clearly defined. Second, the 2008 WHO defined ET include ECMP defined JAK2^{V617F} positive normocellular ET (WHO-ET), prodromal PV and ET.MGM, MPL^{S15} positive ET (Figure 2 middle part in red), as well as JAK2 wild type PMGM (Figure 2 right in blue). There is good evidence that JAK2^{V617F} positive hypercellular ET with no leukoerythroblastosis but with increased megakaryocytic

granulocytic myeloproliferation (ET.MGM) is featured by reduced erythropoiesis and loose clusters of slight to moderate dysmegakaryopoiesis is rather frequent (ET.MGM, Table 2)^[40]. Third, the diagnostic differentiation between JAK2^{V617F} positive ET.MGM without leukoerythroblastosis in Table 2 and JAK2 wild type PMGM without leukoerythroblastosis is clinically relevant and not addressed by the 2008 WHO classification. Fourth, the 2008 WHO classification disregard the importance of increased *vs* normal or decreased erythrocytes, leukocytes, LAP score, platelets and spleen size for diagnosis, classification and staging of thrombocythemia in MPNs of various molecular etiology. Simple tests like blood cell counts including platelets, leukocytes, hematocrit and erythrocytes, spleen size on echogram, EEC, and LAP score are even not taken into account to distinguish the latent (masked), early and overt thrombocytic and erythrocytic stages of PV from the overt trilinear polycythemic stage of classic PV. These shortcomings of the 2008 WHO MPN criteria prompted us to propose integrated WHO-ECMP criteria for the diagnosis of ET (Table 2), PV (Table 3) and PMF or PMGM (Table 4).

THREE STAGES OF ET

Sustained increase of platelet counts ($> 350 \times 10^9/L$) associated with slight splenomegaly on echogram (> 12 cm),

Table 4 World Health Organization and European Clinical, Molecular and Pathological criteria for diagnosis and staging of primary megakaryocytic granulocytic myeloproliferation, or primary myelofibrosis

Michiels JJ	Clinical criteria (2005)	Thiele J	pathological criteria (2005/2008)	
A1	Hypercellular JAK2/MPL wild type ET and no preceding or allied other subtype of myeloproliferative neoplasm: JAK2 ^{V617F} or MPL ^{S15} normocellular ET, prodromal or classical PV, Ph1+ CML or MDS	B1	PMGM and relative reduction of erythroid precursors. Abnormal clustering and increase in atypical giant to medium sized dysmorphic megakaryocytes containing bulky/clumsy (cloud-like) hypolobulated nuclei and definitive maturation defects	
C	Clinical stages	MF	Staging of myelofibrosis	
C1	Early clinical stages Normal hemoglobin or slight anemia, grade 1 : hemoglobin > 12 g/dL No, slight or moderate splenomegaly on ultrasound scan or CT Hypercellular ET, platelets in excess of 400, 600 or even > 1000 × 10 ⁹ /L No leuko-erythroblastic blood picture and/or tear drop erythrocytes	MF 0	Prefibrotic stage PMGM/PMF	RF 0/1
		MF 1	Early fibrotic PMGM/PMF	RF 2
C2	Intermediate clinical stage Anemia grade II: hemoglobin > 10 g/dL Definitive leuko-erythroblastic blood picture and/or tear drop erythrocytes Splenomegaly, increased LDH	MF 2	Manifest fibrotic PMGM/PMF	RF 3 = RCF
		MF 3	Advanced fibrotic PMGM/PMF	RF 4 = RCF
C3	Advanced clinical stage Anemia grade III: hemoglobin < 10 g/L Splenomegaly and increased, normal or decreased platelet count Thrombocytopenia, leukocytosis, leukopenia, increased circulating CD34+ cells	MF 3	Osteosclerosis	

ET: Essential thrombocythemia; PMGM: Primary megakaryocytic and granulocytic myeloproliferation; CT: Computed tomography; LDH: Lactodehydrogenase; PV: Polycythemia vera; CML: Chronic myeloid leukemia; PMF: Primary myelofibrosis; MF: Myelofibrosis; RF: Reticulin fibrosis; RCF: Reticulin/collagen fibrosis.

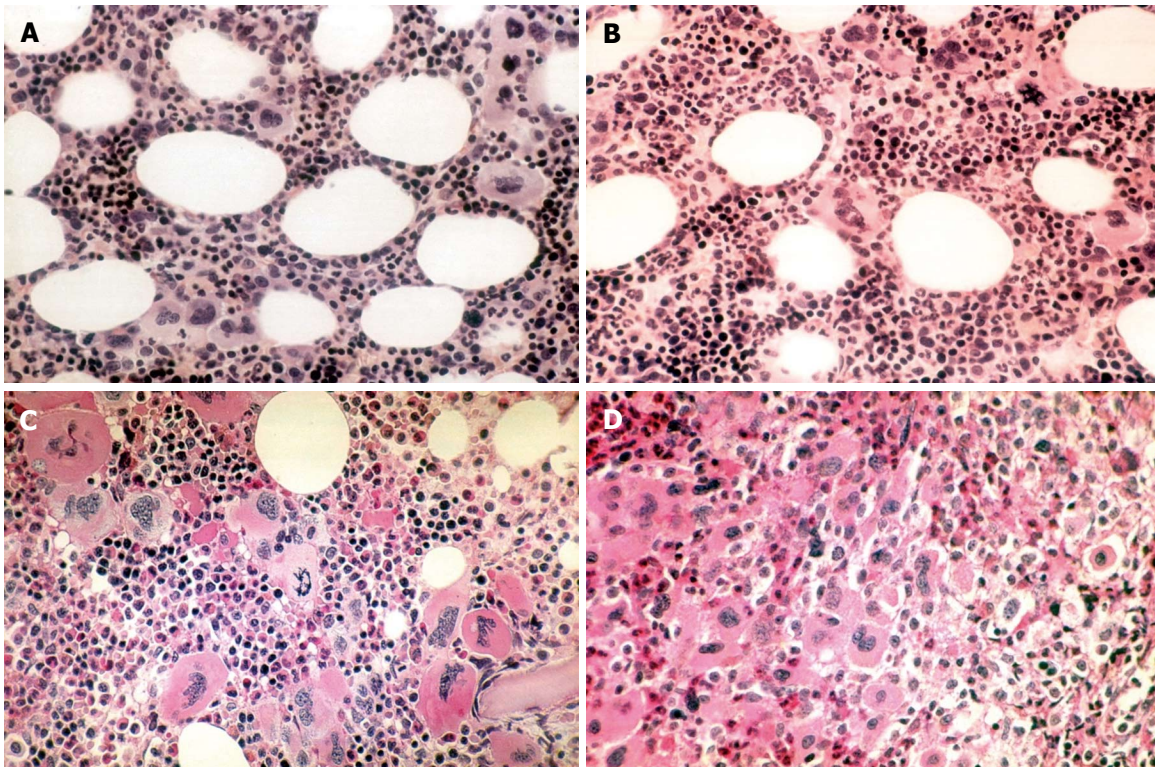


Figure 3 Bone marrow histology features in essential thrombocythemia and polycythemia vera patients. A: Normocellular essential thrombocythemia (ET) bone marrow histology [World Health Organization (WHO)-ET] with increase of clustered pleomorphic megakaryocytes similar as in prodromal and overt polycythemia vera (PV); B: ET/PV bone marrow histology with pleomorphic megakaryocytes and increased cellularity due to increased erythropoiesis as can be seen in WHO and European Clinical, Molecular and Pathological defined prodromal PV and overt PV patients; C: PV bone marrow histology with increased cellularity due to increased erythropoiesis/granulopoiesis and increase of clustered pleomorphic megakaryocytes and no increase of reticulin fibrosis; D: Advanced PV bone marrow histology with dense clustered pleomorphic/dysmorphic megakaryocytes (not cloud-like) and increase in reticulin fibrosis grade 2.

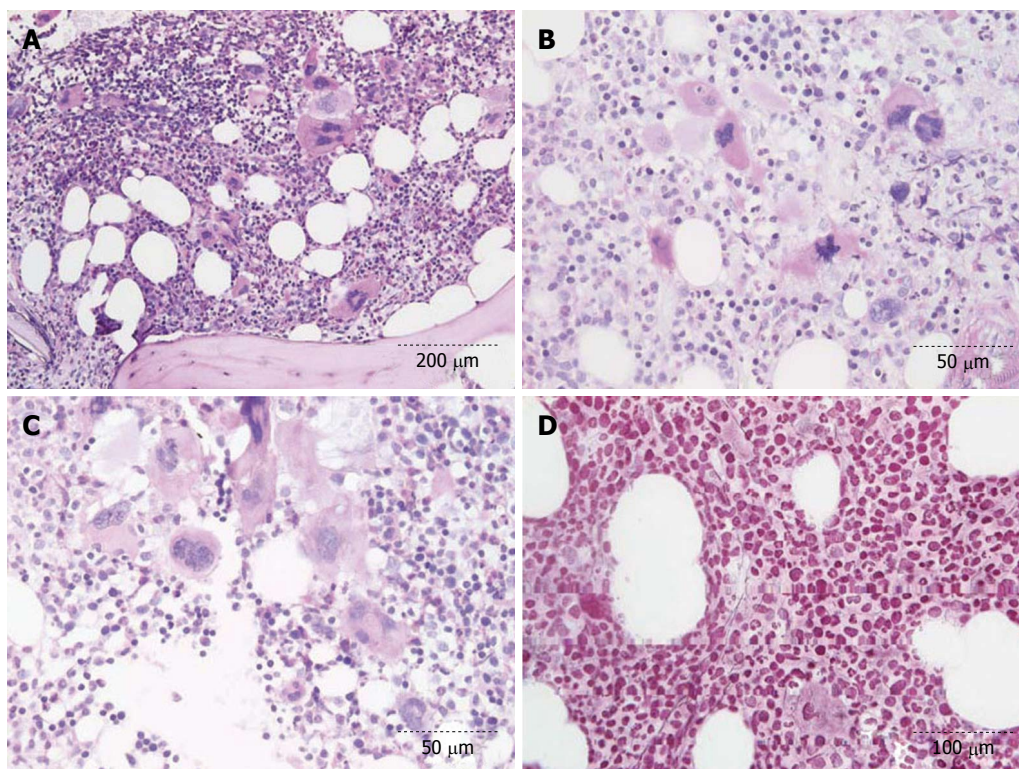


Figure 4 $JAK2^{V617F}$ mutated essential thrombocythemia due to megakaryocytic, granulocytic myeloproliferation with slight splenomegaly (spleen 16 cm on echogram) and a hypercellular megakaryocytic granulocytic bone marrow and clustered pleomorphic clumpy megakaryocytes with dysmorphic (not cloud-like) nuclei: prefibrotic essential thrombocythemia due to megakaryocytic, granulocytic myeloproliferation. A-C: $JAK2^{V617F}$ -positive essential thrombocythemia due to megakaryocytic, granulocytic myeloproliferation (ET.MGM) featured by hypercellular ET due to increased megakaryocytic granulocytic myeloproliferation and the presence of pleomorphic/dysmorphic megakaryocytes (not cloud-like); D: Reticulin fibrosis grade 1, myelofibrosis grade 0.

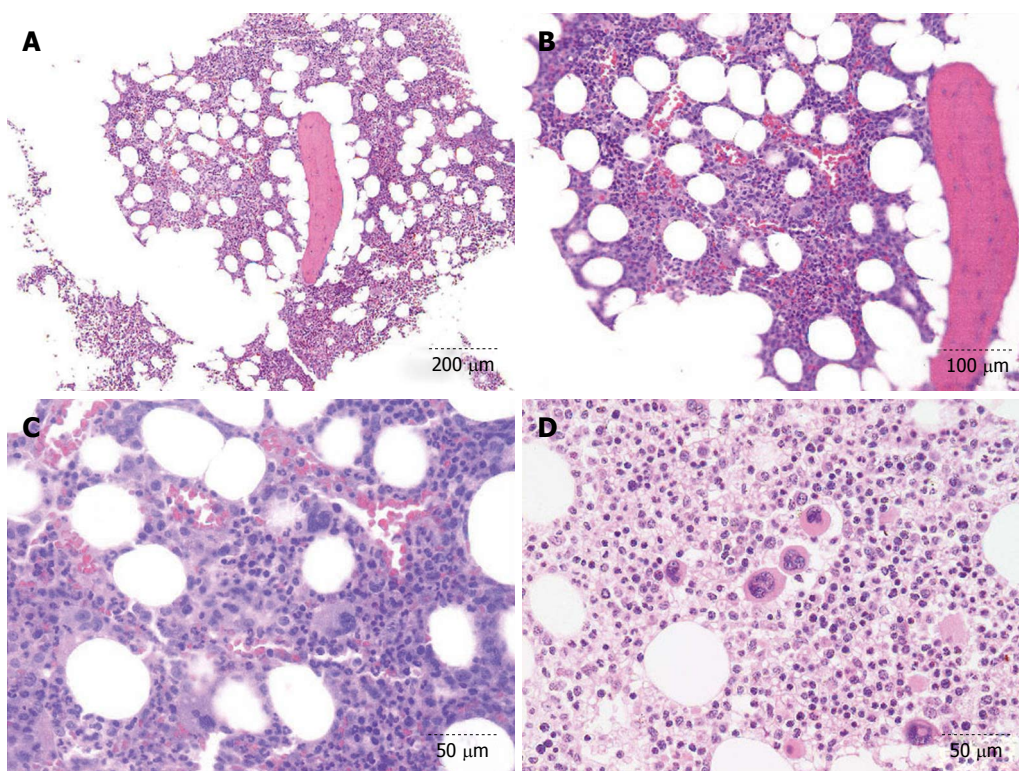


Figure 5 Forty-three-year-old female with positive polycythemia vera (platelets $405 \times 10^9/L$, low serum erythropoietin, leukocyte alkaline phosphatase score 283, hematocrit 0.52, erythrocytes $6.1 \times 10^{12}/L$, increased red cell mass) with a diagnostic essential thrombocythemia/polycythemia vera bone marrow picture. Such essential thrombocythemia (ET)/polycythemia vera (PV) pictures are regularly seen in prodromal PV and overt PV. A-D: $JAK2^{V617F}$ positive early stage PV with a ET/PV bone marrow histology with loose clusters of pleomorphic megakaryocytes.

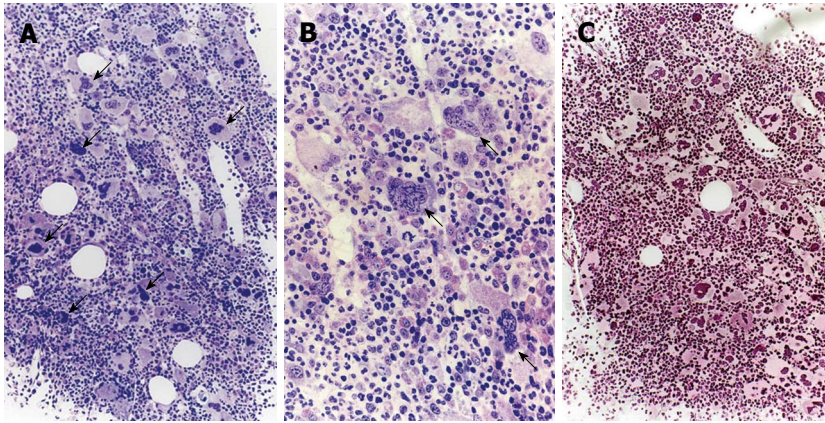


Figure 6 JAK2 wild type hypercellular essential thrombocythemia with platelet counts of $2180 \times 10^9/L$, no splenomegaly, normal lactodehydrogenase and normal white blood cell differential counts with a characteristics picture of prefibrotic primary dysmegakaryocytic granulocytic myeloproliferation. A, B: JAK2 wild type hypercellular essential thrombocythemia with a typical primary megakaryocytic and granulocytic myeloproliferation bone marrow histology with the presence of abnormal clustering and increase in atypical giant to medium sized dysmorphic megakaryocytes containing bulky, clumsy (cloud-like) hypolobulated nuclei and definitive maturation defects; C: Reticulin fibrosis grade 0. Arrows indicate: Immature dysmorphic megakaryocytes with cloud-like nuclei.

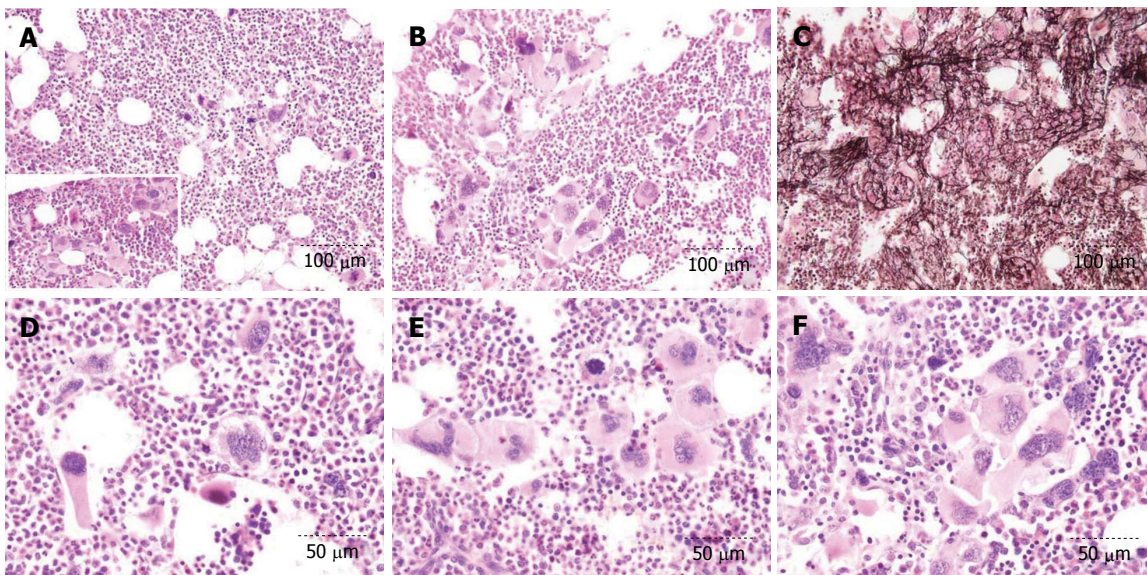


Figure 7 Thirty-seven-years old woman (asymptomatic except fatigue) with JAK2 wild type hypercellular essential thrombocythemia: platelets $1205 \times 10^9/L$, Hb 12.5 g/dL, erythrocytes $4.9 \times 10^{12}/L$, leukocytes $18 \times 10^9/L$, slightly increased lactodehydrogenase, no splenomegaly on palpation as the presenting features of primary megakaryocytic and granulocytic myeloproliferation (Table 5). A, B, D-F: JAK2 wild type hypercellular bone marrow histology due to primary megakaryocytic and granulocytic myeloproliferation with the presence of clustered atypical giant to medium sized dysmorphic megakaryocytes containing bulky (cloud-like) hypolobulated nuclei and definitive maturation defects; C: Reticulin fibrosis grade 2.

normal erythrocytes ($< 6 \times 10^{12}/L$) obviating the need of RCM, normal or increased leukocytes ($> 12 \times 10^9/L$) with normal erythrocyte sedimentation rate (ESR) is suspicious of myeloproliferative ET in the absence of any cause for reactive thrombocytosis. Pre-treatment bone marrow biopsy is needed as the final step in the diagnostic workup to correctly classify the JAK2^{V617F} positive and JAK2 wild type thrombocythemias in various prefibrotic MPDs. The presence of giant platelets in a peripheral blood smear and clustered large or giant mature megakaryocytes in bone marrow smear and biopsy are the pathognomonic clues to the clinical diagnosis of ET. PVSG defined ET without leukoerythroblastosis includes three phenotypes of ET when the WHO-ECMP criteria are applied

(Table 2)^[18-22]. The WHO-ECMP criteria classify clinical ET as normocellular ET (WHO-ET), prodromal PV mimicking ET, and ET associated with a prefibrotic MGM bone marrow picture without features of leukoerythrocytosis and extramedullary hematopoiesis (ET. MGM) and ET associated with PMGM. The screening for JAK2^{V617F} mutation is very helpful in the diagnostic workup of MPN patients^[41-63]. Prodromal PV patients carry the JAK2^{V617F} mutation (Figures 3 and 4). Only half of the patients with PVSG or WHO defined ET carry the JAK2^{V617F} mutation (sensitivity 50% to 60%) and only a very few carry the MPL^{S15} mutation^[59,60]. A typical MPN bone marrow histology (either ET, PV or PMGM) excludes reactive thrombocytosis, congenital or secondary eryth-

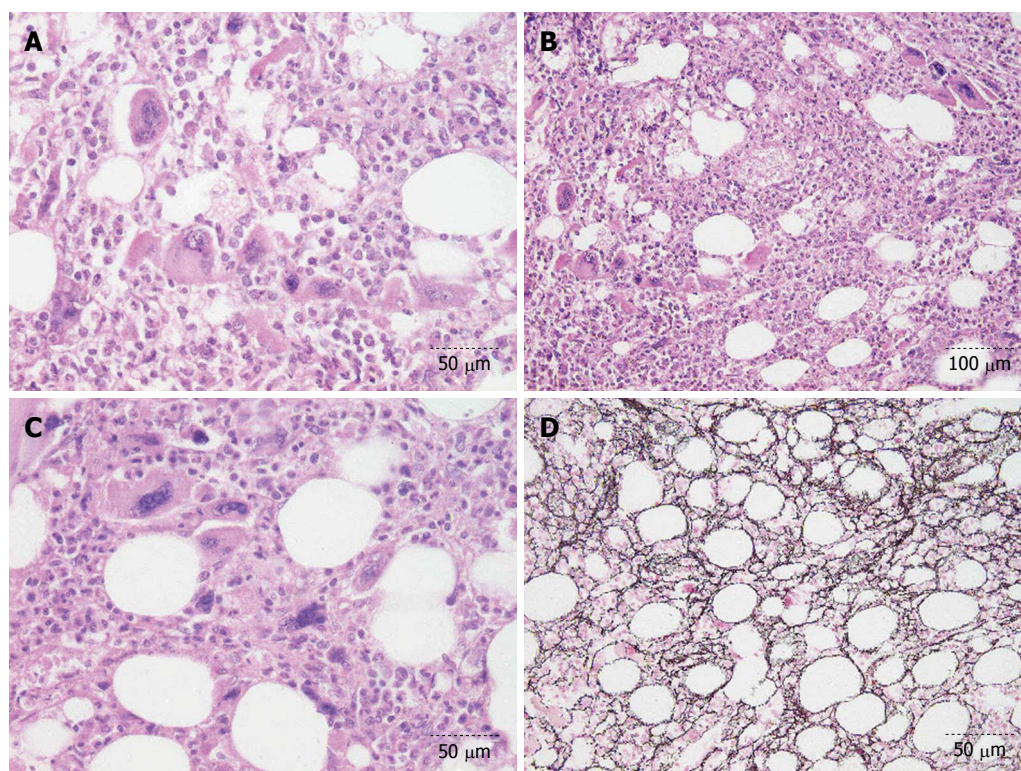


Figure 8 Chronic megakaryocytic granulocytic myelosis according to the Hannover Bone Marrow Classification at time of diagnosis in 1995, and JAK2 wild type primary megakaryocytic granulocytic myeloproliferation according to World Health Organization and European Clinical, Molecular and Pathological criteria in 2006. A-C: Hypercellular bone marrow histology with the presence of Abnormal clustering and increase in atypical giant to medium sized dysmorphic megakaryocytes containing bulky/clumsy (cloud-like) hypolobulated nuclei and definitive maturation defects; D: Reticulin fibrosis grade 2, myelofibrosis grade 1.

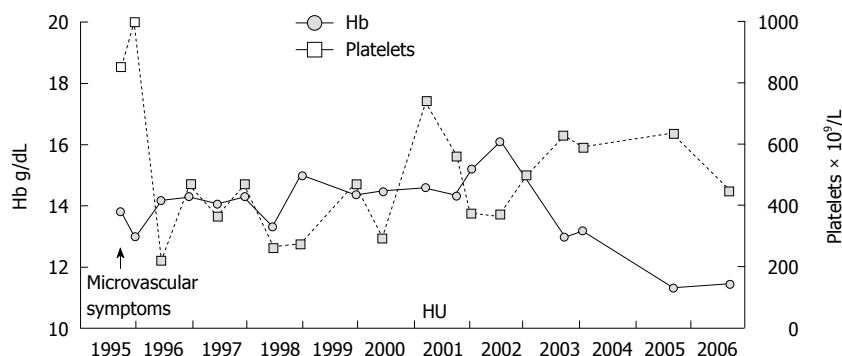


Figure 9 The case of primary megakaryocytic and granulocytic myeloproliferation in Figure 7, who presented in 1995 with microvascular circulation disturbances treated with hydroxyurea for 11 years complicated by mild anemia at platelet counts of $600 \times 10^9/L$ after 10 years of hydroxyurea (HU) for 10 years (1996-2006).

rocytoses, CML, and thrombocythemia associated with refractory anemia with increased ringed sideroblasts^[61-63]. WHO-ECMP defined prefibrotic JAK2 wild type PMGM is featured by a hypercellular bone marrow due to pronounced granulopoiesis and dominated by dense clusters of dysmorphic megakaryopoiesis with atypical immature megakaryocytes which are conspicuously enlarged due to increase of nuclear and cellular size with bulky and irregular, round-shaped (cloud-like) nuclei (Table 4, Figures 6-8).

PV VS PRIMARY OR SECONDARY

Characteristic features suspicious for PV include increased

hematocrit (> 0.51), increased erythrocytes ($> 6 \times 10^{12}/L$), slight splenomegaly, increased leukocytes ($> 12 \times 10^9/L$) or LAP score with normal ESR, an increased platelets ($> 400 \times 10^9/L$) (Table 3). The detection of JAK2^{V617F} in granulocytes with sensitive polymerase chain reaction (PCR) techniques plays a key-role in the diagnostic work-up of patients with suspected PV (Table 3)^[64,65]. In the context of erythrocytosis the presence of the JAK2^{V617F} mutation has a sensitivity of 95% and a positive predictive value of 100% for the diagnosis of PV, and excludes CP and SE without the need of RCM measurement (Figure 2)^[18]. In the context of a JAK2^{V617F} positive erythrocythemia (hematocrit > 0.51 in males and > 0.48 in females) the presence of

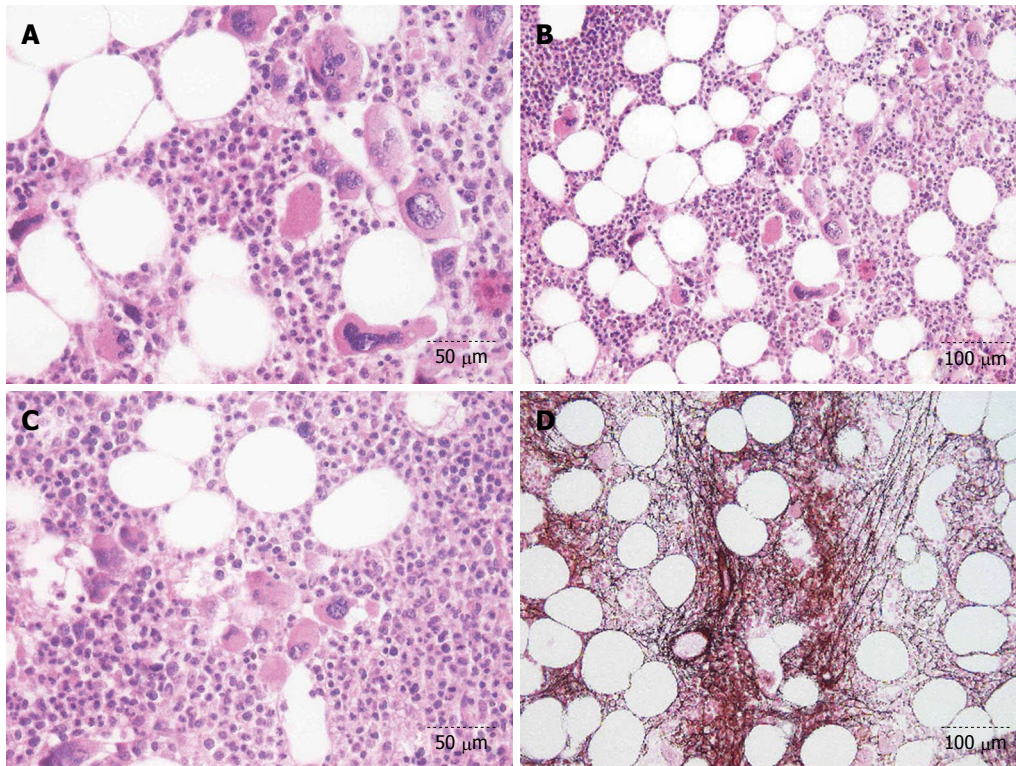


Figure 10 Essential thrombocythemia case diagnosed in 1995 as chronic megakaryocytic granulocytic myelosis, and as JAK2 wild type primary megakaryocytic granulocytic myeloproliferation in 2006 (Figure 8) was complicated by slight anemia and increased bundles of reticulin fibrosis grade 2 after 10 years of hydroxurea treatment (Figure 9). A-C: Bone marrow histology findings in 2006 show tightly clustered immature megakaryocytes with low degree of dysmegakaryopoiesis and cloud-like nuclei. Sometimes the nuclei have an irregular contour and no real hyperchromasia; D: Increase in reticulin fibrosis with many cross-sections grade 2/myelofibrosis grade 1 (Table 5).

large platelets in peripheral blood smear, large megakaryocytes in smears from aspirated bone marrow, low serum EPO, ferritin and slight splenomegaly on echogram are diagnostic for prodromal or overt PV showing a typical ET/PV or PV bone marrow histology picture (Figure 2). As compared to bone marrow histopathology, EEC and serum EPO levels are specific but not sensitive enough to differentiate between myeloproliferative PV, primary erythrocytosis and SE^[60]. EEC and serum EPO levels do not differentiate between prodromal PV (normal RCM and normal erythrocyte count) *vs* classic PV (increased erythrocyte count $> 6 \times 10^{12}/L$ and RCM). EEC in the clinical research setting surely will contribute to a better understanding of the role of JAK2^{V617F} in the etiology of heterozygous *vs* homozygous mutated MPN. Pre-treatment bone marrow histology is very insightful and the most powerful tool to stage PV, and to differentiate trilinear hypercellularity in PV from an isolated increase of erythropoiesis in CP and SE with a specificity and sensitivity approaching 100% (Table 3)^[67-70]. In SE^[69,71] and in CP due to a gain of function mutation in the Epo-receptor, the megakaryocytes are of normal size and morphology and there is no tendency to cluster^[72,73]. Differential diagnosis of JAK2 wild type PV, early erythrocythemic PV and idiopathic erythrocytosis (increased RCM and erythrocytes) is problematic and can best be solved by the combined use of bone marrow histology and molecular screening including JAK2^{V617F}, JAK2 exon and MPL⁵¹⁵

mutations obviating the need to measure RCM. About 5% of WHO defined PV patients are JAK2^{V617F} negative and half of them may carry a JAK2 exon 12 mutation^[57,58]. Scott *et al*^[57] identified JAK2 exon 12 mutations in 10 erythrocytosis patients with increased RCM but negative for the JAK2^{V617F} mutation, which according to PVSG criteria could be diagnosed as PV in 6 and idiopathic erythrocytosis in 4. Pre-treatment bone marrow biopsies in 5 patients carrying one of the JAK2 exon 12 mutations showed characteristic erythroid hyperplasia with slight morphological abnormalities of the megakaryocyte in the study of Scott *et al*^[57]. In another report, 5 cases of JAK2^{V617F} negative PV carrying exon 12 mutation (F537-K539delinsl or N542-E534del) were diagnosed as idiopathic erythrocytosis with increased hemoglobin and hematocrit, low serum EPO, normal platelet and leukocyte counts, no or palpable spleen and a typical hypercellular bone histopathology predominantly due to erythroid hyperplasia and clusters enlarged megakaryocytes with hyperploid nuclei was observed in 2 cases^[58].

ROLE OF JAK2 AND MPL MUTATIONS IN THE ETIOLOGY AND PROGNOSIS OF MPN

Applying allele-specific PCR analysis in PVSG-defined MPD patients, a high frequency of the JAK2^{V617F} muta-

tion of 95% (92%-97%) is described in PV, and a lower frequency of 53% (49%-57%) in ET and 52% (44%-55%) in MF (post PV, post ET and PMF)^[18,74]. Only 3%-4% of ET, 24%-27% of PV and 6%-18% of MF patients are homozygous for the JAK2^{V617F} mutation^[18,72]. Within the JAK2^{V617F}-positive MPNs, good evidences accumulate that the majority of PVSG defined ET patients are heterozygous for the JAK2^{V617F} mutation^[41,42] and behaves as an indolent slow onset myeloproliferation of mature enlarged megakaryocytes with no progression to homozygosity and MF during long-term follow-up^[43-46]. As compared to JAK2^{V617F}-negative ET, the presence of JAK2^{V617F} mutation has been significantly associated with higher hemoglobin level, higher leukocyte counts and less pronounced thrombocytosis^[43-48]. Two studies showed that erythroid burst forming unit colonies are already homozygous for the JAK2^{V617F} mutation in PV patients with a heterozygous pattern of JAK2^{V617F} in their peripheral blood granulocytes^[41,42]. Homozygous JAK2^{V617F} PV and PMF refers to a more rapid onset and slowly progressive disease in about one third during long-term follow-up^[49-52]. The percentage of JAK2^{V617F} mutation and progression from heterozygous to homozygous due to mitotic recombination of chromosome 9p (loss of heterogeneity of chromosome 9p: LOH 9p) is strongly correlated with increased LAP score, with the ability to form spontaneous EEC and with progressive post-PV MF^[50,53]. Homozygous JAK2^{V617F} PV and PMF patients belong to an advanced stage of MPN and displayed significantly higher hemoglobin at time of diagnosis, increased incidence of aquagenic pruritus, higher LAP scores in granulocytes, and higher rate of fibrotic transformation^[49-52]. Homozygous MPN patients are older, had larger spleen, more frequent leukocytosis, and displayed evolution to secondary MF and a significantly higher risk of cardiovascular events as compared to heterozygous and wild type MPN patients. Vannucchi *et al.*^[53] employed quantitative assays for JAK2^{V617F} allele levels in granulocytes in a prospective study of 175 PV patients at time of diagnosis. The JAK2 mutant allele burden could be quantified as 1%-25%, 25%-50%, 50%-75% and 75%-100% in 57, 50, 34 and 32 PV patients respectively at time of investigation. The burden of JAK2^{V617F} allele was directly correlated with abnormally increased levels of hematocrit, white cell and neutrophil count, LDH and LAP score, spleen size on echogram and with decreased values for serum ferritin, and erythropoietin^[53]. The JAK2^{V617F} allele burden nicely correlated with a progressively higher relative risk for aquagenic pruritus, spleen size on echogram, total thrombosis and the need for receiving myelosuppressive therapy^[53]. Mechanisms other than the mutated JAK2^{V617F} in exon 14 is observed in a proportion of PV and MF patients displaying a gain of chromosome 9p, mostly due to trisomy 9^[54-56]. Campbell *et al.*^[56] reported that the JAK2^{V617F} mutation was found in all 10 MPN patients with trisomy 9, and in 28 of 29 MPN patients (PV, ET or CMF) with a 20q deletion. The finding of the JAK2 exon 12 mutations in patients with PV or idiopathic erythrocytosis, but not in ET further confirms the strong

association between the JAK2 mutations and MPN and clearly demonstrates the pivotal role of JAK2 mutations as pathogenic events in variable phenotypes of MPN^[57,58]. MPL^{W515L} and MPL^{W515K} mutations has been found in some ET and MF patients indicating the importance of the MPL signalling pathway in the etiology of clonal MPN^[59,60].

DIAGNOSTIC DIFFERENTIATION AND NATURAL HISTORY OF JAK2V617F MUTATED ET, PV AND PMF

We propose to extend the PVSG and WHO criteria into a broader set of integrated WHO-ECMP criteria not only for the diagnosis and classification but also for staging of MPN burden at the peripheral blood, spleen and blood and bone marrow level. Upon application of the integrated set of WHO-ECMP criteria, the JAK2^{V617F} mutated ET comprises three phenotypic manifestations of ET including normocellular ET (WHO-ET) (Figure 3), ET 2 with early features of PV (prodromal PV, Figure 3) and ET.MGM with loose or dense clusters of pleomorphic to dysmorphic megakaryopoiesis in a hypercellular bone marrow due to increased granulopoiesis (Figure 4).

The bone marrow histology of JAK2^{V617F} mutated ET.MGM show different grades of granulocytic hypercellularity, which can appear to overlap with PV (hematocrit < 0.51) presenting with a hypercellular bone marrow with more or less pronounced increase of granulopoiesis. The histology of ET.MGM bone marrow showing slightly dysmorphic megakaryopoiesis and may overlap with that of ET cases with mild hyperplasia of granulopoiesis and/or a mixture of mainly mild dysmorphic megakaryocytes (Table 3). Therefore, we may predict a significant overlap (grey zones of about 20%-30%) between ET.MGM and PV with increased granulopoiesis due to an inter-observer disagreement among hematopathologists.

The prognostic importance of the WHO bone marrow features and grading of MF (Table 5) has demonstrated by Kvasnicka and Thiele in a large retrospective study of 865 PVSG defined normocellular ET patients with a platelet count in excess of $600 \times 10^9 \text{ mm}^3/\text{L}$ ^[75,76]. In this study, Kvasnicka and Thiele reclassified PVSG defined ET as normocellular true ET (WHO-ET) in 167, and prefibrotic CIMF in 174 and early fibrotic CIMF-1 in 135 according to WHO bone marrow criteria^[75]. WHO-ET patients showed no significant loss of life expectancy compared to significant loss of life expectancy in CIMF 0 and CIMF 1 ($P = 0.0001$). The 15 years relative survival was 84% for WHO-ET compared to 68% for CIMF 0 and 55% for CIMF 1. Interestingly, WHO-ET patients were 10 to 12 year younger compared to CIMF 0 and CIMF 1. A similar, large retrospective study has been performed by Barbui *et al.*^[77]. A total of 1104 PVSG defined ET patients (platelet count > $600 \times 10^9/\text{L}$) from seven centers in Italy and the United States diagnosed between 1975 and 2008 were analyzed retrospectively using the 2008 WHO clinical and

Table 5 Grading of reticulin fibrosis and myelofibrosis

Grading ^[78,79]	Grading of MF ^[80]	Description of RF and RCF in MF as a secondary event in MPN
Normal RF-0	MF 0	No reticulin fibers, occasional individual fibers or focal areas with tiny amount of reticulin fiber network
RF 1	MF 0	Fine reticulin fiber network throughout much of section and no course reticulin fibers
Slight increase		
RF 2	MF 1	Diffuse fine reticulin network with focal collections of thick course reticulin fibers and no collagenisation
Moderate increase		
RF 3 = RCF	MF 2	Diffuse and dense increase in reticulin with extensive intersections, and presence of collagen fibers and no or minor osteosclerosis
Marked increase		
RF 4 = RCF and O	MF 3	Diffuse and dense reticulin with coarse bundles of collagen associated with significant O
OS Dry tap	Sclerotic	

MF: Myelofibrosis; RF: Reticulin fibrosis; RCF: Reticulin/collagen fibers; MPN: Myeloproliferative neoplasms; O: Osteosclerosis.

bone marrow criteria. Bone marrow biopsies were evaluated by one pathologist (Dr. Thiele). In this cohort of 1104 PVSG defined ET patients, 891 (81%) were diagnosed as normocellular WHO-ET (JAK2^{V617F} positive 61%); and 180 (16%) as hypercellular ET with prefibrotic PMF (pPMF) bone marrow histology (JAK2^{V617F} positive 58%). The overall survival curves show the expected differences in overall survival between WHO-ET and pPMF similar as shown by Kvasnicka *et al.*^[76]. When compared to the 2008 Eurostat age- and sex-standardized incidences for all causes of death, there was no or minimal loss of life expectancy in WHO-ET MPN patients. The difference of 15 years overall survival in WHO-ET *vs* pPMF (80% and 59%, respectively) was mainly due to the 15 years leukemia-free survival incidence in WHO-ET and pPMF (0.8% *vs* 12.3%, respectively). There were significant differences in leukocyte counts ($8.6 \times 10^9/L$ *vs* $9.7 \times 10^9/L$), LDH (298 mU/mL *vs* 429 mU/mL) and reticulin grade grade 1 (3% *vs* 24%) at time of diagnosis of WHO-ET and pPMF 0/1 respectively. Evolution of pPMF 0/1 into fibrotic PMF 2/3 (PAMM) after 10 and 15 years increased from 0.8% to 9.3% in WHO-ET and from 9.3% to 16.9% in pPMF 0/1. Unfavorable prognostic factors in pPMF include LDH above the upper limit of normal, increase of CD34+ circulating cells, spleen size growth of 0.5 to 1 cm/year, slight anemia hemoglobin < 13 g/dL, and constitutional symptoms. In this study 60% of WHO-ET and pPMF patients carried the JAK2^{V617F} mutation. Evolution of WHO-defined ET into myeloid metaplasia of the spleen with MF and leukoerythroblastosis is very rare and predicted to be rather frequent in JAK2^{V617F}-positive hypercellular ET/MGM or JAK2 wild type PMGM. Large scale collaborative prospective management study of newly diagnosed MPN patients comparing the various degrees of JAK2^{V617F} and MPL^{S15} mutation load and JAK2 wild type PMGM are needed.

GRADING OF SECONDARY MF IN MPN

The terms RF and reticulin/collagen fibrosis (RCF) are well established in the literature^[24,78-81]. Sequential biopsies indicate that initially there is a diffuse or patchy increase in fine RF fibers admixed with abundant hematopoietic elements in PV and PMGM during long-term follow-

up. In sequential bone marrow biopsies, the marrow in fibrotic PV and PMF is replaced by course collagen fibers with a decreasing number of hematopoietic cells. This progression from RF to RCF in the bone marrow biopsy during long-term follow-up may be analogous to the similar wound healing, in which collagen composition changes as time passes (fine reticulin type III collagen is replaced by course collagen type I collagen). The precise mechanisms by which cytokines from abnormal neoproliferative hematopoietic clone in the various MPNs do stimulate the host's polyclonal fibroblasts to produce excessive amounts of fine RF fibers and course RCF bundles are out of scope in this review.

Two kinds of fiber qualities can easily be distinguished by common staining in light microscopy: RF^[78,79] and RCF^[24,80]. Gomori's silver staining detects early and course RF and do not stain collagen fibers thereby underestimating advanced RCF MF grade 2 and 3. Collagen fibers stain with Mason's trichrome stains, and are negative in the Gomori's silver stain. Consequently both Gomori's stain for RF and trichrome stain for CF are to be used for optimal MF-grading of RF and RCF^[78,79] and for grading of MF^[80] (Table 5). The evolution of RF into RCF as documented by the combined use of silver and trichrome stains simple means a determinative change from reversible normal reticulin (= RF) into progressed pathological collagen scarring (RCF without or with osteosclerosis). Clinically, RCF often results in cytopenia and dry tap, when aspiration is attempted. RF with very early RCF usually do occur without real scarring. Bone marrow aspiration in RF without collagen fibrosis (MF-1) usually does not cause the symptom of dry tap. Advanced MF (RCF = MF 2 and 3^[80]) designates pronounced increased collagen fibrosis visible scarring spotted areas and sometimes with foci or larger areas of atrophic hematopoiesis in the bone marrow in light microscopy.

MF itself is not a disease because RF and RCF is a secondary event induced by polyclonal fibroblasts in response to cytokines released from the clonal granulocytic and megakaryocytic proliferative cells in both PV and PMF. The presence of RF is well documented in ET, PV, PT, PMGM, CML and in many other conditions. Various degrees of RF is rather rare in normocellular ET (WHO-ET) and does occur in about one third of PV and in

Table 6 World Health Organization and European Clinical, Molecular and Pathological staging of prodromal, classical and advanced polycythemia vera related to therapy

PV, ECMP stage	0		1	2	3	4	5
Michiels ECMP	Erythrocy- themic PV	Prodromal PV mimicking ET	Polycythemic PV prefibrotic	Classic PV prefibrotic	Advanced PV PMF stage	Post-PV MF Spent phase PV	Leukemic evolution MDS AL
LAP-score	N/ ↑	↑	↑	↑	↑ / ↑ ↑	Variable	Variable
Red cell mass	↑	N	↑	↑	↑	Variable	N/ ↓
Serum EPO	N/ ↓	N/ ↓	↓	↓	↓	Variable	N/ ↓
Leukocytes × 10 ⁹ /L	< 12	< 12	< 12	N-> 12	> 15	> 20	> 20
Platelets × 10 ⁹ /L	< 400	> 400	< 400	> 400	< or > 1000	Variable	Variable
Hemoglobin g/dL (mmol/L)	> 16 (10)	< 16 (10)	> 16 (10)	> 16 (10)	> 16 (10)	N/> 12	< 12
Hematocrit	> 0.51	< 0.51	> 0.51	> 0.51	> 0.51	Variable	N ↓
Erythrocytes × 10 ¹² /L	> 6	< 6	> 6	> 6	> 6	Variable	N/ ↓
ECMP bone marrow	Early PV	Pro-PV	Early PV	Trilinear PV	Trilinear PV	Myelofibrosis	AML
Bone marrow cellularity (%)	50-80	50-80	60-90	80-100	80-100	Decreased	Increased
Grading myelofibrosis ^[57]	RF 0-1	RF 0-1	RF 0-1	RF 0/1, MF 0	RCF 2/3 MF 1/2	RCF 3/4 MF 2/3	No MF
Splenomegaly on palpation	No	No/+	No/+	+	++/+++	/Large	Large
Spleen size, echogram cm	< 12	< 12-15	12-15	12-18	18-> 20	> 20	> 20
Spontaneous EEC+	+	+	+	+	+	+	No
JAK2 ^{V617F} in granulocytes	+	+	+	+/++	+/++	++	No or +
BFU-e (exon 12)	+(++)	+(++)	+(++)	++	++	++	No
Therapeutic implications	Low risk	Low risk	Low risk	Intermediate risk PV	High risk PV	Post-PV MF Spent phase	Acute leukemia
First line treatment option ^[82,83]	Aspirin	Aspirin	Phlebotomy	Phlebotomy ¹	If IFN	JAK2 inhibitor	Chemotherapy
Asp/Phleb ^[82,83]	phlebotomy	phlebotomy low	Aspirin	Aspirin	resistant→	→Bone marrow	Bone marrow
IFN ^[84-86]		dose IFN?	Low dose IFN	IFN→	HU or	transplantation	transplantation?
MPN reductive treatment			→	if resistant	HU first line	Aspirin?	Supportive
Hydroxyurea ^[83]			Complete response	→HU			
JAK2 inhibitor ^[87-90]							

¹ ↑ : Increased; ↓ : Decreased; N: Normal; +: Present or heterozygous; ++: Homozygous. ET: Essential thrombocythemia; PV: Polycythemia vera; MPN: Myeloproliferative neoplasms; MF: Myelofibrosis; AMM: Agnogenic myeloid metaplasia; IFN: Interferon; EEC: Endogenous erythroid colony; ECMP: European Clinical, Molecular and Pathological; LAP: Leukocyte alkaline phosphatase; RCF: Reticulin/collagen fibrosis; RF: Reticulin fibrosis.

the majority of patients with PMGM during long-term follow-up^[24,25]. The easiest way in grading of RF using the reticulin silver stain has been performed by the PVSG and in the recent UK study^[78,79]. A scoring system of MF based on morphometric analysis (point intersection with an ocular grid) and quality of fibers (reticulin and collagen fibers) and the bone marrow fiber density (fine or course reticulin and some or course bundles of collagen) has been proposed by Thiele *et al*^[80] 2005 (Table 5).

CONCLUSION

The underreported early stages of MPN are currently detected by the combined use of clinical, molecular and pathological markers as recommended by integrated WHO-ECMP MPN criteria for the classification and staging of ET, PV and PMGM (Tables 3-6). A wide diffusion and implementation of WHO-ECMP criteria are awaited to clarify their value in recognizing the prefibrotic stages of MPN and in predicting significant differences in long-term prognosis between JAK2^{V617F} normocellular WHO-ET and ET.MGM *vs* JAK2 wild type hypercellular ET associated with PMGM. The urgent need of prospective evaluation of integrated WHO-ECMP criteria include complete blood cell counts (erythrocytes, leukocytes, platelets, LAP score), spleen size on echogram, JAK2 mutation screening, JAK2 mutation load, serum EPO fol-

lowed by bone marrow biopsy (Tables 2-4 and 6, Figure 2). This integrated approach by clinicians, scientists, molecular biologists and pathologists thereby creates the great advantage to detect all early thrombocytic and erythrocytic stages of ET and PV several to more than 10 years earlier. The proposed WHO-ECMP classification and staging of patients with MPN will be very helpful in predicting the natural history of JAK2^{V617F} mutated ET, PV and ET.MGM patients (Table 6), *vs* MPL⁵¹⁵ mutated ET, *vs* JAK2 wild type PMGM. The WHO-ECMP criteria surely will have important implications in choosing proper treatment options for the management and prevention of thrombotic and bleeding complications and serious complications of progressive MPN disease burden in prodromal PV and classical PV (Table 6)^[81-90]. A primary rigid venesection regimen according to Dameshek^[3] aiming at a hematocrit below 0.45 in males and below 0.42 in females according on top of low dose aspirin will reduce the cumulative incidence of minor and major thrombosis from above 50% to less than 2% per patient/year during long-term follow-up^[16,81,82]. According to current insights, interferon is the treatment of choice in intermediate stage PV patients^[82-86]. High risk PV in terms of high JAK2^{V617F} allele burden, progressive MPN disease, splenomegaly and constitutional symptoms are candidates for myelosuppressive (hydroxyurea) or myeloreductive (JAK2 inhibitor) treatment^[87-90].

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Management of adult Langerhans cell histiocytosis based on the characteristic clinical features

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begun with single ($n = 3$) or multiple ($n = 1$) spinal bone lesion(s) in 4 patients (all males), with multiple bone lesions in 3 patients (1 male and 2 females), with single skull lesion in one female patient and with ambiguous symptoms including hypothyroidism in the remaining one male patient. We also recognized the correlation between pregnancy/childbirth and LCH in 4 patients. In terms of treatment, 9 patients received systemic immuno-chemotherapy alone, of which the majority received vinblastine-based chemotherapy while 4 received 2-chlorodeoxyadenosine. Five had a combination of immuno-chemotherapy with surgical resection or radiotherapy, 2 had immunotherapy alone, 2 had surgical resection followed by observation alone to date. Three patients received hematopoietic stem cell transplantation after extensive chemotherapy. In terms of outcome, 15 patients are alive (9 with active disease, 6 without active disease), with a median of 66 mo (range 17-166 mo), two died of disease while the remaining 1 lost to follow-up. Based on these results, we think that early diagnosis and rapid introduction of appropriate treatment are essential, in order to overcome the problems relevant to adult LCH.

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Key words: Langerhans cell histiocytosis; Adult; Immuno-chemotherapy; 2-chlorodeoxyadenosine; Childbirth

Abstract

To find out the most appropriate management, clinical features of 18 cases of adult multisystem langerhans cell histiocytosis (LCH) have been analyzed. The patients comprising of 9 males and 9 females were median age of 36 years, ranging from 18-53 years at diagnosis. Regarding the initial symptoms, 7 patients (2 males and 5 females) showed central diabetes insipidus (CDI) and other endocrine symptoms with thickened pituitary stalk or a mass at the hypothalamic region. Additional 2 patients initiated the disease with CDI with no immediate diagnosis. In the remaining patients, the disease

Core tip: Clinical features and treatment in a total of 18 adult patients with langerhans cell histiocytosis (LCH) were reviewed. We found two major groups regarding the initial symptoms; one was central diabetes insipidus and other endocrine symptoms ($n = 9$) and the other bone diseases ($n = 8$; 1 skull, 4 spinal and 3 multiple). We also recognized the correlation between pregnancy/childbirth and LCH in 4 patients. Based on the clinical features and outcomes, early diagnosis and rapid introduction of appropriate treatment are essential, in order to overcome the problems relevant to adult LCH.

Imashuku S, Shimazaki C, Tojo A, Imamura T, Morimoto A. Management of adult Langerhans cell histiocytosis based on the characteristic clinical features. *World J Hematol* 2013; 2(3): 89-98 Available from: URL: <http://www.wjgnet.com/2218-6204/full/v2/i3/89.htm> DOI: <http://dx.doi.org/10.5315/wjh.v2.i3.89>

INTRODUCTION

Langerhans cell histiocytosis (LCH) is a rare lymphoproliferative and granulomatous disease comprising of CD1a-positive LCH cells, T-cell lymphocytes, macrophages, eosinophils and other cells^[1-3]. About two-thirds of LCH cases are seen in childhood, with the remaining occurring in adulthood^[4]. Information on clinical features in adult LCH patients is limited^[5,6]. While pediatric LCH has been extensively investigated, adult LCH remains poorly scrutinized, except for characteristic pulmonary diseases, which is closely related to smoking^[7,8]. Epidemiology of non-pulmonary adult LCH is still poorly understood. LCH is an enigmatic disease that is thought to be caused by a pathological combination of oncogenesis and immune dysregulation^[1-3]. Aricò *et al*^[5] analyzed 274 biopsy-proven adult LCH cases from 13 countries; the mean ages at the onset and diagnosis of disease were 33 years and 35 years, respectively, with single-system LCH (SS-LCH) 31.4% and multisystem disease LCH (MS-LCH) 68.6%. Central diabetes insipidus (CDI) was found in about one third (29.6%) of the patients. The probability of survival in these cases at 5 years post-diagnosis was 92.3%, indicating that the adult LCH is not necessarily a fatal disease, but the highly problematic in adults is the impaired quality of life associated with active disease, particularly of MS-LCH. Based on our previous literature review on 43 Japanese adult cases^[4], age distribution showed a peak at 20-40 years of age (31/43 cases, 72%), particularly in females in the 3rd decade. Five cases (11.6%) were older than 55 years. In terms of involved organs, beside lungs ($n = 15$) which are the most frequently involved organ, bone ($n = 11$), CDI ($n = 10$), skin ($n = 8$), followed by various organs were noted in these adult cases. The LCH in adults excluding isolated pulmonary disease could be comparable with those in childhood; however, the characteristics in adult disease remain elusive. Here, we present a case series on recently treated 18 adult MS-LCH and review clinical features with specific issues relevant to adult patients, including imaging of characteristic findings. Regarding the treatment/outcome, there seems to be a significant progress in recent cases described here, compared to the cases in our previous literature review^[4]. In this case series, we also attempted to find out the appropriate therapeutic measures in adult LCH patients.

CASE REPORT

Patients and methods

Adult (> 18 years) LCH cases referred to the authors for

treatment and/or consultation during the period of 1999-2012 have been analyzed for clinical characteristics and treatment. Cases of isolated pulmonary LCH were excluded in the analysis. The patients comprising of 9 males and 9 females were median age of 36 years, ranging from 18-53 years at diagnosis. The diagnosis of LCH was confirmed immunohistochemistry [S100 (+), CD1a (+) or CD207 (+)] on the biopsied or resected tumors in all patients. In terms of treatment, besides surgery/radiotherapy or immuno-chemotherapy, all patients with CDI were given nasal desmopressin acetate hydrate (DDAVP) and those with other endocrine symptoms received hormonal replacement therapy. Outcome at the last follow-up was defined as alive with no active disease (ASAD), alive with disease (AWAD), or died. The follow-up period of the 15 patients excluding 1 lost-to follow up and 2 deceased was a median 66 mo (range 17-166 mo). Of these cases, Cases 13-16 were previously published focused on the endocrine problems^[9]. Case 17 were included in an analysis of LCH-related neurodegenerative disease^[10] and Cases 4 and 17 were also included in a therapeutic trial of LCH^[11]. Case 10 was briefly reported as a case of multiple bone lesions^[12].

Case 1

A 27-year-old female complained of amenorrhea and polyuria/polydipsia after her second childbirth. She was found to have a mass at the hypothalamic pituitary region (HPR), the biopsy of which revealed LCH. She was treated with radiotherapy (20 Gy) to the CNS mass, oral prednisolone (PSL) and DDAVP. No other LCH lesions developed in this case, thus no other systemic chemotherapy was given. In terms of outcome, she has been lost to follow-up.

Case 2

A 25-year-old female developed amenorrhea and polyuria/polydipsia after her second childbirth. She had a mass at the HPR, and granulomatous lesions at the nasal alar parts as well as thyroid mass, which biopsy revealed LCH. At first she was treated with hydrocortisone, DDAVP and levothyroxine sodium. Four years later, multiple LCH lesions were noted at her scalp, perineal-anal regions, subcutaneous abscess-like lesions at the lumbar area, cervical subcutaneous mass. She was treated with systemic chemotherapy (cyclophosphamide/etoposide/PSL), but remained in a partial remission. Particularly, her recalcitrant fistula-forming subcutaneous lesions were probably related to her poorly controlled diabetes mellitus. Eventually, she died of sepsis-induced disseminated intravascular coagulation (DIC).

Case 3

A 35-year-old female developed multiple masses after childbirth at the right subauricular and subscapular regions and right 5th rib, which biopsy revealed LCH. She then developed multiple bone lesions at the spine (C2, C4, C5, Th3-4), clivus, occipital bone, as well as subcutaneous

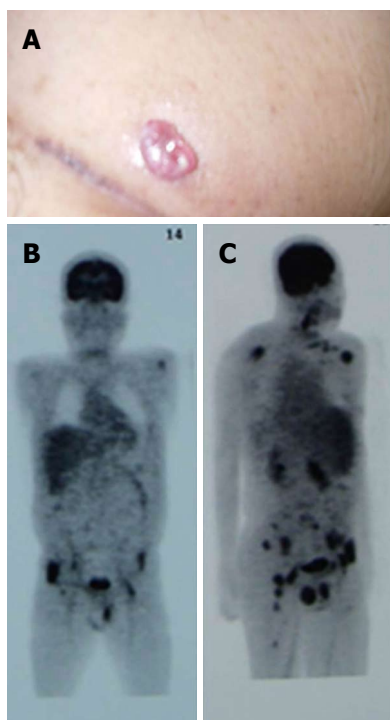


Figure 1 Langerhans cell histiocytosis skin lesions and systemic positron emission tomography imaging. A: Soft papular skin lesion is found at the inguinal area. Biopsy of the lesion revealed the histology of langerhans cell histiocytosis; B, C: Positron emission tomography scan shows multiple lesions, at the scapulas, plevic bones, cervical and inguinal lymph nodes: frontal view (B), lateral view (C).

masses (at her left upper arm and elbow). The lesions at the Th3-4 caused paresis due to spinal compression, which needed laminectomy. She was initially treated with bisphosphonate alone, but thereafter with intensive systemic chemotherapy (JLSG-96 protocol)^[13], including vincristine and cytosine arabinoside. However, 2 years later, she needed allogeneic hematopoietic stem cell transplantation (HSCT) because of progressive disease. The patient has currently been alive without active disease (ASAD).

Case 4

A 37-year-old male was found to have CDI, in association with hyper-prolactinemia (seum PRL 56.1 ng/mL). A thickened pituitary stalk was demonstrated on brain magnetic resonance imaging (MRI), which biopsy revealed LCH. Since then he developed multiple bone lesions at spines (cervical, lumbar), bilateral ilium, left sacroiliac joint, and bilateral ribs as well as skin lesions (Figure 1A) and cervical lymphadenopathy. He was treated first with PSL and bisphosphonate followed by systemic chemotherapy (JLSG-96 protocol)^[13]. One year later, because of progression of the disease (Figure 1B and C), he received HSCT and has currently been in a state of ASAD.

Case 5

A 40-year-old male complained first of mandibular pain. He was found to have multiple bone lesions (left man-

dible, left temporal, occipital, bilateral femurs, cervical spines and right petrous bone. Biopsy of the temporal bone lesions revealed LCH. He was treated with irradiation (6 Gy) to the left petrous bone and systemic chemotherapy, including vinblastine (VBL) and PSL followed by the JLSG-96 protocol^[13] and bisphosphonate; however, reactivation occurred to C1, C2, right mandible, Th5, bilateral femur distal end. Thereafter, he responded well to the chemotherapy with 2-deoxychloroadenosine (2CDA; cladribine). The patient has currently been in a state of ASAD.

Case 6

A 46-year-old female, who was first noted to have thyroid cysts at age 43, complained of back pain and found to have a compression fracture of Th4. A year later she complained of right rib pain. Positron emission tomography (PET) scan revealed hot spots (SUVmax; 7.0) at the both thyroid lobe (Figure 2A) as well as other numerous hot spots at the deep cervical lymph nodes, right scapula, right ilium as well as right rib. Another year later, right lobe of the thyroid was removed, which was diagnosed as LCH. She also received a resection of right rib, which was also found to be LCH. Since then, she was treated with VBL and PSL; however, treatment was stopped because of VBL neurotoxicity. Thereafter, new bone lesions at the frontal bone, rib, ankle, knee, *etc.* She gave births two children; however, the initiation of LCH was not relevant to her childbirth. The patient has continued to have active disease (alive with active disease, AWAD).

Case 7

A 40-year-old male complained of nuchal and left-shoulder pain without any triggering events. With MRI, he was found to have a mass at the left upper and lower articular processes and part of the left articular arch of C3, which was positive for Ga⁶⁷ bone scintigraphy. This mass was totally resected. Nearly 2 years later, he had a tender and soft swelling at the right sided parietal bone (Figure 2B), which was also positive for Ga⁶⁷ scintigraphy. This tumor was again resected. Both tumors were diagnosed as LCH. Currently at age 46, the patient has been followed up without receiving any systemic chemotherapy and in a state of ASAD.

Case 8

A 40-year-old male was found to have a mass at the left articular arch to the spinous process of C6, which was significantly hot (SUVmax; 6.6) with PET scan (Figure 3). The mass was biopsied, which was diagnosed to be LCH. The CT scan showed the lymphadenopathy at the bilateral axillary as well as inguinal area. Thus, an inguinal node was biopsied, which also showed LCH with complex karyotypes. Past history showed he had a severe atopic dermatitis since age of 20, treated with Protopic (tacrolimus hydrate) ointment. He was treated with systemic chemotherapy [VBL/methotrexate (MTX)/PSL/bisphosphonate]. The patient has currently been treated for active C6 lesion.

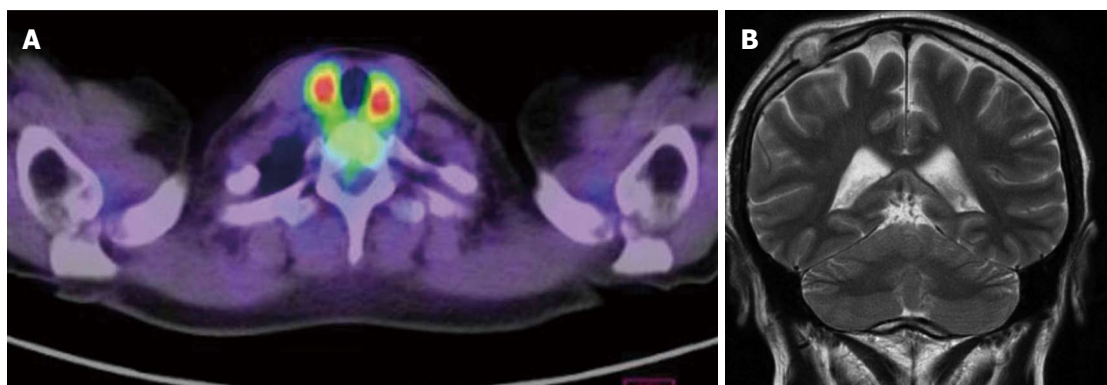


Figure 2 langerhans cell histiocytosis thyroid (A) and skull (B) lesions. A: Positron emission tomography scan shows hot spots (SUVmax = 7.0) at both lobes of thyroid; B: Magnetic resonance imaging (T2W2) shows a mass at the right parietal region.

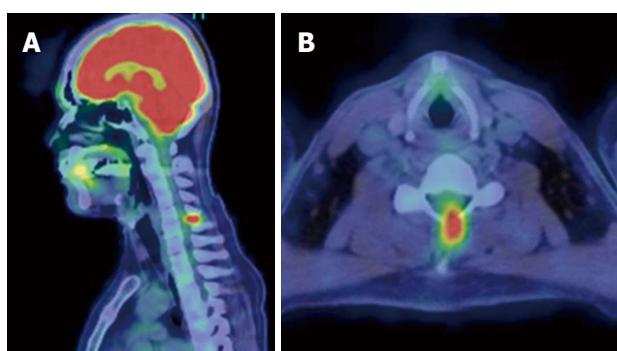


Figure 3 langerhans cell histiocytosis bone lesion at cervical vertebra 6. Positron emission tomography scan shows a hot spot (SUVmax = 6.6) at the spinous process of C6. A: Sagittal view; B: Axial view.

Case 9

A 27-year-old female, who had been treated for atopic dermatitis since childhood, was noted to have a swelling at the right orbit. MRI showed a mass lesion at the right temporal bone extended to the adjacent muscle, which consisted of heterogeneous components (Figure 4A and B). The mass was extensively removed by surgery and the diagnosis of LCH was made. PET scan did not reveal any other suspected lesions. Thus, she was put on observation alone. The patient has been followed up longer than two years without reactivation of the disease. No CDI has occurred and the patient has been in a state of ASAD.

Case 10

A 31-year-old male was first noted to have 1st thoracic spine lesion at age 25, which was biopsied and diagnosed as LCH. Two years later the systemic bone survey revealed a skull lesion, which was surgically removed. Two more years later, he had difficulty in opening mouth and was diagnosed to have a lesion at the mandible bone. Since then he was treated with PSL and bisphosphonate. Nevertheless, he complained of right sided lumbago two more years later due to the involvement of newly appeared iliac bone lesions. Currently the patient has had multiple bone lesions as well as CDI. He needed further systemic chemotherapy for active disease.

Case 11

A 53-year-old female first developed CDI at age of 41. Two years later, she was noted to have a right mandibular bone mass which was totally resected; however, diagnosis remained unknown. Three more years later she developed bone lesions at the frontal to left temporal skull, which caused a diffuse bone defect, including skull and facial bones (Figure 4C), when LCH was diagnosed. With systemic CT scan, she also was noted to have multiple osteolytic lesions at the skull, spine, scapula, rib and pelvis. She received LCH-A1 protocol^[6], consisting of VBL and PSL for a year, which induced a new bone synthesis at the frontal bone. More recently, 12 years after the onset of initial symptoms, she has had newly-developed left mandibular osteolysis. She has currently been treated for active disease.

Case 12

A 36-year-old female was noted to have amenorrhea even after stopping milk feeding for her first-born baby. Administration of Gonadotropin-releasing hormone was successful in resuming a menstrual cycle and she gave birth of second child; however, soon after second childbirth she complained of symptoms compatible with CDI, when brain MRI showed a loss of bright spot at the pituitary posterior lobe; in addition, almost a year later, she was found to have a hypothalamic mass with a gadolinium (Gd)-enhanced MRI (Figure 5A). Biopsy of the mass confirmed the diagnosis of LCH. Since the probable compression of cerebrum due to a huge mass caused impaired consciousness, the patient received systemic chemotherapy, first with VBL followed by 2CDA.

Case 13

A 20-year-old girl first complained of polyuria/polydipsia and amenorrhea. Eight months later, she was detected a Gd-enhanced mass at the HPR by a brain MRI. An immediate open biopsy of the mass confirmed the diagnosis of LCH. Initially, irradiation (21 Gy) to the CNS markedly reduced the size of mass. Two years later, the patient was noted to have re-growth of hypothalamic mass, continued amenorrhea, poorly-controlled CDI and generalized cutaneous LCH, which was confirmed by

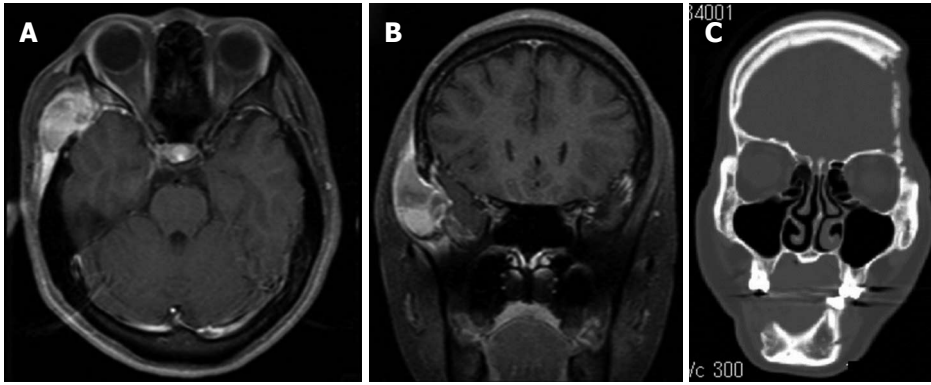


Figure 4 Langerhans cell histiocytosis skull mass (A, B) and lytic skull (C) lesion. A, B: Gadolinium-enhanced magnetic resonance imaging (T1W1) shows a mass with heterodensity at the right temporal area. axial view (A), coronal view (B); C: Computed tomography scan shows extensive lytic bones, at the left temporal bone and mandible. Defect of the right mandible is due to surgical resection.

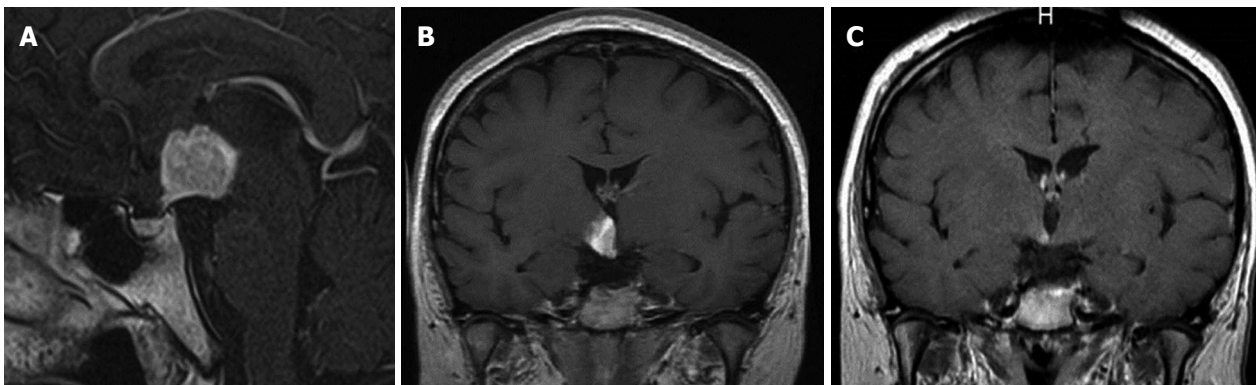


Figure 5 Langerhans cell histiocytosis mass at the hypothalamic area (A) and hypothalamic–pituitary area, comparison of pre and post chemotherapy (B, C). A: Gadolinium-enhanced magnetic resonance imaging (W1T1) shows a large mass at the hypothalamic area. Pituitary stalk is enlarged; B, C: Gadolinium-enhanced magnetic resonance imaging (T1W1) shows (B) a large mass before chemotherapy and (C) a residual mass post chemotherapy. The size of mass was significantly reduced after 2-deoxychloroadenosine treatment.

biopsy. Re-institution of systemic chemotherapy significantly (> 50%) reduced the size of hypothalamic mass. The patient has currently been in a state of ASAD.

Case 14

A 36-year-old female developed CDI in association with amenorrhea. However, the thickened pituitary stalk detected by MRI was put on under observation and not immediately treated. After progression of the thickened pituitary stalk into a significant Gd-enhanced mass at the HPR, a biopsy was performed to reveal typical LCH histology, when the patient had amenorrhea, fatty liver, reduced glucose tolerance. Systemic chemotherapy with 2CDA significantly (> 50%) reduced the mass size (Figure 5B and C). LCH lesions outside the CNS were not found. The patient remains markedly obese and diabetic, with residual active disease.

Case 15

A 38-year-old male was first diagnosed with primary hypothyroidism. Nineteen months later, he developed the symptoms of CDI, fatigue and disturbed consciousness along with disorientation and abnormal behaviors. A brain MRI revealed a Gd-enhanced mass at the HPR,

which biopsy did not confirm the diagnosis of LCH. Two years later, osteolytic bone lesions appeared on the right femur and left clavicle, when the LCH was eventually diagnosed by a bone biopsy. The patient received systemic chemotherapy with 2CDA, but the mass size was reduced minimally (< 50%), and he remained significantly obese and diabetic. Four years from the initial CDI symptom, the patient developed retropharyngeal B-cell lymphoma. He has had active diseases of LCH as well as lymphoma.

Case 16

A 46-year-old male first presented with decreased libido and erectile dysfunction seven years after total gastrectomy for gastric adenocarcinoma. Four years later, a Gd-enhanced mass at the HPR was detected. Until then, he had ignored his polydipsia/polyuria symptoms, thus the diagnosis of CDI was delayed. Biopsy of the CNS mass led to a diagnosis of LCH. A systemic MRI survey also revealed multiple spinal involvements. The patient also had loss of concentration and short term memory deficits suggesting mild neurodegenerative disease, which findings were confirmed with brain MRI examination. Eventually, the patients received systemic chemotherapy



Figure 6 Langerhans cell histiocytosis spinal lesions. Non-enhanced magnetic resonance imaging (T1W1) shows high and low signals at multiple vertebral bodies (arrows).

with 2CDA, which markedly ($> 50\%$) reduced the CNS mass size. However, currently, he has been treated for the regrowth of the CNS mass.

Case 17

A 23-year-old young adult was noted to have cerebellar ataxia and dysarthria. Past history revealed that at age of 16, he had been diagnosed as CDI; however, the exact cause was unidentified. At age 20, he was found to have polycystic lung disease with pneumothorax, followed by a mild ataxia. At age 23, he suffered a traffic accident when he was incidentally found to have a brain disease with a mass at the HPR as well as neurodegenerative disease on MRI performed at the emergency hospital. Eventually, LCH was diagnosed from the biopsy of lung tissues. He received monthly intravenous immunoglobulin therapy for neurodegenerative disease with dexamethasone^[10]; however, the patient declined further treatment. Currently, he has remained with progressive neurological symptoms and with active lung disease.

Case 18

An 18-year-old man initially complained of low back pain and cervical mass. MRI revealed multiple spinal bone involvement (Figure 6). The initial diagnosis of LCH was made from the histology obtained by excisional iliac biopsy. A year later, he developed swelling of left cervical lymph nodes. CT scan of the chest also revealed a nodule in the right lung and the enlargement of left upper mediastinal lymph nodes. Histopathology of the biopsied cervical lymph node showed coexistence of two tumorous components; one was LCH and the other tissue of Hodgkin disease with Reed-Sternberg/Hodgkin cells being positive for CD30. The disease responded temporarily to irradiation (36 Gy) and systemic chemotherapy, but became refractory with relapses to the lungs and lymph nodes. Despite autologous followed by allogeneic HSCTs, he died of refractory Hodgkin disease at age of 23.

Summary of the cases

As summarized in Table 1, cases consisted of 3 SS-LCH

(all CNS disease) and 15 MS-LCH. Regarding the initial symptoms, 7 (2 males and 5 females) of the 18 patients had CDI and other endocrine symptoms with thickened pituitary stalk or a mass at the HPR. Additional 2 patients initiated the disease with CDI with no immediate diagnosis. In the remaining patients, the disease begun with single ($n = 3$) or multiple ($n = 1$) spinal bone lesion(s) in 4 patients (all males), with multiple bone lesions in 3 patients (1 male and 2 females), with localized skull/muscle lesion in one female patient and with ambiguous symptoms including hypothyroidism in one male patient. Thyroid mass was noted in 2 patients. In terms of treatment, 9 patients received systemic immuno-chemotherapy alone, of whom 3 with CNS disease and 1 with multiple bone lesions received 2CDA. Five patients had a combination of immuno-chemotherapy with surgical resection or radiotherapy, 2 had immunotherapy alone, 2 had surgical resection followed by observation alone to date. Three patients received HSCT after extensive chemotherapy. In terms of outcome, 15 patients are alive (9 with active disease; AWAD, 6 without active disease; ASAD) with a median follow-up of 66 mo (range 17-166 mo) and 2 died of disease; 1 from sepsis-induced DIC and the other from progression of Hodgkin disease. The remaining 1 patient is lost to follow-up. As late sequelae, CDI ($n = 9$), neurodegenerative disease ($n = 2$) and obesity/diabetes mellitus ($n = 3$) are noted.

DISCUSSION

Clinical features

To date, adult LCH cases have mostly been reported as case series^[14-18]. Here, we add another case series describing the clinical features and discussing the issues specifically relevant to adult LCH. Adult patients may have LCH as a recurrence of childhood LCH as well as *de novo* LCH developing first in adult life. Here described is all the latter type of LCH. None had a history of LCH in childhood.

Two major clinical features of non-pulmonary LCH in adults

It is apparent that there are two major groups; one is a CNS mass with endocrine problems (9/18) and the other is recalcitrant bone lesions (7/18). Both types of disease are histologically non-malignant, but extensive disease causes various impairments leading to the decreased quality of life, and limiting to achieve normal daily life activity.

Endocrine problems

Adult patients are often noted first with endocrine problems such as CDI, amenorrhea, loss of libido and obesity^[9]. Particularly, hypothalamic-pituitary disease is the most common CNS manifestation of LCH, which leads to CDI and anterior pituitary hormone deficiencies. CDI is diagnosed from the finding that MRI scan shows absence of the bright spot of the posterior pituitary on

Table 1 Summary of 18 cases of non-pulmonary adult langerhans cell histiocytosis

Cases	Age (yr)/sex	Initial symptoms/signs	Subsequent symptoms/ disease progression	Treatment			Outcome	Follow-up (mo)
				Surgical resection	Radiation	Immune-chemo Rx/HSCT		
1	27/F	CDI/endocrine	None	Y	Y	Y ⁴	LTF	-
2	25/F	CDI/endocrine	Nasal/thyroid/skin masses			Y	DOD ¹	216
3	35/F	MBL	MBL			Y/Y	ASAD	132+
4	37/M	CDI	MBL/skin lesions			Y/Y	ASAD	128+
5	40/M	MBL	MBL		Y	Y	ASAD	133+
6	46/F	Thyroid mass/spine (Th4)	MBL/LNs	Y		Y	AWAD	27+
7	40/M	Spine (C3)	Parietal bone	Y			ASAD	72+
8	40/M	Spine (C6)	Inguinal LNs			Y	AWAD	17+
9	27/F	Temporal bone	None	Y			ASAD	26+
10	31/M	Spine (Th1)	MBL			Y ⁴	AWAD	108+
11	53/F	CDI	MBL	Y		Y	AWAD	166+
12	36/F	Endocrine	HPR mass			Y ³	AWAD	28+
13	20/F	CDI/endocrine	Skin		Y	Y	ASAD	156+
14	36/F	CDI/endocrine	None			Y ³	AWAD	52+
15	38/M	Hypothyroid	CDI/endocrine			Y ³	AWAD	48+
16	46/M	Endocrine	CDI/MBL/ND-CNS			Y ³	AWAD	60+
17	23/M	CDI	Lungs/ND-CNS			Y ⁴	AWAD	72+
18	18/M	Spine (multiple)	LNs/Lungs		Y	Y/Y	DOD ²	72

¹From disseminated intravascular coagulation; ²From Hodgkin disease; ³Systemic chemotherapy with 2-deoxychloroadenosine; ⁴Steroid alone with or without bisphosphonate. CDI: Central diabetes insipidus; MBL: Multiple bone lesions; HPR: Hypothalamic pituitary region; LNs: Lymph nodes; ND-CNS: Neurodegenerative central nervous system disease; Y: Yes; LTF: Lost to follow-up; ASAD: Alive without active disease; AWAD: Alive with active disease; DOD: Died of disease.

the T1-weighted sequences^[9,19]; however, it is common that patients are taken care and followed up about these problems at the Endocrinology Unit until when Gd-enhanced MRI reveals a thickened pituitary stalk and/or a hypothalamic mass. Generally, it takes a year or longer for the mass to be biopsied and correct diagnosis be confirmed. Even when the diagnosis is confirmed, there are occasions that it takes time for the patient to be referred to hemato-oncologists for chemotherapy. Whenever the diagnosis and the introduction of treatment are delayed, the patient may develop not only endocrine problems but also cognitive impairment such as memory deficits as well as consciousness disturbances, as shown in our cases (Cases 12, 16, 17).

LCH in association with childbirth

The development of LCH in association with childbirth has not been well recognized. Regarding LCH occurring during pregnancy, only a few sporadic cases have been described previously^[20,21]; however, no information is available how childbirth influenced on the development of LCH. In our series, the correlation between pregnancy/childbirth and LCH in adult female patients was noted in 4 cases (Cases 1-3, 12). Sex hormones are believed to participate in immune responses, as estrogens have been found to serve as enhancers in humoral immunity while androgens/progesterone appears to act as natural immune-suppressors^[22]. For examples, postpartum thyroiditis/diabetes mellitus is speculated to be a consequence of the immunological flare that occurs after the lifting of the pregnancy-related immune suppression^[23,24]. Moreover, pregnancy and the post-partum period are associated with

increased breast cancer aggressiveness^[25]. Thus, the hormonal imbalance in the postpartum period may trigger the development of LCH. Detailed examination of pregnancy and childbirth history in female LCH patients may clarify whether the associated hormonal changes influence the pathogenesis and the development of LCH.

LCH in association with various diseases/events

Two patients (Cases 16, 17) were noted to have cognitive disturbance due to LCH-related neurodegenerative CNS disease^[10]. Additionally, two patients (Cases 15, 18) developed malignant lymphoma; one with concurrent LCH and Hodgkin disease and the other developed B-cell lymphoma after systemic chemotherapy for LCH. The association of LCH and other malignant lymphoid neoplasms has been well recognized^[26-28]. In this series two patients (Cases 8, 9) with severe atopic dermatitis were found to develop LCH. This is an interesting topic considering the antigen-stimulation in the skin. It is also cautioned that recalcitrant or clinically atypical skin eruptions must be differentiated from LCH and other rare disorders^[29] but no data is available that incidence of LCH is higher in patients with severe atopic dermatitis. Four patients (Cases 3, 5, 7, 8) were diagnosed to have LCH from spinal bone lesions. Particularly, of whom two had single spine (C3 or C6) involvement, not in the spinal body but in the arch. Spinal lesion should be searched for any adult who complained of cervical pain^[30]. Intriguingly, discovery of LCH was triggered by road traffic accident in 2 patients (Cases 8, 17), although such reports are rarely found^[31]. In Case 8, LCH lesion at the cervical spine was identified at the emergency hospital. In Case 17, traffic accident

incidentally led to the diagnosis of CNS- and pulmonary-LCH in the patient.

Importance of CT/MR/PET imaging for the diagnosis

To determine the precise biopsy/excision site of LCH, CT/MRI findings are inevitable. Particularly, bone scintigraphy for multiple bone lesions and Gd-enhanced MRI for CNS lesions are essential for the diagnosis of LCH^[9,19]. However, more recently, ¹⁸F-FDG PET is recommended. In one large study it was concluded that whole body FDG-PET scans can detect LCH activity and is useful to evaluate early response to therapy with greater accuracy than other imaging modalities (MRI, CT, plain films) in patients with LCH lesions in the bones and soft tissues^[32]. Also, it is a useful tool for the monitoring of CNS disease activity in LCH^[33,34]. It is said that ¹⁸F-FDG PET might be useful to detect an early neurodegenerative lesions before MRI abnormalities appear, where bilateral hypometabolism is shown in the cerebellum and the basal ganglia (caudate nuclei) areas^[34].

Therapeutic measures for adult LCH

In this case series, four patients received surgical resection of LCH mass without immuno-chemotherapy. Four patients received irradiation to the CNS-mass ($n = 2$), bone ($n = 1$) and lungs ($n = 1$), in association with immuno-chemotherapy. In the majority, systemic immuno-chemotherapy was given, mostly with a conventional combination of VBL/PSL or JCSL-96 protocol including VCR/cytosine arabinoside (AraC)/PSL^[13] for induction. In 3 cases with CNS-LCH, 2CDA was employed. Previously proposed A1 protocol for adult LCH^[6] was used only in one case in this series. With these measures, 6 ASAD cases were obtained, but necessity for further improvement of treatment for adult LCH seems apparent. As future trials, we have to scrutinize how efficiently we can employ AraC, 2CDA, clofarabine, and other novel agents for adult LCH patients. In the past, treatment reports on adult LCH cases were very limited^[35,36]. In particular, the usefulness of intravenous 2CDA for CNS-LCH as well as for systemic MS-LCH was described in adult patients^[37-40]. Windebank *et al*^[41] also reported the usefulness of subcutaneous 2CDA treatment ($5 \text{ mg/m}^2 \times 5 \text{ d}$, $q4 \text{ wk}$, for up to 6 cycles) in LCH. Effectiveness of the combination of 2CDA/AraC was described for extremely refractory cases^[42]. More recently, effectiveness of clofarabine ($25 \text{ mg/m}^2 \times 5 \text{ d}$, $q4 \text{ wk}$) has also been reported^[43,44]. Particularly, Simko *et al*^[44] demonstrated usefulness of clofarabine for multifocal skull lesions. On the other hand, Morimoto *et al*^[11] reported the usefulness of Special C regimen of JLSG for treating adult LCH patients on ambulatory basis. Intriguingly, for the treatment of multiple bone LCH lesions in adults, Cantu *et al*^[45] reported that AraC alone is an effective and minimally toxic, while VBL/PSL results in poor overall responses with excessive toxicity. Considering the fact that about 50% of LCH possess BRAF V600E mutation, molecular targeting treatment with vemurafenib has been proposed

Table 2 Therapeutic options in the treatment of adult langerhans cell histiocytosis

Protocol	Drugs	Ref.
A1 protocol	VBL/PSL	[6]
JLSG-96	VCR/AraC/MTX/6MP/PSL	[13]
Cladribine-based	2CDA/PSL, 2CDA/AraC	[37-41]
Clofarabine-based	Clofarabine	[43,44]
JLSG-special C	VBL/MTX/6MP/PSL	[11]
Others	AraC alone	[45]
Molecular targeting	Vemurafenib	[46]
Bone therapy	Zoledronic acid	[47]

VBL: Vinblastine; PSL: Prednisolone; MTX: Methotrexate; 6MP: 6-mercaptopurine hydrate; 2CDA: 2-deoxychloroadenosine.

more recently^[46]. As bone therapy regimen, Zoledronic acid as bisphosphonate is available, although its effectiveness on LCH bone lesions is still elusive^[47]. Allogeneic HSCT for adult LCH is not within a scope of this article, although a few reports on pediatric LCH cases have been described^[48,49]. As well recognized, in the recipients of allogeneic HSCT, care must be taken for the transplant related adverse events. In Table 2, a list of candidate systemic immuno-chemotherapy regimens is summarized, which we think is useful in choosing regimens for adult LCH patients. In practice, for an adult case of LCH with persistent minimal disease and systemic involvement, we prefer once a month or twice a month treatment, like Special C regimen of JLSG^[11]. However, with these regimens, some adult patients may still show VBL neurotoxicity, MTX hepatotoxicity, or neutropenia due to mercaptopurine hydrate (6MP); such events make it difficult to achieve the entire regimens as planned. Although we recognize that 2CDA is highly effective and could be useful in adult LCH, it is often difficult persuading the patients to stay in the hospital for the 5-d continuous treatment. If subcutaneous 2CDA is available at the outpatient care, this agent could be more employed in the treatment of adult LCH. In any case, it is important to make a most appropriate treatment plan for each patient individually. In summary, for adult patients with two major types of LCH, *i.e.*, recalcitrant multiple bone lesions and/or a mass at the HPR, early introduction of systemic immuno-chemotherapy using conventional regimens including AraC or alternative 2CDA or clofarabine regimens is recommended to overcome the disease-related impairment of quality of life.

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Pseudohemophilia of Erik von Willebrand caused by homozygous one nucleotide deletion in exon 18 of the VW-factor gene

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Author contributions: Michiels JJ, Berneman Z and Schroyens W analysed the clinical features of congenital severe type 1 and 3 VWD and obligate heterozygous carriers; Gadisseur A and Michiels JJ analysed the molecular characteristics of severe type 1 and 3 VWD patients and wrote the manuscript.

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Abstract

The original description of a novel severe bleeding disorder as "Hereditary Pseudohemophilia" by Erik von Willebrand can currently be labelled as von Willebrand disease (VWD) type 3. VWD type 3 is autosomal recessive caused by homozygous or double heterozygous null mutations in the von Willebrand factor (VWF) gene and typically characterized by prolonged bleeding time and APTT, FVIII: C levels below 2%, undetectable VWF: Ag, VWF: RCo and VWF: CB and absence of ristocetin induced platelet aggregation (RIPA). Autosomal recessive von Willebrand disease type 3 VWD with virtual complete VWF deficiency are homozygous or compound heterozygous for two null alleles (gene deletions, stop codons, frame shift mutations, splice site mutations, and absence of mRNA). Reports on severe

recessive VWD compound heterozygous for a null allele and a missense mutation and homozygous or double heterozygous for missense mutations are associated with very low but measurable FVIII and VWF: Ag and should be reclassified as severe recessive type 1 VWD. Homozygous missense or compound missense/null mutations related to recessive severe type 1 VWD have been identified in the VWF prosequence D1 and D2 domains, the D4, B1-3, C1-2 domains, and only a very few in the dimerization site (D3 domain). The detection of even tiny amounts of VWF: Ag after desmopressin acetate (DDAVP) or in hidden sites like platelets allows the differentiation between patients with VWD type 3 and homozygous or double heterozygous recessive severe type 1. Carriers of a null allele related to VWD type 3 or a missense mutation related with severe recessive type 1 VWD may present with mild VWD with low penetrance of bleeding in particular when associated with blood group O. Heterozygous obligatory carriers (OC) of a null mutation or a missense mutation related to recessive VWD type 3 or severe type 1 both present with asymptomatic or mild VWD type 1 in particular when associated with blood group O. The response to DDAVP of OC of either a nonsense or a missense mutation appears to be abnormal and diagnostic with a 3-times higher response of FVIII: C as compared to VWF: Ag. In contrast, the responses to DDAVP of FVIII: C and VWF: Ag are equally good in individuals with low VWF levels related to blood group O and a normal VWF gene and protein (pseudo-VWD). These observations are completely in line with and extend the original observations of von Willebrand in a large family with VWD type 3 and asymptomatic or mild true type 1 VWD in OC.

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Key words: Autosomal recessive von Willebrand disease type 3 and 1; Molecular etiology; Carrier of von

Willebrand disease null or missense allele; Desmopressin acetate responses

Core tip: The novel lethal bleeding disorder described as “Hereditary Pseudohemophilia by von Willebrand (VW) in 1926 is caused by a homozygous nonsense mutation (one nucleotide deletion in exon 18) of the VW-factor gene consistent with autosomal recessive VW disease (VWD) type 3. Heterozygous carriers presented with VWD type 1 with variable penetrance of mild mucocutaneous bleeding manifestations. The present editorial reviews the clinical, laboratory and molecular features of severe recessive type 1 and 3 VWD and obligate heterozygous carriers of VWF nonsense and missense mutations.

Gadisseur A, Berneman Z, Schroyens W, Michiels JJ. Pseudohemophilia of Erik von Willebrand caused by homozygous one nucleotide deletion in exon 18 of the VW-factor gene. *World J Hematol* 2013; 2(4): 99-108 Available from: URL: <http://www.wjgnet.com/2218-6204/full/v2/i4/99.htm> DOI: <http://dx.doi.org/10.5315/wjh.v2.i4.99>

INTRODUCTION

In 1926 Erik von Willebrand first described a novel severe bleeding disorder which he named “Hereditary Pseudohemophilia” in at least 4 affected members of the original large family S, living on the Föglö Aland Island^[1-3]. In this report we review the available clinical, laboratory and molecular features of the original family S, which can now be diagnosed as autosomal recessive VWD type 3 caused by a homozygous null mutation (one nucleotide deletion of exon 18) and of mild VWD type 1 with variable penetrance of bleeding manifestations in heterozygous carriers.

CLINICAL FEATURES

The pedigree of family S, originally described by Erik von Willebrand in 1926, has been updated and numbered by Blombäck in 1999 (Figure 1)^[4].

The proband Hjordis S, case 16, aged 5 years was admitted on April 29 1924 to the hospital Diakonistalsten in Helsinki, Finland. At the age of 1 year, her bleeding tendency was observed after falling and hurting her nose and bled unusually long. At 3 years of age, she fell and had a deep cut in the upper lip. She bled heavily for 3 d and became bloodless and almost unconscious. She had to be confined to bed for 10 wk before recovering. After this she had frequent bruising, and regular episodes of epistaxis and gingival bleeding. An ankle distortion was followed by a severe articular bleeding with intense pain for some weeks (hemarthrosis). When a bleeding time according to the Duke method was performed she continued to bleed for 2 h and this had to be stopped by compression. Erik von Willebrand never visited the

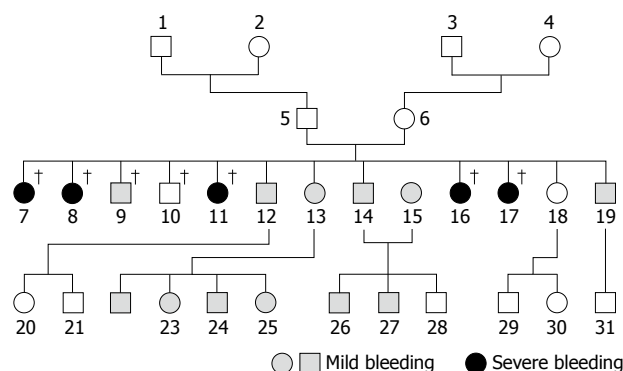


Figure 1 Pedigree of the original family S described by von Willebrand updated by Blombäck and Nilsson in the 1990s.

Aland Islands himself. Eight years later at the age of 13, Hjordis bled to death during her fourth menstrual period.

Her mother, Mrs Augusta, case 6, aged 44 years, had a history of frequent and persistent nose bleedings during her entire youth, but not lately. Menstruation (menarche) begun at age 16 years and had a duration of 6 d or more, always copious, especially lately. She had experienced normal deliveries without severe bleeding. The bleeding time was normal. Augusta, the mother of Hjordis, (case 4, Figure 1), and four of 10 siblings in the family had an increased bleeding tendency.

Her father Mr Oskar S, case 5, aged 48 years, had rather sturdy nose bleedings, when he was young, and did not bruise more easily than other people. Neither of his parent (cases 1 and 2) had been bleeders. One of his sisters and several of her children (family E) had a moderate bleeding tendency.

Her oldest sister, Dagny S, case 7, had her first severe nose bleeding after a slight trauma at the age of 1 year. After this she had several nose bleedings and died from intestinal bleeding at the age of 2 years.

Her sister Anna S, case 8, began to have frequent nose bleeds from the age of 1 year. When she was 4 years old, she fell and two teeth penetrated her tongue. She had a bleeding that could not be stopped and died.

Her sister Dagny S, case 11, had thrush when she was a few weeks old. When her mother tried to loosen the membranes, there was a bleeding that almost would not stop. She bled much after insect bites and died at the age of 2 years from intestinal bleeding.

Her younger sister, case 17, aged 3 years, bruised easily since the age of 1 year and experienced heavy nose bleeds for the first time at age 1.5 years. Thereafter nose bleeds fairly often recurred. The bleeding often started spontaneously, and once went on for a whole week. The bleeding time was considerable prolonged. She had prolonged bleedings after insect bites. At the age of 5 she developed influenza, started to vomit blood and died within 20 h.

Her younger brother, case 19 (proven heterozygous for del 18 in 1990s), suffered in his youth from a slightly increased bleeding tendency, although he did not report

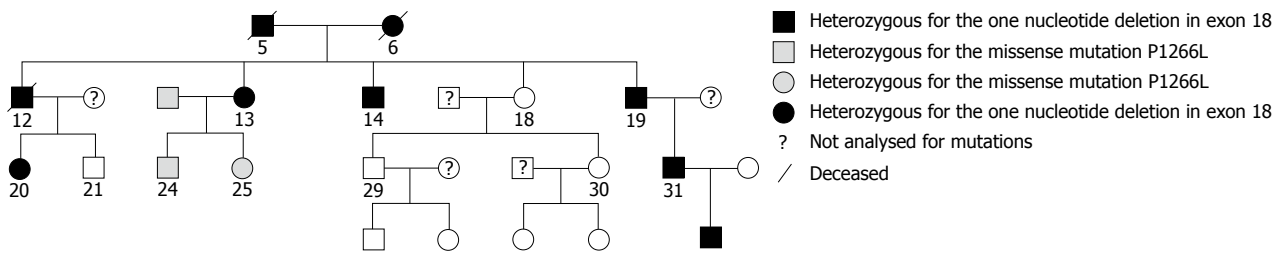


Figure 2 Results of molecular investigations in the original family S in the 1990s reveal a heterozygous nonsense mutation in seven affected members with mild bleeding diathesis^[4].

Table 1 FVIII: C and von Willebrand factor levels in family S together with finding of heterozygous non-sense mutation, deletion exon 18, consistent with mild von Willebrand disease type 1

Family S, Number	Mutation	FVIII: C IU/dL	VWF: Ag IU/dL	VWF: RCo IU/dL	RCo/Ag Ratio
5		0.93			
6		0.51			
12	del 18	0.81	0.80	0.73	0.91
13	del 18	0.53	0.56	0.46	0.82
14	del 18	0.35	0.35	0.32	0.91
18	negative	0.96	0.86	0.65	0.76
19	del 18	1.10	0.67	0.81	1.20
22	unknown	0.50	0.38	0.25	0.66
23	unknown	0.73	0.39	0.39	1.00
24	P1266L	0.39	0.34	0.42	1.23
25	del 18/P1266L	0.69	0.20	0.30	1.5

VWF: von Willebrand factor; VWD: von Willebrand disease; FVIII: Factor VIII.

any problems in 1992.

LABORATORY FEATURES

The inheritance of the disease in Family S could be followed through four generations^[1-5]. In the female bleeders, the bleeding diathesis is manifested in a mild form or in a severe form, whereas the males show only the mild form of bleeding diathesis (Figure 1). Among female affected members with severe bleeding five deaths in one generation had occurred, four in childhood and one shortly after menarche (Figure 1). The women are of two types, those with a single and those with double trait. The former (heterozygotes) may had a milder form of bleeding, the latter (homozygotes) a severe lethal form. There was no opportunity to study the so-called homozygote women because they died from fatal bleeding before the reproductive age and long before FVIII and the von Willebrand factor were identified as causes of hemophilia and VWD more than 3 decades later in the late 1950s. This means that data on the level of FVIII: C and VWF parameters are lacking in those deceased women with the double trait (Table 1). This may explain why Jurgens *et al*^[6] have falsely interpreted the bleeding disorder as a constitutional thrombopathy mainly based on the very prolonged bleeding times. Jurgens *et al*^[7] mentioned several years later a deficiency of an antihemophilic factor (FVIII) in heterozygous affected family members with mild VWD, but the cases with severe bleeding could not studied anymore at that time.

Studies by Nilsson *et al*^[5] showed decreased values for VWF: Ag and VWF: RCo and normal ratios for VWF: RCo/VWF: Ag in the affected heterozygous members of family S with mild VWD type 1 (Table 1) leaving the molecular etiology of the “homozygotes” elusive at that time.

MOLECULAR STUDIES

The DNA samples were screened for mutations with PCR, followed by direct sequencing in the “hot spot” regions in exons 18, 28, 32, 43 and 45 found in the Swedish-Finnish patients^[8,9]. In the original family S one nucleotide deletion in exon 18 was identified in heterozygous carriers, which had been found to be a “hot spot” in the majority of VWD type 3 patients in Sweden^[9,10]. This mutation interrupts the reading frame leading to an early translational stop (null allele). Five individuals (numbers 13, 14, 19, 20 and 25 in Figure 2 and Table 1) all having VWD type 1 were found to be heterozygous for the deletion. The deceased family member 12 must also have carried the same deletion, as his daughter is a carrier. In the third generation, at least four individuals (numbers 12, 13, 14, 19, Figure 2) carry the deletion. These results indicated that the deletion originated from the parents of family S (number 5 and 6, Figures 1 and 2) who are thought to be heterozygous. All five daughters, who died from uncontrolled bleedings, very likely would have been homozygous for the deletion in exon 18 consistent with pseudohemophilia, now called VWD type 3.

Table 2 Reports of autosomal recessive severe type 1 von Willebrand disease caused by homozygous missense or double heterozygous missense/null mutations in the D1 or D2 domain

Mutation	F/M (yr)	BT (min)	VIII: C (U/dL)	VWF: Ag (U/dL)	VWF: RCo (U/dL)	Domain/VWF	VWD type
D141Y/null ^[19]	F/63	> 30	0.03	< 1	< 1	D1	Severe 1
C275S/null ^[19]	F/26	> 30	0.03	< 1	< 1	D1	Severe 1
R273W/R273W ^[20]	Boy	15	0.20	0.06	0.06	D1	Severe 1
R273W/R273W	Boy	15	0.33	0.09	0.04	D1	Severe 1
R273W/R273W	Boy	> 20	0.09	< 0.01	< 0.01	D1	Severe 1
W377C/W377C ^[12]	Child	> 20	0.02	0.03	0.03	D1	No data
C570S/C570S ^[21]	Boy	↑↑	0.12	0.05	0.05	D2	Severe 1
Q77X/splice site Intron ^[24]		> 30	0.20-0.31	0.04-0.06	0.03-0.06	D1/D2	Severe 1

VWF: von Willebrand factor; VWD: von Willebrand disease.

The three siblings, Harald, Sylvia and Runar, (cases 12, 13 and 14, proven heterozygous for del 18) had more less severe nose bleeds, especially in their youth but never experienced a pronounced bleeding tendency during life-long follow-up. These data demonstrate that the original family S, described by Erik von Willebrand as pseudohemophilia A, has to be diagnosed as autosomal recessive VWD type 3 caused by a homozygous null mutation (one nucleotide deletion of exon 18). In the family S in addition to the deletion in exon 18, two mutations at S1263 and P1266 in exon 28 were identified in two siblings (numbers 24 and 25, Table 1, Figure 2) with an unrelated and clinically normal father, who married into the family S. The transition G→A at S1263 is neutral, and the other C→T at P1266L results in an amino acid substitution of proline to leucine (P1266L). P1266L is a frequent mutation in Sweden and has been described as VWD type 1 Malmö with increased ristocetin induced platelet aggregation (RIPA) (mild 2B).

DIAGNOSIS AND MOLECULAR BIOLOGY OF RECESSIVE TYPE 3 VWD

The inheritance of a pronounced bleeding tendency in subsequent reports of families with VWD type 3 is autosomal recessive^[11-15]. Patients with VWD type 3 are typically characterized by prolonged bleeding time (BT) and APTT, FVIII: C levels below 2%, undetectable VWF: Ag, VWF: RCo and VWF: CB levels before and after desmopressin acetate (DDAVP) and absence of RIPA^[15]. VWD type 3 patients with FVIII: C levels above 2% and detectable levels of VWF: Ag and response of FVIII: C to DDAVP should be reclassified as severe recessive type 1 VWD caused by double heterozygous for a nonsense/missense mutation or homozygous or double heterozygous missense mutation causing a severe secretion defect^[12,13].

In 31 cases diagnosed as VWD type 3, (age 2 to 80, median 15 years) described by Schneppenheim *et al.*^[12] bleeding manifestations were recorded as easy bruising and prolonged epistaxis in 31 (100%), spontaneous joint bleeding in 23 (76%), muscle bleeding in 7 (22%) and gastrointestinal bleedings in 3 (10%). The bleeding

manifestations and complications of childbirth have been nicely evaluated in 385 Iranian patients diagnosed as autosomal recessive type 3 VWD (SSC-ISTH classification) and compared to age matched severe hemophilia A^[14]. Among patients with type 3 VWD the incidences of spontaneous hemarthrosis (37%) and muscle bleedings (52%) are lower most likely because FVIII: C levels are higher (1%-9%) as compared to severe hemophilia A (≤ 1%).

Type 3 VWD with virtual complete VWF deficiency (severe VWD) and absence of FVIII: C (pseudohemophilia) are homozygous or compound heterozygous for two null alleles (gene deletions, stop codons, frame shift mutations, splice site mutations, and absence of mRNA) in the majority and rarely compound heterozygous for a null allele and a missense mutation or homozygous for a missense mutation^[11-18]. The null alleles are located all over the VWF gene in nearly all exons 3-52^[18]. The data base of the SSC of the ISTH reports 58 null alleles and 14 missense alleles involved in the etiology of type 3 VWD^[18]. Missense mutations related to severe recessive VWD type 1 are mainly located in the D1-D2 domains (D47H, S85P, Y87S, D141Y, D141N, C275S, W377C, I427N, and in the D4, B1-3, C1-2, CK domains (P2063S, C2174G, C2362F, N2546Y, C2671Y, C2754W, and C2804Y), but not in the D3, A1 and A2 domains except one (C1071F/null)^[18]. Consequently, some so-called type 3 VWD patients, who are compound heterozygous for a null allele and a missense mutation and may have detectable but very low VWF levels, are incorrectly diagnosed as VWD type 3 and should be reclassified as recessive severe type 1 VWD^[19-27].

DIAGNOSIS AND MOLECULAR BIOLOGY OF RECESSIVE SEVERE TYPE 1 VWD

A considerable number of missense mutations related to autosomal recessive severe type 1 VWD have been identified in the VWF prosequence (D1 and D2 domains), and the D4, B1-3, C1-2 and CK (dimerization) domains, but only a very few in the dimerization site (D3 domain (Tables 2 and 3)^[19-26]. There are two reports on double heterozygous missense/null mutation D141Y/

Table 3 Laboratory features of recessive severe type 1 due to a double heterozygous missense mutation in the CK domain of the von Willebrand factor gene

Mutation	Age (yr)	Gender	BT	FVIII: C	VWF: Ag	VWF: RCo	VWF: RCo/Ag	RIPA	VWD type
C2754W/C2754W ^[31]	13	F	> 20	0.12	< 0.05	< 0.05	-	nt	3
Father C2754W ^[31]	-	M	5	0.54	0.33	0.38	1.15	nt	Mild 1
Mother C2754W ^[31]	-	F	5	0.55	0.38	0.43	1.13	nt	Mild 1

VWF: von Willebrand factor; VWD: von Willebrand disease; M: Male; F: Female; FVIII: Factor VIII; RIPA: Ristocetin induced platelet aggregation.

null and C275S/null associated with VWD severe type 1 and not type 3 with documented hemarthros in one of them (Table 2)^[19]. Expression studies the missense mutation D141Y and C275S showed a severe secretion defect of mainly dimers while higher molecular weight bands like tetramers and hexamers were barely detectable^[20]. Homozygotes for the missense mutations W377C^[12] and for R273W^[20] in the propeptide D1 domain have been described to be associated with autosomal recessive severe type 1 (not type 3) VWD phenotype (Table 2). Homozygous missense mutation C570S in the D2 domain has been described as the cause of recessive severe type 1 VWD laboratory phenotype mimicking a type 2C (II C) VWF multimers^[21].

The multimeric pattern of homozygous R273W and C570S clearly showed the absence of high molecular weight multimers and a pronounced monomeric band mimicking type 2C (II C) subtype VWD^[20]. Expression studies of recombinant R273W, W377C and C570S showed a severe secretion defect mainly consisting of dimers and failed to form intermediate and high molecular weight multimers^[19-21]. These findings demonstrate that mutations in the D1 and D2 VWFpp domain completely abolishes multimerization of VWF. Heterozygous asymptomatic carriers of such missense mutation are asymptomatic or diagnosed as mild type 1 VWD with borderline values of VWF parameters around 0.50 U/dL. Heterozygotes for a missense mutation in the D1 or D2 domain typically show a pronounced VWF dimer band.

Homozygous missense mutation C2364F in the B1-3 domain and double heterozygous C2364F/null has been reported to be associated with severe type 1 VWD featured by FVIII: C levels of 12 to 32 U/L, very low but detectable VWF: Ag and undetectable VWF: RCo^[22-26]. C2364F heterozygous carriers were asymptomatic, had normal or slightly prolonged BT, subnormal values for VWG: Ag and VWF: RCo with a normal VWF: RCo/Ag ratio, and a normal VWF multimeric pattern in a low 0.8% or 0.9% agarose resolution gel (asymptomatic “dominant” VWD type 1)^[22]. However, analysis of VWF in plasma from cases with severe autosomal recessive VWD homozygous for a missense mutation C2362F or compound C2362F/null (exon 42 of the B1-3 domain) as well as heterozygous carrier of C2364F all showed a heightened proteolytic pattern with marked increase of 176 and 140 kDa degradation products mimicking type 2A (II A) VWD^[26]. Other causes of severe autosomal recessive type 1 VWD include homozygous C2364Y (B1-3 domain) or double heterozygous C2364Y/intron 13

splice site^[23], homozygous C2671Y (exon 49) or double heterozygous missense mutation C2671Y/del (exon 49) of the VWF gene^[27]. DDAVP in recessive VWD severe type 1 induces a poor for VWF: Ag and VWF: RCo but significant increase of FVIII: C levels. In some cases of autosomal recessive severe type 1 VWD patients FVIII: C, VWF: Ag and VWF: RCo reached values of > 0.50, 0.11 and 0.09 U/L respectively after DDAVP^[22].

RECESSIVE SEVERE TYPE 1 VWD DUE TO MUTATIONS IN THE CK DOMAIN

The replacement of cysteine residues in the CK dimerization domain of the VWF gene causes two completely different laboratory phenotypes of VWD either severe VWD type 3 or VWD type 2D (II D)^[28-36]. Homozygous or double heterozygous loss of cysteines mutations C2739Y, C2754W, C2804 and C2806 results in severe autosomal recessive type 1 VWD with nearly complete absence of VWF^[29,35]. Homozygous C2754W mutation is associated with VWD severe type 1 and a mild type 1 VWD in heterozygous carriers (Table 3). Expression studies of C2754W show intracellular production of mainly monomers and dimers (indicating a dimerization defect) with no secretion of mutant VWF indicating that homozygous C2754W mutation indeed will lead to severe type 1 or 3 VWD^[35].

Experimental and clinical data are in line with the concept that loss of a single disulfide band in the CK domain of VWF leads to a recessive quantitative VWF deficiency with very low VWF: Ag (VWD type 3) if an intrachain disulfide band is involved (C2739Y or C2754W), and to a dominant-negative qualitative defect of VWF with abnormal multimers if an interchain-disulfide bond is involved, which leads to the characteristic type dominant type VWD type 2D (II D) multimeric pattern (Figure 3)^[35,36].

RECESSIVE TYPE 1 VWD DUE TO A HOMOZYGOUS MISSENSE MUTATION IN THE D2 DOMAIN

The 1534C > A mutation in the consensus splicing site of intron 13 (D2 domain) induces exon 14 skipping with the introduction of a premature termination after codon 586, resulting in a truncated VWF^[37]. Moreover, the 1534C > A mutation induces the activation of a cryptic

Patients	Blood group	¹ BT min	aPPT s	² RIPA	FVIII (U/dL)	VWF: Ag (U/dL)	VWF: RCo (U/dL)	VWF: CB (U/dL)	Plat. VWF: Ag (U/dL)	Mutation
I -1 (father)	O	7	34.3	89%	120	101.2	70	82.9	48.1	1543-3C > A/N
I -2 (mother)	O	-	31.2	87.5%	123	64.6	44	56.4	56.4	1543-3C > A/N
II -1 (proband)	O	20	37.8	5.4%	51	14.5	12.5	9.8	9	1543-3C > A/1543-3C > A
II -2 (sister)	O	6	34.2	85.6%	146	76.5	77	86.7	56.8	1543-3C > A/N
Normal range		2-9	30-40	60%-84%	60-160	60-160	60-130	65-150	70-140	

¹BT (bleeding time) was performed using Ivy method; ²RIPA was performed with 1.2 mg/mL ristocetin; RIPA: Ristocetin induced platelet aggregation; VWF: von Willebrand factor; aPPT: activated partial thromboplastin time.

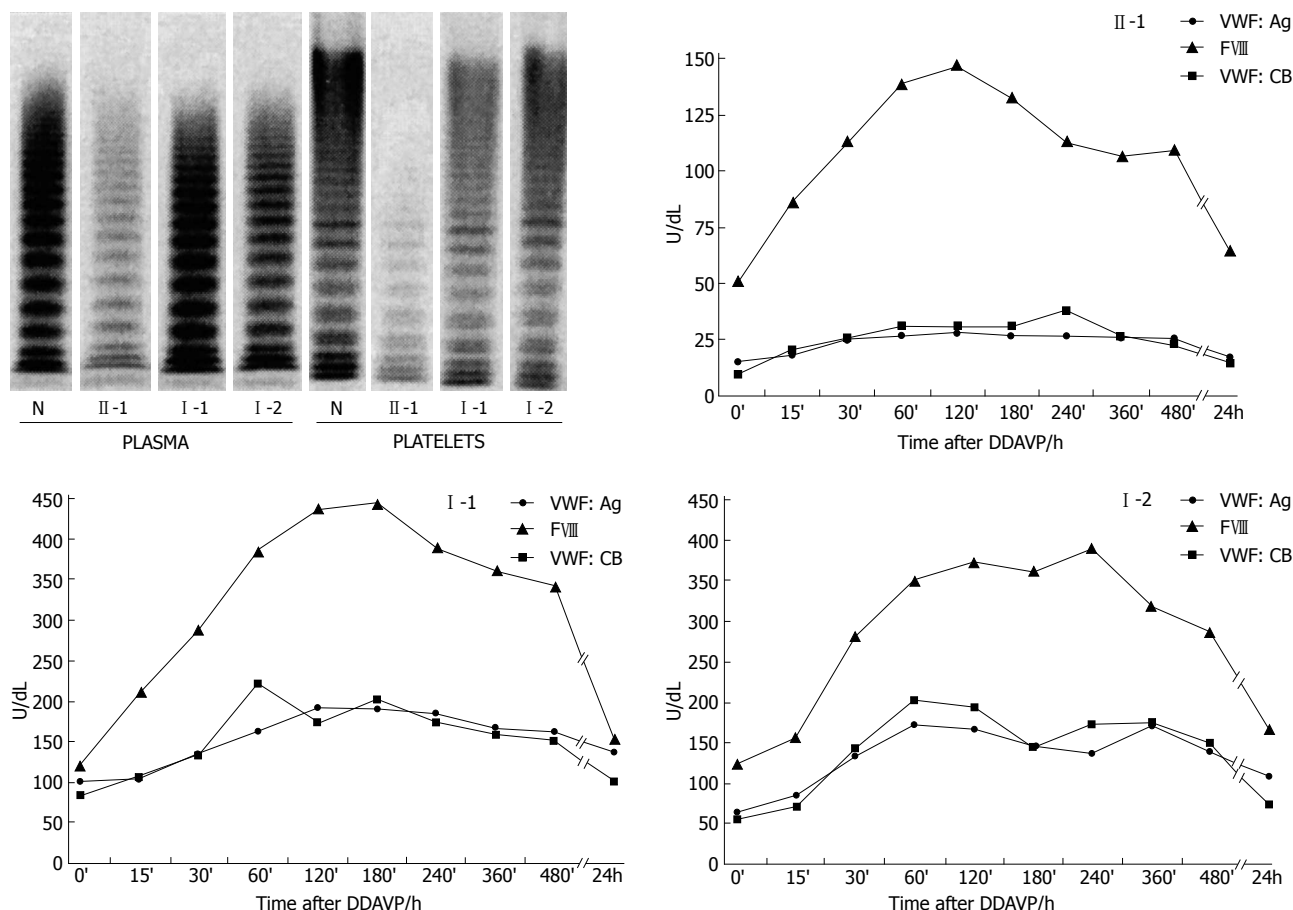


Figure 3 State of the Art characterization of a family with autosomal recessive von Willebrand disease type 1 by Casonato and co-workers using a complete set of laboratory assays related to von Willebrand disease diagnosis, von Willebrand factor multimeric analysis in plasma and platelets and the response of FVIII, VWF: Ag and VWF: RCo to desmopressin acetate before and at several time points after desmopressin acetate according to standardized recommendations anno 2006. RIPA: Ristocetin induced platelet aggregation; VWF: von Willebrand factor; VWD: von Willebrand disease; DDAVP: Desmopressin acetate.

splicing site, 62 nucleotides upstream from the normal site^[37]. The spliceosome produces a normal transcript of normal VWF. Gallinaro described a family with autosomal recessive type 1 VWD caused by homozygous intron 13 splicing site mutation 1534-3C > A (Figure 3)^[17]. The proband, a 34-year old man had a history of severe epistaxis requiring blood transfusion and recurrent mild epistaxis and gingivorrhagia not requiring medical attention. The proband had VWF values between 10 to 15 IU/dL with normal ratios of VWF: RCo/VWF: Ag (0.86), decreased VWF: CB/VWF: Ag ratio (0.6), and increased ratio of FVIII/VWF: Ag (3.5) indicating a secretion defect. (dot) The plasma VWF multimeric pattern showed a homogenous decrease in all oligomers with a subtly loss of large VWF multimers and a more pronounced loss of platelet VWF multimers (VWD type 1 plasma low/platelet low). The response

to DDAVP was restricted for VWF parameters but good for FVIII: C thereby confirming a severe secretion defect. After DDAVP VWF: Ag increased from 15 to 28 IU/dL (1.9x) and FVIII from 51 to 146 IU/dL (2.9x) followed by normal half life times for VWF and FVIII (Figure 3). The father (I -1) and sister (II -2) heterozygous for the 1534-3C > A mutation never bled whereas the heterozygous mother suffered from mild menorrhagia, hematomas and bleeding after delivery. The plasma VWF values of affected family members were in the low normal range and relatively decreased as compared to FVIII (Figure 3). Interestingly the response of FVIII: C to DDAVP was rather good but severely restricted for all VWF parameters as compared the completely normal increase of FVIII to values above 3.0 IU/dL consistent with carrier state of a missense mutation related to a secretion defect (Figure 3)^[37].

Table 4 Laboratory phenotype and clinical symptoms in 69 patients with true von Willebrand factor deficiency type 1 heterozygous for the von Willebrand factor null allele (parents of type III von Willebrand disease)

Author		Zhang <i>et al</i> ^[13]		Eikenboom <i>et al</i> ^[15]	
Number of patients		25	17	14	6
Blood Group		A	O	A	O
FVIII: C (%)	Mean	81	74	93	81
	Range	37-121	11-128	69-138	58-93
VWF: Ag	Mean	45	32	61	
	Range	13-94	12-70	37-98	40-66
VWF: RCo	Mean	-	-	56	53
	Range	-	-	30-92	39-68
Mild bleedings ¹		13	11	1	1
		52%	65%	7%	17%

¹Mild bleeding was defined by one or two bleeding symptoms mainly epistaxis, bruises and/or prolonged menstruations without abnormal bleeding after both extraction or surgery, hemarthros or muscle bleeding. VWF: von Willebrand factor.

Table 5 Von Willebrand factor antigen (VWF: Ag) levels in heterozygous carriers for a null allele related to pseudohemophilia A-von Willebrand disease type 3 and for the mutation C2364F related to severe recessive type 1 von Willebrand disease

Carriers	Number of patients	VWF: Ag mean $\pm$ SD (IU/dL)	VWF: Ag range (IU/dL)
Null allele:			
Blood group O	15	43.2 $\pm$ 10.8	30-66
Blood group non-O	15	61.3 $\pm$ 23.6	25-98
C2364F:			
Blood group O	8	35.2 $\pm$ 16.2	25-55
Blood group non-O	15	61.5 $\pm$ 26.6	30-140

VWF: von Willebrand factor; VWD: von Willebrand disease.

DETECTION OF SYMPTOMATIC AND ASYMPTOMATIC OBLIGATORY CARRIERS OF RECESSIVE VWD TYPE 3 AND SEVERE TYPE 1

The only objective and correct way to characterise true type 1 VWF deficiency heterozygous for the VWF null allele or missense mutation is to analyse the bleeding manifestations and FVIII: C/VWF parameters in obligate heterozygous parents of recessive type 3 or severe type 1 VWF patients. In the study of 27 patients with congenital type 1 VWF deficiency associated with one null allele analysed by Schneppenheim *et al*^[12], 20 were asymptomatic and only 7 presented very mild bleeding, mainly bruising and epistaxis. All except one, had a normal BT. The mean values for FVIII: C, VWF: Ag, and VWF: RCo were 0.76, 0.39 and 0.39 U/mL respectively with an increased FVIII: C/VWF: Ag ratio of 1.9 and a normal VWF: RCo/Ag ratio of 1 consistent with true type 1 VWF deficiency. In the study of Zhang *et al*^[13] including 25 patients heterozygous for the VWF null allele

and blood group non-O, 12 had no history of bleeding and 13 presented with very mild bleeding (one or two bleeding symptoms mainly epistaxis, bruises and/or prolonged menstruations with no abnormal bleeding after tooth extraction). The mean values for FVIII: C and VWF: Ag were 0.81 and 0.45 respectively with an increased ratio for FVIII: C/VWF: Ag of 1.8 (Table 4). In the same study of Zhang *et al*^[13] out of 17 patients heterozygous for the VWF null allele but having blood group O, 8 had no bleeding history and 11 presented minor bleedings (65%, Table 4). The mean values for FVIII: C and VWF: Ag were 0.74 and 0.32 respectively with an increased ratio for FVIII: C/VWF: Ag of 2.3 (Table 2). In the study of Eikenboom the values VWF: Ag and VWF: RCo in carriers of a null allele ranged from 0.30 to 0.98 IU/mL (Table 4)^[15]. In these studies^[12,13,15], there is a wide range of values from 0.11 to 1.28 U/mL for FVIII: C, from 12 to 0.94 for VWF: Ag, with ratios of FVIII: C/VWF: Ag ranging from normal to increased above 2 indicating the difficulty to distinguish true congenital type 1 VWF deficiency from VWF deficiency related to blood group O.

Using the recently developed sensitive bleeding score assessment, Castaman *et al*^[38] compared the severity of bleeding symptoms in 70 OC of recessive type 3 VWD, 42 OC of recessive type 1 VWD and in 215 normal controls. OC of VWD type 3 with a null mutation had clearly less severe bleeding than patients diagnosed as type 1 VWD. OC of type 1 VWD with a missense mutation were distinct from normal controls, presenting more epistaxis, cutaneous bleeding and usually did not significantly bleed after surgery, further pointing to the wide heterogeneity of VWD as a heterozygous congenital disorder of the VWF gene mutations (Table 5).

Obligatory carriers (OC) of a nonsense mutation related to VWD type 3 and OC of missense mutation related to severe recessive VWD type 1 in the population are asymptomatic or manifest mild bleeding, and have VWF levels at 50% of normal (true type 1 VWD according to the law of Mendel). Such OC of a null allele or missense mutation may become more symptomatic when associated with blood group O or another modifier of the VWF level. Castaman and Eikenboom demonstrated that ABO blood group significantly influences the VWF: Ag levels in OC of a null allele related to VWD type 3 or the missense mutation C2364F related to severe recessive VWD type 1 (Table 5)^[39]. From a genotypic point of view, OC of a null allele in type 3 VWD are very similar to asymptomatic or mild type 1 VWD patients with a single missense allele.

Based on careful analysis of reports on recessive VWD we proposed in 2006 the Antwerp Classification of recessive VWD type 3, recessive severe type 1 VWD, and true type 1 VWD heterozygous for a null allele or missense mutation with variable penetrance of bleeding manifestations but symptomatic when associated with blood group O (Table 6)^[40]. In subsequent studies the variable penetrance of bleeding manifestations mild VWD type 1 is clearly related to blood group O^[41,42].

Table 6 The 2006 Antwerp Classification of recessive von Willebrand disease type 3, recessive severe on Willebrand disease type 1 and obligatory carriers of a null or missense allele with asymptomatic or mild on Willebrand disease type 1 and variable penetrance of bleeding tendency

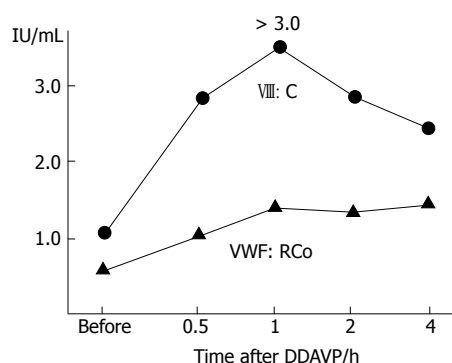
Category VWD	BT	FVIII: C (%)	VWF (%) Ag	RCo	RIPA	Bleeding type	VWF gene mutation
Severe type 3	↑↑↑	1-9	zero	zero	zero	Severe	Double
Recessive						Hemophilia	Nonsense
Severe type 1	↑↑↑	9-40	1-10	0-6	zero	Moderate	Double
Recessive VWD						Severe	Missense
Blood group O (30-32)	N	35-150	35-150	35-150	N	Asymp	None
(Pseudo-VWD)						Very mild	
Carrier type 3	N↑	30-140	15-90	15-90	N	Asymp	Single
Minor influence (-10%) of bloodgroup O						Very mild	Non-sense (null allele)
Carrier type 1	N	N	N	N	N	Asymp	Single
(polymorphism)							Missense
Mild type 1	N↑	20-80	20-50	20-50	N	Mild	Mis/Non-sense or Y1584C/
Recessive or variable penetrance and multigenetic background							Bloodgroup O ^[19]
Dominant type 1	N↑	20-80	10-40	0-30	N	Mild	Single
Secretion defect	↑/↑↑	5-20	5-20	5-20		Moderate	Missense
Dominant type 1							
Vicenza	N/↑	< 15	< 15	< 15		Moderate	Single ^{R126H} Missense

VWF: von Willebrand factor; VWD: von Willebrand disease; RIPA: Ristocetin induced platelet aggregation.

Table 7 Response of FVIII: C and von Willebrand factor parameters to DDAVP (0.3 ug/kg) in an obligatory carriers of a null allele heterozygous for the nonsense splice site mutation IV7 + 1G > A in intron 7. (0874 + 1G > A) in intron 7

DDAVP	Before	1	2	4	6	H post-DDAVP
FVIII: C	0.84	5	5.4	5.3	4.9	IU/mL
VWF: Ag	0.64	1.3	1.7	1.5	1.4	IU/mL
VWF: RCo	0.67	1.8	2	1.35	1.2	IU/mL
FVIII: C/VWF: Ag ratio	1.3	3.8	3.1	3.5	3.5	Carrier of null allele
VWF: RCo/Ag ratio	1.05	1.38	1.17	0.9	0.86	Mild type 1 VWD

VWF: von Willebrand factor; VWD: von Willebrand disease; DDAVP: Desmopressin acetate.

**Figure 4** Good response of FVIII: C and restricted response of von Willebrand factor: RCo to desmopressin acetate (0.3 ug/kg) in a carrier of a null allele (Q2470X/normal). VWF: von Willebrand factor; DDAVP: Desmopressin acetate.

Similarly, the C1584 variant of mild VWD type 1 is associated with a slight decrease of VWF and FVIII: C levels,

especially in combination with bloodgroup O^[43,44].

We studied a consanguineous family with type 3 VWD. The proband was a boy with VWD type 3, who presented with mucocutaneous bleeding and recurrent hemarthrosis of an ankle. Laboratory analysis found F VIII: C < 1% and an absence of VWF: Ag due to the homozygous nonsense splice site mutation IV7 + 1 G>A in intron 7 (0874 + 1G > A)^[45]. Both parents were heterozygous for the nonsense mutation and completely asymptomatic with near normal to normal values for F VIII: C, VWF parameters and normal ratios for FVIII: C/VWF: Ag (Table 7)^[45]. After DDAVP, the FVIII: C levels rose to much higher levels as compared to VWF: Ag, VWF: RCo and VWF: CB levels. FVIII: C/VWF: Ag ratios were 1.3 before DDAVP but more than 3 after DDAVP consistent with a carrier of a VWF null allele (Table 7). The VWF: RCo/Ag ratio was normal before and after DDAVP consistent with true congenital type 1 VWD disease (Table 7). This demonstrates that an increased ratio FVIII: C/VWF: Ag ratio after DDAVP is typically and diagnostic for true VWF deficiency type 1 heterozygous for a null allele. This important diagnostic clue to true congenital type 1 VWD has also been demonstrated by Lethagen *et al.*^[46] in a carrier of a null allele (Q2470X/normal, Figure 4).

From this analysis of the literature and personal experiences in VWD we conclude that heterozygous carriers of a null mutation related to VWD type 3 and a missense mutation related to recessive VWD severe type 1 both do present with asymptomatic or mild VWD type 1 in particular when associated with blood group O. The response to DDAVP of OC of either a nonsense or a missense mutation related to VWD type 3 or severe type 1 appears to be abnormal and diagnostic with a 3-times

higher response of FVIII: C as compared to VWF: Ag. In contrast, the responses to DDAVP of FVIII: C and VWF: Ag are equally good in individuals with low VWF levels related to blood group O and a normal VWF gene and protein (pseudo-VWD). These observations are completely in line with and extend the original observations of Erik von Willebrand in a large family with VWD type 3 and asymptomatic or mild true type 1 VWD in OC.

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Blood groups, hemoglobin phenotypes and clinical disorders of consanguineous Yansi population

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Abstract

AIM: To study frequency of blood groups, prevalence of sickle-cell anemia trait and glucose-6-phosphate dehydrogenase deficiency (G6PD), among consanguineous Yansi tribe.

METHODS: A total of 525 blood samples were collected, of which 256 among the Yansi population, and 269 for the unrelated control group in the Bandundu

province of Democratic Republic of Congo. Blood group antigens were determined in the following systems: ABO, Rh, Kell, Duffy, Kidd and MNS. Blood grouping and extended phenotype tests were performed according to standard immunohematological procedures. Spot tests and tandem mass spectrometry were used respectively for the assessment of G6PD and sickle-cell anemia trait.

RESULTS: The frequency of ABO phenotypes conformed to the following order O>A>B>AB with notably 62.5%, 23.8%, 12.1% and 1.6% for the Yansi, and 54.6%, 27.5%, 14.1% and 3.7% for the unrelated control group, respectively ($P = 0.19$). As for the Rh phenotypes, the most frequent were ccD.ee, ccD.Ee, CcD.ee, corresponding to 71.5%, 12.1% and 12.1% for the Yansi, and 70.6%, 15.6% and 8.2%, for the unrelated control group ($P = 0.27$). The frequency of MN and Ss phenotypes were statistically different between groups ($P = 0.0021$ and $P = 0.0006$). G6PD was observed in 11.3% of subjects in the Yansi group, and in 12.4% of controls ($P = 0.74$). The sickle-cell anemia trait was present in 22.4% of Yansi subjects and 17.8% in the control group ($P = 0.24$). Miscarriages and deaths in young age were more common among Yansi people.

CONCLUSION: This study shows a significant difference in MNS blood group distribution between the Yansi tribe and a control population. The distribution of other blood groups and the prevalence of hemoglobinopathies did not differ in the Yansi tribe.

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Key words: Blood group antigens; Blood group phenotype; Glucose-6-phosphate dehydrogenase deficiency; Sickle-cell anemia; Hemoglobin electrophoresis; Clinical disorders; Consanguinity; Yansi

Core tip: Assessment of blood group frequencies is important to evaluate the risk of alloimmunization after transfusion or pregnancy. Few documented studies have been published about the frequency of blood groups and extended phenotypes in the Congolese people in general and the consanguineous Yansi tribe in particular. This is also the case for the prevalence of glucose-6-phosphate dehydrogenase deficiency and sickle-cell trait. We show that the distribution of MNS blood groups is different in the Yansi tribe, compared to the general population. The Yansi also present with a higher frequency of medical disorders. This study may help in sensitizing the Congolese population about the medical risks associated with consanguineous unions and in building up a database of genetic diseases in the population.

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INTRODUCTION

The antigens of the ABO system were the first to be discovered among the blood group antigens and were known as the first human genetic markers^[1]. Nowadays, there exist 308 antigens of red blood cells described in 30 systems, 12 collections, a 700 series, and a 901 series^[2,3]. Apart from the ABO and Rh, there are many other systems of transfusion significance, such as Kell, Duffy, Kidd and MNS^[4].

The blood groups antigens are most important in transfusion, pregnancy and transplantation because of their immunogenic capacity^[5], and also in human genetics^[6]. The frequency of blood groups antigens differs between populations^[7].

A “genetic” population or a “mendelian” population is influenced by the way the procreating couples are constituted. It is characterized by a genetic pool and by cultural rules that govern the formation of those couples (rule of belonging to a community)^[8]. The genes coding for the blood groups’ markers are, like all genes, inherited from parents and their phenotypic expression is subjected to hereditary laws^[9].

In the Yansi tribe from the Bandundu province in the Democratic Republic of Congo, the constitution of couples lies on two types of marriage: the ordinary marriage and the preferential or consanguineous marriage of “kitiul” type. Very few documented studies have been performed about the constitution of the preferential marriage among Yansi people. Mfukala and Hohegger mention nearly 60% of preferential marriages in which the grandfather marries his granddaughter to the son of his brother’s daughter^[10,11].

Consanguinity increases the probability that both spouses become heterozygote for the same recessive genes causing an increase of homozygosity for that gene in their descent^[12,13]. Therefore, consanguinity increases the risk of developing genetic diseases such as hemoglobinopathies with the possibility of increasing the frequency of the deficient allele for glucose-6-phosphate dehydrogenase deficiency (G6PD)^[14,15].

The G6PD deficiency is the most common enzymatic disorder in humans. It is a sex-linked genetic disease affecting more particularly African, Mediterranean, and Far Eastern people with an incidence of 25%^[16].

The sickle-cell anemia is an example of recessive autosomic disease^[17]. More than 90% of people with sickle-cell anemia are born in Africa where its prevalence varies in some regions between 10%-40%. In the Democratic Republic of Congo, prevalence of sickle-cell anemia trait varies between 15%-20%^[18].

It is in this context that we have determined the frequency of antigens of ABO and Rh blood groups, and of the other blood systems such as Kell, Duffy, Kidd and MNS. We also evaluated the frequency of G6PD deficiency and sickle-cell anemia trait among the Yansi tribe practicing consanguineous marriage compared to an unrelated control group.

MATERIALS AND METHODS

A total of 525 blood samples were drawn, of which 256 among the consanguineous Yansi, and 269 among the control group. Subjects of all ages and of both sexes were selected in the scope of the study. The male/female ratio was not different with 44.9% and 52.4% of male subjects, 55.1% and 47.6% of female subjects in Yansi subjects and control group, respectively. The control subjects were younger than the Yansi subjects: the mean age was 40.86 ± 17.11 years (range: 3-79 years) in Yansi subjects and 28.87 ± 17.20 years (range: 0-79 years) in the control group. Our samples were collected in September 2010 and in June 2011 in the Bandundu province of Democratic Republic of Congo. The Yansi subjects were located in the district of Kwilu whereas control subjects were located in the city of Kikwit, both in the Bandundu province. Controls subjects did not belong to the Yansi tribe but lived in the same region of Congo. Information about subjects was collected through a questionnaire and blood samples were harvested for biological analyses.

For blood groups, samples were collected in EDTA tubes, whereas for G6PD and hemoglobin phenotypes, blood was collected on Whatman 903 filter paper. Blood grouping and extended phenotyping were achieved according to standard immunohematological procedures based on erythrocyte agglutination with specific antisera followed, when necessary, by the addition of antiglobulin reagent^[19]. The tube technique was used for all analyzes of blood grouping using monoclonal and polyclonal reagents in accordance with manufacturer’s procedures. The phenotypes ABO and D were performed by the

double method of Beth-Vincent and Simonin. The ABO and D antigens were typed using monoclonal antisera of Pelikloon (Sanquin). Typing of C, c, E, e, Fya, Fyb, Jka, Jkb, M antigens was carried out with antisera from Immundiagnostika (Seraglu) while K, N, S, s antigens were typed using antisera from Seralone (Biotest).

The detection of the sickle-cell anemia trait was carried out by tandem mass spectrometry^[20,21] whereas assessment of the rate of G6PD deficiency was measured by the Beutler fluorescent spot test technique^[22]. The principle of determination of G6PD deficiency is based on the visualisation of nicotinamide adenine dinucleotide phosphate produced in a blood spot sample. The reagent is prepared by mixing glucose-6P, β -nicotinamide adenine dinucleotide phosphate, saponine, Tris HCl and glutathione (all from Sigma). The reaction is observed under a ultraviolet lamp on Greiner Plate 655191.

Results are presented as frequencies and comparisons of variables between groups were done by a χ^2 test. Results were considered significant at the 5% level ($P < 0.05$). Calculations were done using SAS[®] version 9.3 (SAS[®] Institute; Cary, NC, United States).

RESULTS

We have studied the frequency of blood phenotypes among the consanguineous Yansi population that was compared with a population of unrelated subjects. Concerning the ABO phenotype, the distribution did not differ between the two groups ($P = 0.19$). These frequencies were determined as follows for the phenotypes O, A, B and AB, respectively: 62.5%, 23.8%, 12.1% and 1.6%; for the Yansi; 54.7%, 27.5%, 14.1% and 3.7% for the control group (Table 1). In a study based on a cohort of 2536 blood benevolent donors in the Democratic Republic of Congo, the frequency of ABO phenotypes was O>A>B>AB with 1427 (56.3%) for O, 558 (22.0%) for A, 464 (18.3%) for B and 87 (3.4%) for AB^[23]. Overall the comparison of these data with our control group did not present significant difference ($P = 0.12$) but there was a significant difference ($P = 0.024$) between the Yansi and blood donors such that the blood group O frequency was higher in the Yansi (62.5%).

The frequency of Rh phenotypes did not significantly differ ($P = 0.27$) between the Yansi and the control group (Table 1), ccD.ee: 71.5% and 70.6%; ccD.Ee: 12.1% and 15.6%; ccD.EE: 0% and 1.1%; CcD.ee: 12.1% and 8.2%; CcD.Ee: 1.2% and 0.4%; CCD.ee: 0.4% and 0.4%; ccddee: 2.7% and 3.7%, respectively. Both populations were 90% Rh positive with a predominance of ccD.ee subgroup. Rh negative subjects accounted for 2.7% among the Yansi and 3.7% in the control group. Few documented studies have been published about the frequency of RHD antigen and RH phenotypes in the Congolese people. Two studies carried out on blood donors report frequencies of 98.9% and 99.5% for RHD^[23,24]. As for the RH Cc and Ee phenotypes, the frequencies reported in blood donors was ccD.ee: 69.1%; ccD.Ee: 14.2%; ccD.EE: 1.2%; CcD.ee: 13.7%; CcD.Ee:

Table 1 ABO, Rh and other phenotype frequencies in Yansi population and control subjects *n* (%)

	Yansi <i>n</i> = 256	Control <i>n</i> = 269	<i>P</i> value
ABO phenotype			0.19
A	61 (23.8)	74 (27.5)	
B	31 (12.1)	38 (14.1)	
O	160 (62.5)	147 (54.7)	
AB	4 (1.6)	10 (3.7)	
Rh phenotype			0.27
ccD.ee	183 (71.5)	190 (70.6)	
ccD.Ee	31 (12.1)	42 (15.6)	
ccD.EE	0	3 (1.1)	
CcD.ee	31 (12.1)	22 (8.2)	
CcD.Ee	3 (1.2)	1 (0.4)	
CCD.ee	1 (0.4)	1 (0.4)	
ccddee	7 (2.7)	10 (3.7)	
Kell phenotype			
K+k-	0	0	
K+k+	0	0	
K-k+	256 (100)	269 (100)	
Duffy phenotype			
Fy (a+b+)	0	0	
Fy (a-b+)	0	0	
Fy (a+b-)	0	0	
Fy (a-b-)	256 (100)	269 (100)	
Kidd phenotype			0.096
Jk (a+b+)	96 (37.5)	81 (30.1)	
Jk (a+b-)	141 (55.1)	173 (64.3)	
Jk (a-b+)	19 (7.4)	15 (5.6)	
MN phenotype			0.0021
MM	94 (36.7)	124 (46.1)	
MN	123 (48.1)	89 (33.1)	
NN	39 (15.2)	56 (20.8)	
Ss phenotype			0.0006
SS	10 (3.9)	35 (13.0)	
Ss	39 (15.2)	38 (14.1)	
ss	204 (79.7)	187 (69.5)	
S-s-	3 (1.2)	9 (3.4)	

1.5%; CCD.ee: 0.2% et ccddee 0.1%^[22]. These data are significantly different from our results in Yansi and unrelated subjects ($P < 0.0001$). This can be explained by the small number of samples. Larger studies are needed for the constitution of a reliable database of ABO blood groups and Rh phenotype.

The determination of extended phenotypes focused on the frequency of the most immunogenic systems, such as Kell, Duffy, Kidd and MNS (Table 1). In both populations, all subjects were Kell negative. In the Duffy system, the Fy (a-b-) phenotype was found in all subjects in both groups. The prevalence of Kell and Duffy antigens, which are strongly immunogenic, is low in the Democratic Republic of Congo^[23]. This is also the case of Africans who reach 98% kk, 2% Kk and rare cases for KK^[25].

The frequencies of MN and Ss phenotypes presented a significant difference ($P = 0.0021$ and $P = 0.0006$) between the two groups. These frequencies were respectively in Yansi and unrelated subjects 36.7% *vs* 46.1% for the MM phenotype; 48.1% *vs* 33.1% for MN; 15.2% *vs* 20.8% for NN; 3.9% *vs* 13% for SS; 15.2% *vs* 14.1% for Ss and 79.7% *vs* 69.5% for ss (Table 1). The frequencies

of the ss phenotype are close to data reported previously in Africans (68%)^[26]. In some studies carried out in Africa, the frequencies of the MNSs antigens reveal major differences between ethnicities. This was observed in Tanzania in three ethnic groups who have shown significant differences in the distribution of MNS phenotype^[27], in Benin for the Ss phenotype^[28], among Africans of Cape in South-Africa^[29] and this is also the case for various ethnic groups including Somalia^[30]. This system should be studied in a larger sample because this disparity in the distribution of antigens presents a high risk of alloimmunization during transfusions and pregnancy. Of note, the extended phenotype is not carried out in standard pre-transfusion workup in Africa.

DISCUSSION

We note the presence of S-s- phenotype in both populations with frequencies of 1.2% among the Yansi and 3.4% among the control group. The reported frequency of S-s- phenotype in Africans is around 1.5%^[26]. There are individuals devoid of the U antigen who do not have both the S and s antigens. The U- phenotype is of low incidence in Africans and is absent in Caucasians^[26]. Immunologic challenge of S-s- persons most often stimulates the production of anti-U, which is known to cause decreased survival of transfused antigen-positive red blood cells^[26,31].

As for the Kidd system, there was no significant difference (Table 1) with 37.5% of Jk (a+b+) among the Yansi and 30.1% among the control group; 55.1% of Jk (a+b-) among the Yansi and 64.3% of Jk (a+b-) among the control group. Reported distribution in Africans is as follows: 34% for Jk (a+b+), 57% for Jk (a+b-) and 9% for Jk (a-b-)^[32].

The distribution of AA and AS hemoglobin did not differ between groups ($P = 0.24$). Among the Yansi population, 78.1% and 21.9% of subjects exhibited AA and AS phenotype, respectively. Among the control group, 82.2% and 17.8% of subjects presented with AA and AS phenotype, respectively (Table 2). It is assumed that SS individuals were not found because of early mortality. In the Democratic Republic of the Congo, the sickle-cell anemia trait has a frequency ranging from 15% to 20%, which is also the case of Central Africa^[18,33-35].

As for the G6PD phenotype, there was no significant difference between groups ($P = 0.74$). Among the Yansi, we noted 11.7% of subjects with G6PD deficiency, and among the control group, 12.3% were G6PD deficient (Table 2). Among the control group, the distribution according to sex showed a significant difference ($P = 0.023$): the proportion of males was much higher in the G6PD deficient group compared to the G6PD normal group (Table 3). The high frequency of G6PD deficiency in males is explained by the fact that the coding gene for G6PD is located on the X chromosome, and therefore, the deficiency is fully expressed in the boys affected in the hemizygote manner^[36]. The distribution according to sex shows a non-significant difference ($P = 0.83$) in

Table 2 Distribution of glucose-6-phosphate dehydrogenase and hemoglobin phenotype in Yansi population and control subjects n (%)

	Yansi $n = 256$	Control $n = 269$	P value
G6PD			0.74
Normal	226 (88.3)	235 (87.4)	
Deficient	30 (11.7)	34 (12.3)	
Hemoglobin			0.24
AA	200 (78.1)	221 (82.2)	
AS	56 (21.9)	48 (17.8)	

G6PD: Glucose-6-phosphate dehydrogenase deficiency.

Table 3 Distribution of glucose-6-phosphate dehydrogenase deficiency in relation to gender in Yansi population and control subjects n (%)

Population	Male	Female	P value
Yansi ($n = 256$)			0.83
G6PD normal	101 (44.7)	125 (55.3)	
G6PD deficient	14 (46.7)	16 (53.3)	
Control ($n = 269$)			0.023
G6PD normal	117 (49.8)	118 (50.2)	
G6PD deficient	24 (70.6)	10 (29.4)	

G6PD: Glucose-6-phosphate dehydrogenase deficiency.

the Yansi: among G6PD deficient subjects, 46.7% were males and 53.3% were females. This may be due to selection of mutant alleles by consanguinity. Indeed, many variants of G6PD deficiency have been described with more than 140 known mutations, with variable enzymatic activity^[36,37]. Two possibilities may arise: first, double heterozygous women carrying a completely silent allele and a partially silent allele may present with overall enzyme activity below the test cut-off. In this case they are categorized as deficient and their frequency will increase compared to controls. Second, men with a partially silent allele may be categorized as non deficient if the enzyme activity is above the test cut-off. In this case the frequency of G6PD deficient subjects will decrease compared to controls. On the other side, in normal G6PD subjects of the Yansi population, 44.7% were males and 55.3% were females (Table 3).

We noticed during our survey that several subjects of the population Yansi died prematurely. That can be explained by the presence of congenital defects and/or malformative disorders associated to consanguinity^[38,39], which has been reported in several populations^[40,41]. Such occurrences generate a bias in the population Yansi as miscarriage and infant mortality are superior in Yansi than within control population (Table 4). However, our data do not provide a causal link between the occurrence of these disorders and consanguinity. To get more reliable information on the consequences of consanguinity, biological parameters should be tested at birth, through newborn screening. This may help in sensitizing the Congolese population about the medical risks associated

Table 4 Clinical disorders observed in the two populations *n* (%)

Clinical disorders	Yansi <i>n</i> = 256	Control <i>n</i> = 269	<i>P</i> value
	M = 115; W = 141	M = 141; W = 128	
Asthma	8 (3.1)	-	-
Rheumatism	20 (7.8)	-	-
Paralysis	2 (0.8)	-	-
Sterility	1 (1.4)	-	-
Sexual impotence	1 (0.4)	-	-
Epilepsy	13 (5.1)	-	-
Deafness	1 (0.4)	-	-
Miscarriage	46 (32.6) ¹	3 (2.3) ¹	< 0.0001
Stillbirth	4 (2.8) ¹	-	-
Death in young age	62 (24.2)	3 (2.3)	< 0.0001

¹Percentage among women of childbearing age. M: Men; W: Women.

with consanguineous unions and in building up a database of genetic diseases in the population.

In conclusion, we present in this paper an overview of the blood group phenotypes, hemoglobin S and G6PD deficiency among populations for which few studies have been done to date. In view of our results we find that the different studied markers are not affected by consanguinity in general because the frequency of different variables of consanguineous subjects is not statistically different from that of the unrelated subjects. Nevertheless, the fact that level of MNS blood groups presented relevant difference in Yansi, and also the high frequency of clinical disorder show us that consanguinity brings genetic disorders in this population. Screening of a wider cohort could bring more clarification on the effects of consanguinity on the phenotypes under study in the present paper.

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COMMENTS

Background

The frequency of ABO and Rh blood groups, and of the other blood systems such as Kell, Duffy, Kidd and MNS differs between populations. Blood groups antigens are most important in transfusion, pregnancy and transplantation because of their immunogenic capacity. Distribution of blood groups is not known in Yansi tribe practicing consanguineous marriage. Consanguinity increases the risk of developing genetic diseases. Therefore this study also evaluated the frequency of glucose-6-phosphate dehydrogenase deficiency (G6PD) deficiency and sickle-cell anemia trait.

Research frontiers

The comparison between consanguineous Yansi and unrelated subject showed relevant difference in distribution of MNS blood groups. Authors also found high frequency of clinical disorders in Yansi population.

Innovations and breakthroughs

It is known that bloods group phenotypes, sickle-cell anemia trait and G6PD

deficiency are significant on the medical and anthropological level but also that consanguinity brings genetics disorders in the population. Several studies have been conducted in various African countries but few studies have been done to date in Congolese population in general and in the Yansi tribe in particular. To our knowledge, this study is the first to report data on this specific population.

Applications

This study will contribute to build a database of genetic diseases in the population and it will help in sensitizing the population about the medical risks associated with consanguineous unions.

Peer review

The study aimed to determine the frequency of blood groups antigens in the ABO, Rh and other systems as well as the prevalence of sickle-cell anemia's trait and the G6PD among the consanguineous Yansi tribe in the Democratic Republic of the Congo. The study revealed a significant difference in MNS blood group distribution between the Yansi tribe and a control population. Thus new data has been provided that consanguinity brings genetic disorders in the population. The paper is interesting and timely.

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Gemcitabine cures metastatic hepatic carcinoma and bone metastasis

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cluded. Therefore, the patient was treated with Gemcitabine (1500 mg/wk), as the suggested treatment schedule, for 24 wk in opioid dependency program. Sequential abdominal CT during follow up showed the disappearance of liver metastasis and shrinkage of the pancreatic tumor. Repeated ERCP after treatment showed re-channelization of the pancreatic duct. During 11 years of follow up, 5 CT scans disclosed not only the disappearance of the hepatic tumor but also no cancer recurrence. Progressive shrinkage of pancreatic head was also noted. Therefore, we can say this malignant case was cured by monotherapy with gemcitabine.

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Key words: Gemcitabine; Gemza; Cure; Metastatic hepatic carcinoma; Undifferentiated carcinoma; Bone metastasis

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Abstract

We report a case of a 59-year-old gentleman who had suffered from low back pain for several months. Abdominal sonogram showed multiple heteroechoic nodules in the bilateral liver and an enlarged pancreatic head. Abdominal computer tomography (CT) favored pancreas head tumor with liver and bone metastasis. Endoscopic retrograde cholangiopancreatography (ERCP) disclosed pancreatic duct invasion over the distal portion of the pancreatic duct with prestenotic dilatation. Liver biopsy showed undifferentiated carcinoma. As suggested by the pathologist, the nasopharyngeal area was checked by the ear, nose and throat doctor, was negative and nasopharyngeal carcinoma was ex-

INTRODUCTION

Gemcitabine is a nucleoside analog with a structural similarity to cytarabine. Initially, it has a low objective response rate of around 6%-11% in chemotherapy-naïve patients with pancreas cancer who are given the single agent gemcitabine (800 mg/m² IV weekly for 3 of every 4 wk)^[1].

CASE REPORT

In 1999, a 59-year-old gentleman was referred from another hospital due to an unknown malignancy with liver and bone metastasis. He suffered from lower back pain

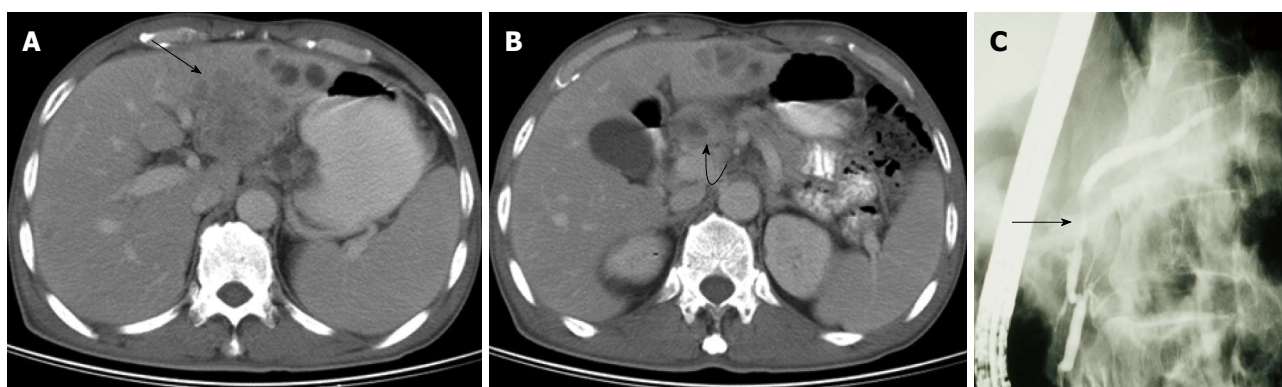


Figure 1 Diagnosis of pancreas adenocarcinoma. A: Multiple liver metastasis (arrow); B: Enlarged pancreas head (curved arrow); C: Focal stenosis over the distal portion of the pancreatic duct (arrow, apple-core like).

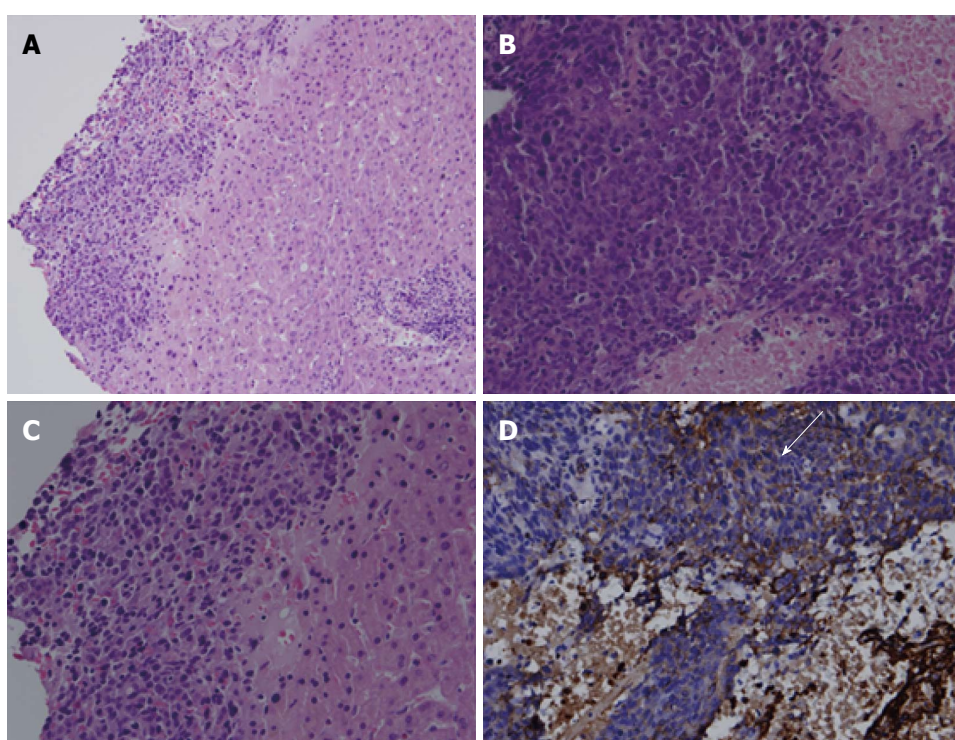


Figure 2 Liver aspiration. A: Liver tissue with tumor arranged in solid sheet on the left side of the picture (HE stain, x 200); B: High power view shows tumor cells with hyperchromatic nuclei and high nucleus-to-cytoplasm (N/C) ratio (HE stain, x 400). Two patches of necrosis are found in the lower and right upper part; C: Tumor cells in solid nest with hyperchromatic nuclei and high N/C ratio. The right side is normal liver tissue (HE stain, x 400); D: AE1/AE3 (IHC stain, x 400). Focal cytoplasmic positive reaction (white arrow).

and loss of body weight, about 10 kg within 3 mo. There was no familial history of malignancy. Physical examination showed pale conjunctiva, knocking pain over the lumbar spine and a mass lesion over the right sternoclavicular junction. The laboratory data showed only normocytic anemia and the hemoglobin was 10.4 mg/dL. The tumor markers, such as carcinoembryonic antigen and CA 19-9, were within normal limits. Abdominal sonogram showed many heterogeneous isoechoic tumors in bilateral lobes of the liver with enlargement of the pancreas head. Abdominal computed tomography (CT) showed suspicion of pancreas head cancer with liver and L3 metastasis (Figure 1A and B). The bone scan showed suspicion of metastasis

over the right sternoclavicular area, L-spine and ischium. Liver biopsy was done. The pathologist reported undifferentiated carcinoma (Figure 2) because of the focally weakly positive result with cytokeratin AE1/AE3, a sole marker of the epithelial differential, and a nasopharyngeal check-up to rule out the possibility of nasopharyngeal carcinoma was suggested. The ear, nose and throat (ENT) doctor disclosed a smooth nasopharyngeal mucosa and biopsy showed no malignancy. Endoscopic retrograde cholangiopancreatography (ERCP) showed focal irregular stenosis, favoring tumor invasion over the pancreatic duct (Figure 1C). Clinically, pancreas cancer with liver and bone metastasis was highly likely.

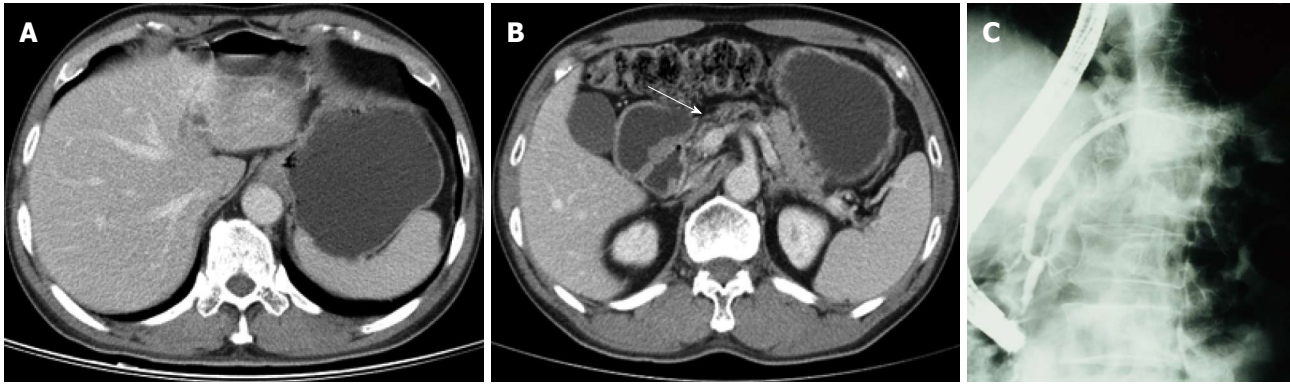


Figure 3 Tumor shrinkage post-chemotherapy. A: Liver nodules disappeared; B: The pancreas head was atrophied (white arrow); C: No stenosis of the pancreatic duct was noted after chemotherapy.

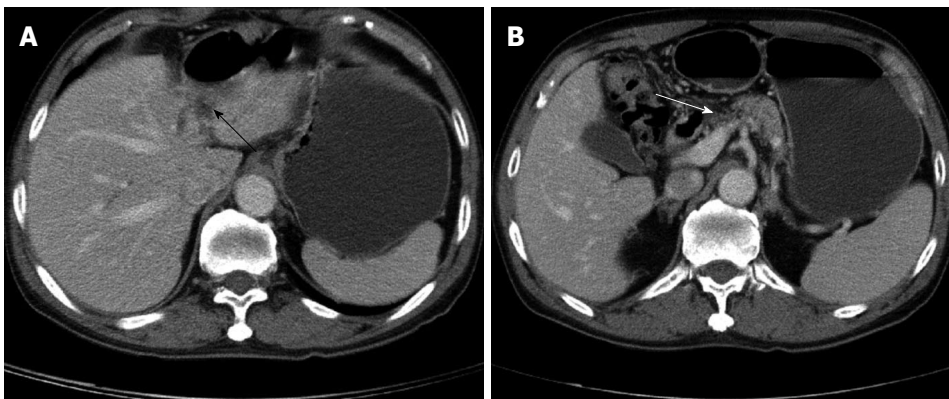


Figure 4 Shrinkage of left lobe of the liver (A, black arrow) and pancreatic head (B, white arrow).

The patient was treated with monotherapy of gemcitabine 1500 mg/wk in GI-OPD. The dose was 1000 mg/m² body surface area and medication was infused in 30 min. The patient received gemcitabine every week for the first 7 wk and then rested for 1 wk. Thereafter, he received treatment weekly for 3 wk and then rested for 1 wk. In total, the patient received 19 doses within 24 wk. Throughout, the patient tolerated the treatment well and blood transfusion was not necessary. Repeat CT scan showed disappearance of the liver mass (Figure 3A) and shrinkage of the pancreatic head (Figure 3B). Repeat ERCP showed re-channelization of the pancreatic duct (Figure 3C). The mass over the right sternoclavicular joint became softer and smaller gradually. Lumbago improved gradually during gemcitabine therapy and finally vanished after the treatment course. In the following 11 years, 5 CT scans showed progressive shrinkage of the left lobe of the liver (Figure 4A) and pancreatic head (Figure 4B). No cancer recurrence was noted. Also, repeat CA19-9 was normal. In 2010, 11 years after treatment, the patient had senile dementia but was still cancer free. Therefore, we can say this patient was cured by gemcitabine.

DISCUSSION

Undifferentiated carcinoma with multiple metastasis is

common, about 20%-25%, in cancer of an unknown primary site^[2]. In our case, NPC was excluded by biopsy on grossly normal mucosa of the nasopharyngeal area. In clinics, the mid or lower third portion of thoracic esophageal carcinoma with intra abdominal metastasis is also common, with an incidence of around 15.0%-17.4%^[3,4]. However, the gastroscop was negative too. The positive reaction with immunohistochemistry stain of AE1/AE3 was specific for carcinoma but carcinoma with pancreatic duct metastasis is very rare. Image studies of CT and ERCP, together with liver biopsy, resulted in the diagnosis of pancreas adenocarcinoma with multiple metastases.

In 1997, the study of Burris *et al*^[5] showed that the clinical benefit and survival of gemcitabine (1000 mg/m² weekly for 7 wk followed by 1 wk of rest, then weekly for three out of every 4 wk) was approved for first-line therapy of metastatic pancreatic cancer. However, there was no evidence to show that gemcitabine has the ability to cure advanced pancreas adenocarcinoma, even in cases with multiple organ metastases. But in our case, the liver mass disappeared and atrophy of left lobe of liver was noted in the following abdominal CT after treatment with gemcitabine. The repeated ERCP also showed the re-channelization of the irregular stenotic pancreatic duct. There was no cancer recurrence during the eleven years of follow-up. It was amazing that gemcitabine could cure

metastatic liver carcinoma.

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