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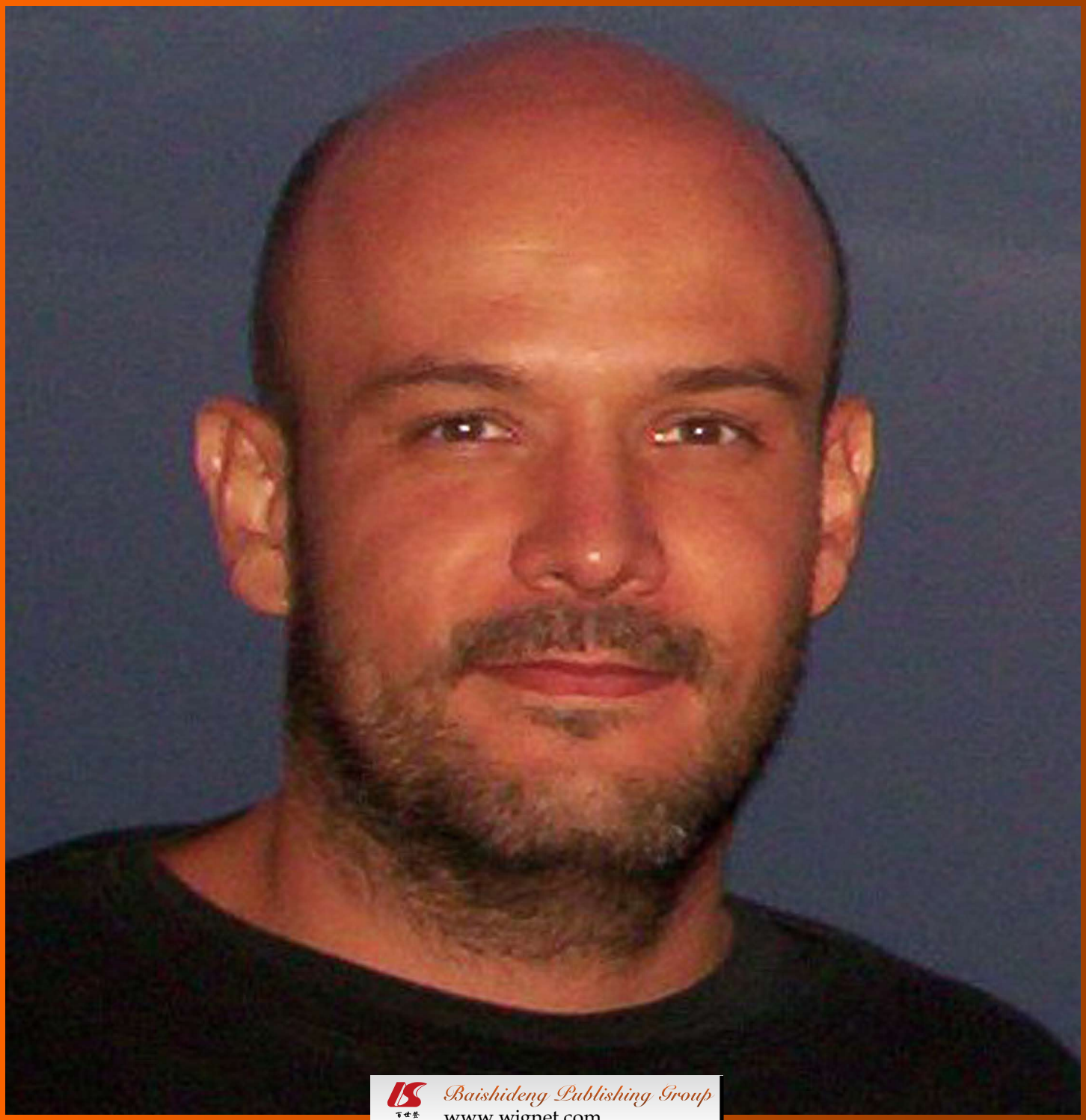

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Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Hematology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: wjhematol@wjgnet.com
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EDITORIAL

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

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

Marie-Christine Kyrtsonis, Dimitrios Maltezas, Efstathios Koulieris, Tatiana Tzenou, Haematology Section of 1st Department of Propaedeutic Internal Medicine, Athens Medical School, Laikon University Hospital, 11527 Athens, Greece
Stephen J Harding, The Binding Site Group Ltd., Birmingham B15 1QT, United Kingdom
Author contributions: Kyrtsonis MC and Harding SJ designed research; Kyrtsonis MC, Maltezas D, Koulieris E, Tzenou T and Harding SJ wrote the paper.
Correspondence to: Marie-Christine Kyrtsonis, MD, PhD, Assistant Professor of Hematology, Haematology Section of 1st Department of Propaedeutic Internal Medicine, Athens Medical School, Laikon University Hospital, Agiou Thoma 17, 11527 Athens, Greece. mck@ath.forthnet.gr
Telephone: +30-210-7462183 Fax: +30-210-7462183
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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.

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.

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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; lymphoplasmocytes 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 × 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.*, IgGκ, IgGλ, IgAκ, IgAλ, IgMκ and IgMλ. 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 is 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 albuminurea 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

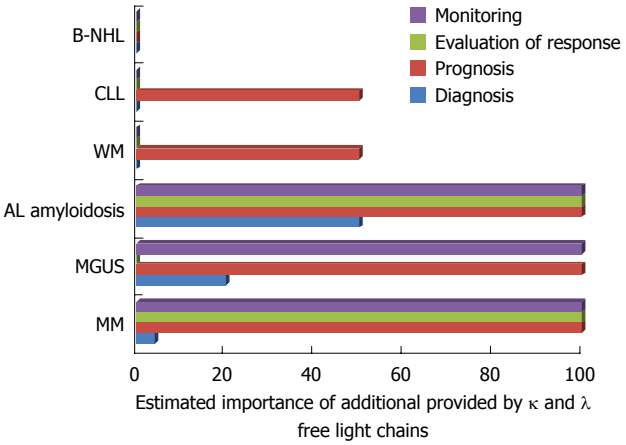

Figure 1 Estimated importance of additional information provided by free κ and λ free light chains for the diagnosis, prognosis, evaluation of response and monitoring of multiple myeloma, monoclonal gammopathy of undetermined significance, amyloid light-chain amyloidosis, chronic lymphocytic leukemia,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

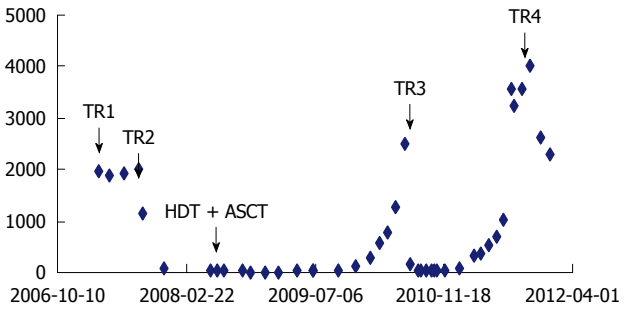

Figure 2 κ free light chain fluctuations in a light-chain κ multiple myeloma patient, during disease course. κ-free light chain fluctuations in an LC-κ multiple myeloma patient that presented mild anaemia and bone pains and no serum or urine paraprotein by serum protein electrophoresis and immunofixation.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

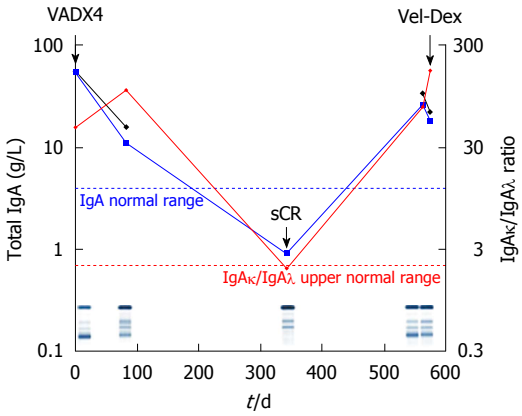

Figure 3 Heavy chain fluctuations in the course of a immunoglobulin A multiple myeloma patient. An immunoglobulin (Ig)Aκ multiple myeloma patient achieved stringent complete remission (sCR) after second line treatment with velcade-Dexamethasone. At relapse, he was retreated with velcade-Dex. A second 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 IgAκ/IgAλ 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), IgAκ/IgAλ ratio (red line) and IgAκ/IgAλ ratio normal range (upper limit, red dashed line).

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, β2-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 IgGκ, IgGλ, IgAκ and IgAλ, along with their deriving ratios: IgGκ/IgGλ, IgGλ/IgGκ, IgAκ/IgAλ and IgAλ/IgAκ[1,22], were recently introduced; their utility was shown by several groups. It was preliminary reported that increased HLC-IgG and -IgA ratios 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 β2-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

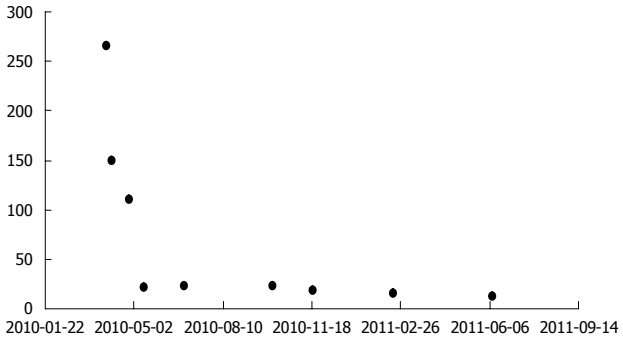

Figure 4 Lambda free light chain fluctuations in a amyloid light-chain-amyloidosis patient, in response to treatment. Total immunoglobulin (Ig)G and IgA were decreased, immunofixation showed only κ monoclonality. λ-FLC fluctuations in response to treatment, in a LC-λ AL amyloidosis patient with bone 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 IgAκ, 3 IgAλ, and 4 IgGκ 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 β2M, 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 (κ + λ) value above 60.6 mg/mL was superior compared to FLCR; thus, a model, based on four variables, namely sFLC (κ + λ) 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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EDITORIAL

Emerging immunological concepts in the pathogenesis of myelodysplastic syndromes

Claudio Fozza

Claudio Fozza, Department of Biomedical Sciences, University of Sassari, 07100 Sassari, Italy
Author contributions: Fozza C ideated and wrote the manuscript.
Correspondence to: Claudio Fozza, MD, Department of Biomedical Sciences, University of Sassari, Viale San Pietro 12, 07100 Sassari, Italy. cfozza@uniss.it
Telephone: +39-79-228282 Fax: +39-79-228282
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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.

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 riole in 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 a-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 astransplantation 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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EDITORIAL

Circulating microparticles and microRNAs as players in atherosclerosis

Nicoleta Alexandru, Adriana Georgescu

Nicoleta Alexandru, Adriana Georgescu, Petru Poni Institute of Macromolecular Chemistry, R-6600 Iasi, Romania
Nicoleta Alexandru, Adriana Georgescu, Department of Pathophysiology and Pharmacology, Institute of Cellular Biology and Pathology Nicolae Simionescu of Romanian Academy, 050568 Bucharest, Romania
Author contributions: Alexandru N and Georgescu A contributed equally to this paper.
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Correspondence to: Adriana Georgescu, PhD, Department of Pathophysiology and Pharmacology, Institute of Cellular Biology and Pathology Nicolae Simionescu of Romanian Academy, 8, BP Hasdeu Street, PO Box 35-14, 050568 Bucharest, Romania. adriana.georgescu@icbp.ro
Telephone: +40-21-3194518 Fax: +40-21-3194519
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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 interest 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.

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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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 then 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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FRONTIER

Aspirin responsive platelet thrombophilia in essential thrombocythemia and polycythemia vera

Jan Jacques Michiels, Fibo WJ Ten Kate, Peter J Koudstaal, Perry JJ Van Genderen

Jan Jacques Michiels, Department of Medicine, Erasmus Medical Center, Erasmus University Rotterdam, 3015 GE, Rotterdam, The Netherlands
Jan Jacques Michiels, Department of Hematology University Hospital Antwerp, B-2650 Edgem, Antwerp, Belgium
Jan Jacques Michiels, European Working Group on Myeloproliferative Neoplasms, Goodheart Institute and Foundation, 3069 AT, Rotterdam, The Netherlands
Fibo WJ Ten Kate, Department of Pathology, Erasmus Medical Center, Erasmus University Rotterdam, 3015 GE, Rotterdam, The Netherlands
Peter J Koudstaal, Department of Neurology, Erasmus Medical Center, Rotterdam, Erasmus University Rotterdam, 3015 GE, Rotterdam, The Netherlands
Perry JJ Van Genderen, Department of Internal Medicine, Haven Hospital, 3011 TD, Rotterdam,The Netherlands
Perry JJ Van Genderen, Erasmus Medical Center, Rotterdam, 3015 GE, Rotterdam, The Netherlands
Author contributions: Michiels JJ wrote the manuscript, discovered the platelet-mediated microvascular disturbances in thrombocythemia and conducted the studies on the etiology of platelet thrombophilia in thrombocythemia; Ten Kate FWJ performed the pathology study on skin biopsies from erythromelalgic areas and bone marrow histology for detection of early stage essential thrombocythemia and polycythemia vera; Van Genderen PJJ performed the platelet kinetic, platelet function and coagulation studies; Koudstaal PJ analyzed and interpreted the neurological, ocular and visual manifestations as part of the broad spectrum of aspirin responsive microvascular disturbances in thrombocythemia.
Correspondence to: Jan Jacques Michiels, MD, PhD, European Working Group on Myeloproliferative Neoplasms, Goodheart Institute and Foundation, Erasmus Tower, Veenmos 13, 3069 AT, Rotterdam, The Netherlands. goodheartcenter@upcmail.nl
Telephone: +31-62-6970534 Fax: +31-10-4212054
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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 thrombomoduline (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.

Key words: Erythromelalgia; Migraine-like cerebral transient ischemic attacks; Platelets; β-thromboglobulin; Thrombomoduline; 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 (thromboangiits 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				
Thrombomoduline (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	-

[1]Significant 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 × 10^9/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

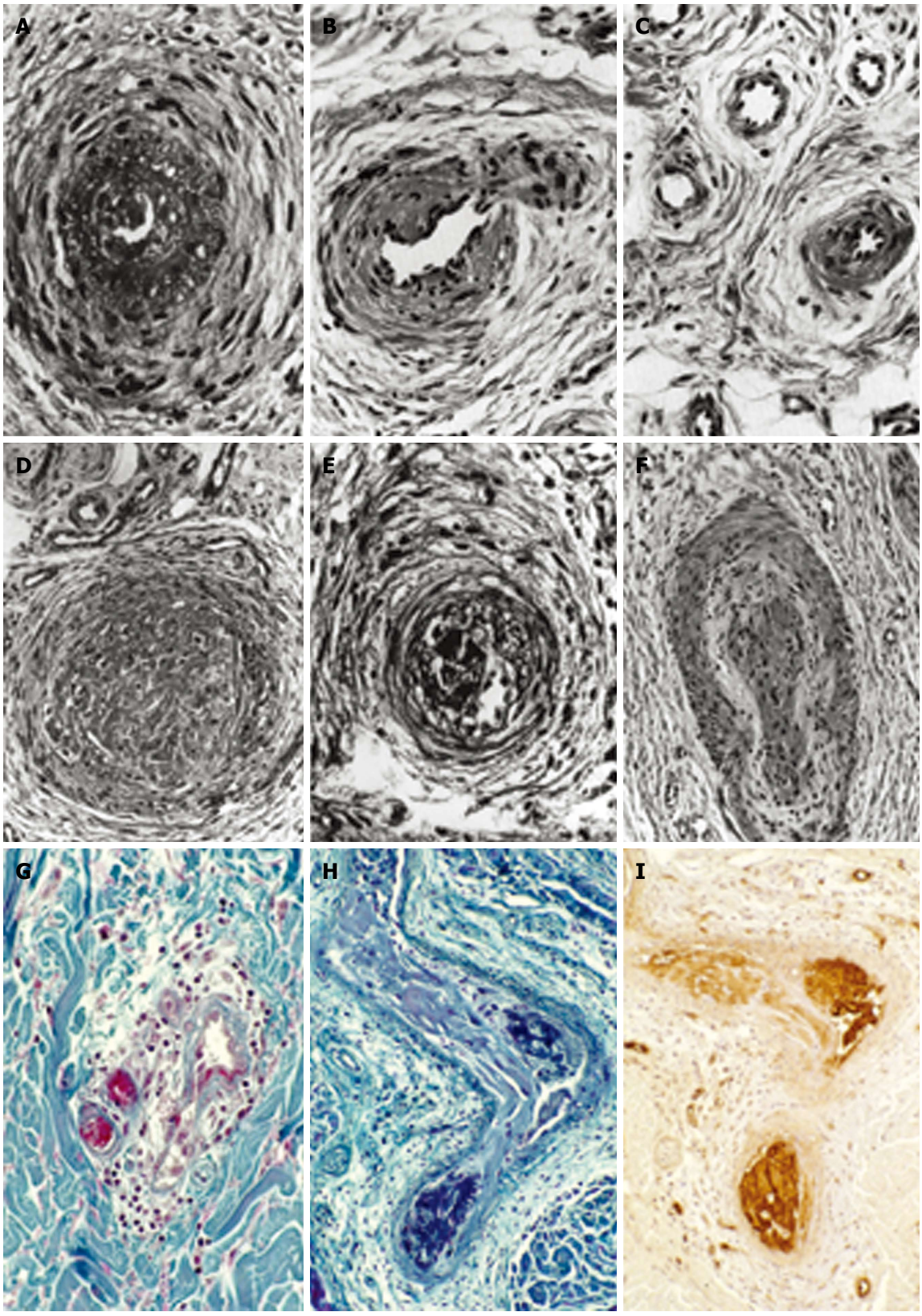


Figure 1 The histopathological appearances of a skin punch biopsy from areas of recently relapsed erythromelalgia 1 wk after aspirin discontinuation arteriolar l fibromuscular intimal proliferation, swollen endothelial cells, slight intravascular and perivascular infiltration with inflammatory cells, 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 trombi (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 antipholipid 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].

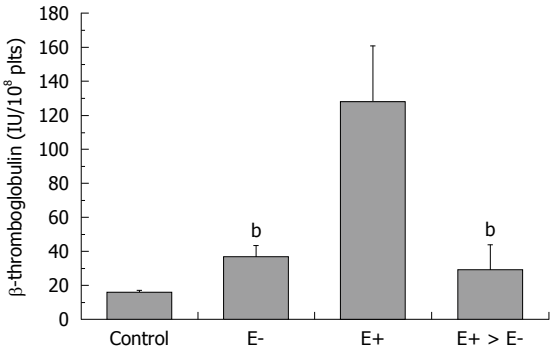


Figure 2 β-thromboglobuline levels in healthy control, and in essential thrombocythemia (E) either asymptomatic (E-), complicated by erythromelalgia (E+) and after treatment of E+ with aspirin (E+ → E-)[24]. [b]$P < 0.01$, significant differences between E- and E+ > E- as compared to E+.

[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 thrombomoduline (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 (Duesseldorff-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 10^5 platelets (ng/10^5 pL) was significantly lower in the untreated ET patients (0.48 ng/10^5 pL) compared to normal controls (0.92 ng/10^5 pL) and increased to a level (0.60 ng/10^5 mL) 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 10^5 platelets (ng/10^5 pL) was 4-fold and significantly higher (1.0 ng/10^5 pL) in the untreated ET patients compared to normal controls (0.25 ng/10^5 pL, $P = 0.04$). The mean value of plasma TXB2 (0.86 ng/10^5 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/10^5 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 nubmer																
	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

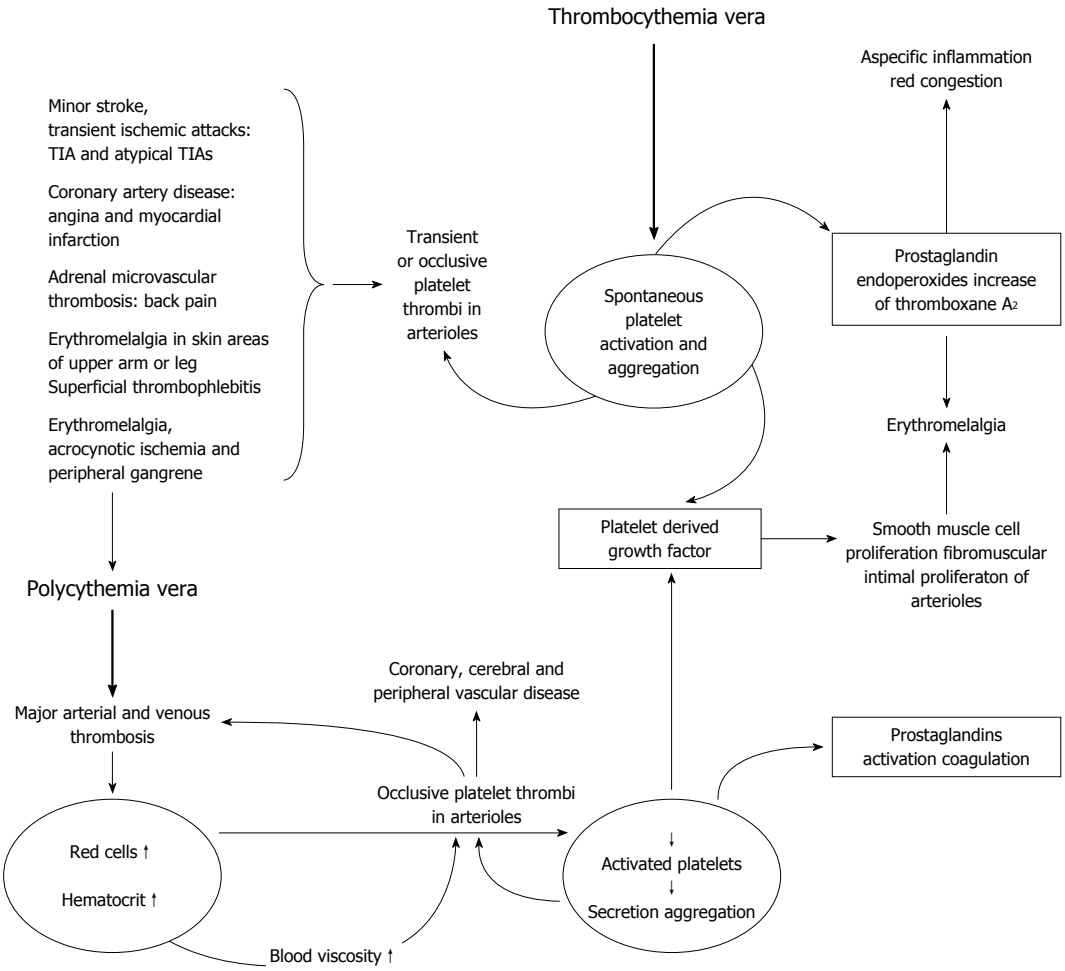


Figure 3 Etiology and pathophpysiology of platelet-mediated fibromuscular intimal proliferation and platelet thrombi in erythromelalgia and its acrocyanotic complications in thrombocythemia vera (essential thrombocythemia and polycythemia vera) and etiology of major thrombosis on top of erythromelagic 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 coexistent 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	17 (30)
or hemiparesis arm and/or leg	
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 × 10^9/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) underwent 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 × 10^9/L. Five patients in maintained complete remission of ET (platelet counts < 350 × 10^9/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 (ETT)] 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-

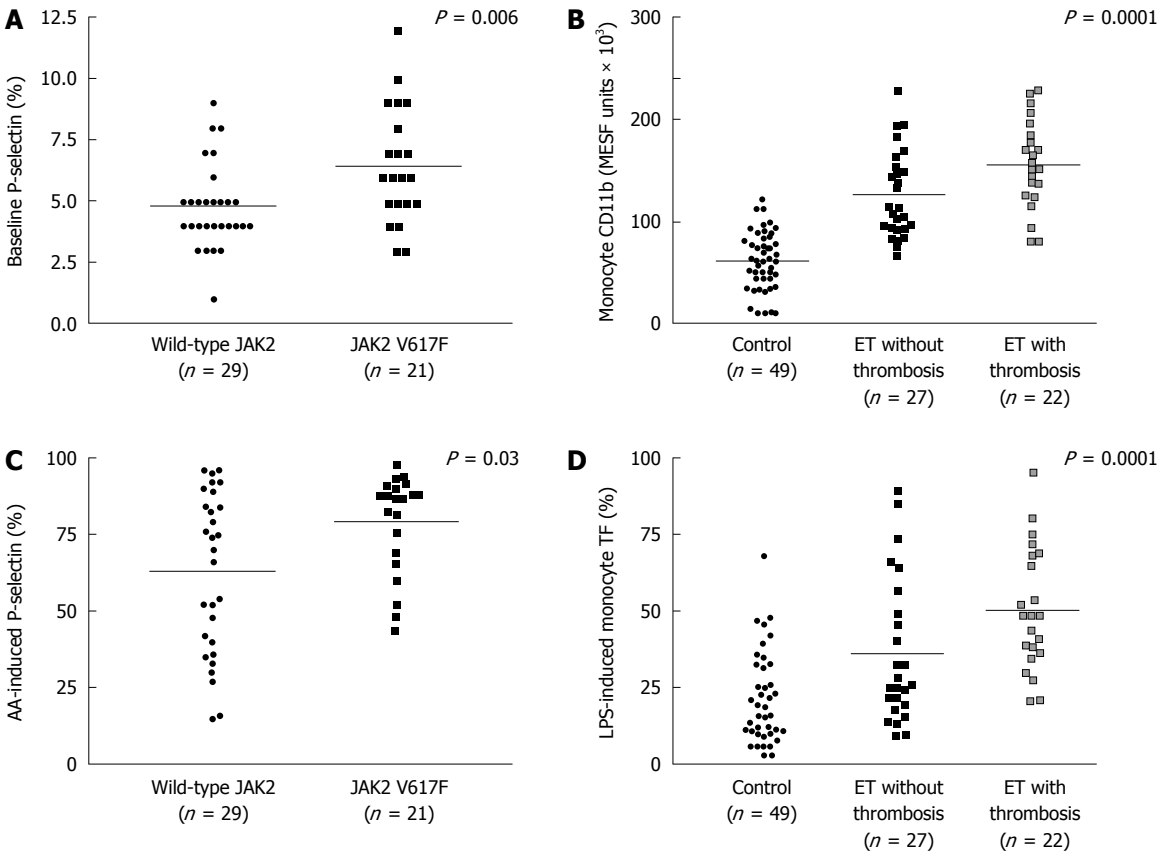


Figure 4 Baseline P-selectin levels (A), and arachidonic acid-induced P-selectin levels (C) in JAK2V617F mutated essential thrombocythemia and JAK2 wild type essential thrombocythemia; monocyte CD11b (B), and LPS-induced monocyte tissue factor expressions (D) in controls, essential thrombocythemia without 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 [$P \geqslant 0.0001$, 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^{V616F} 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 × 10^{11}/L) are dispersed and loosely clustered with predominance of large and giant forms (Figure 5). Following anagrelide monotherapy for about 1 year (platelet count near to normal, 4.5 × 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 ploidization and/ 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.

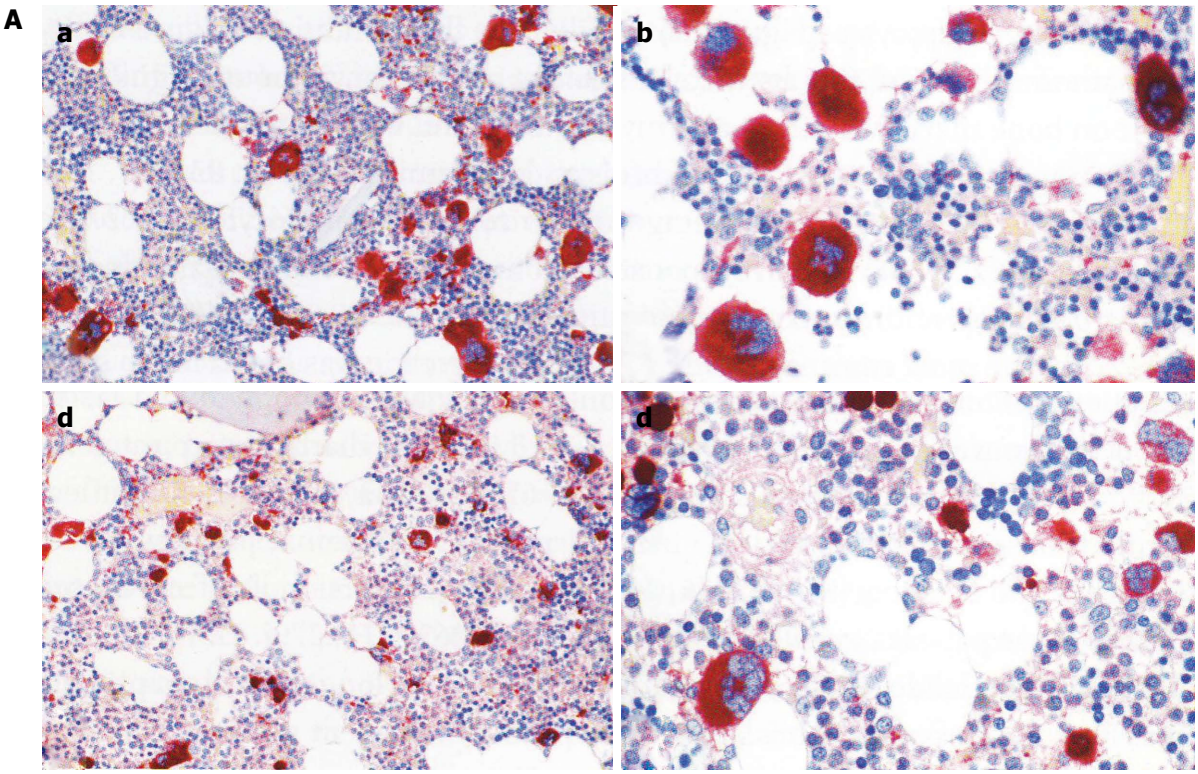
Table 5 Incidence of thrombosis in polycythemia vera and essential thrombocythemia patients

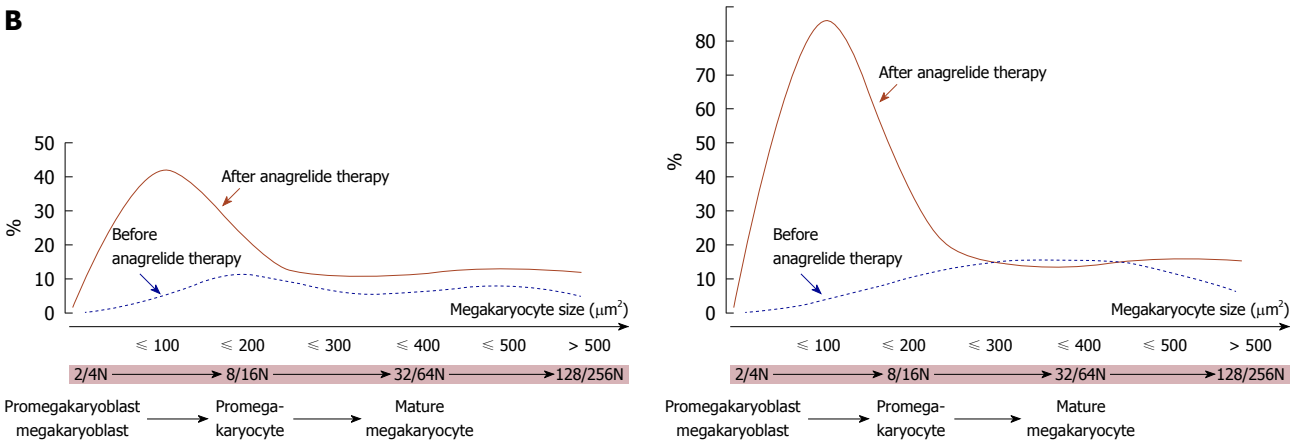
	PV		ET	
	GISP	Marchioli	Cortelazzo	Carobbio
Patients number	1213	1638	100	439
Age group (yr)				
< 40	1.8	2.1	1.7	NA
40-60	2.8	NA	6.3	NA
> 65	5.1	4.9	15.1	2.3
Previous Thrombosis	4.8	5.0	NA	2.3

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].

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





Correlation between CD61⁺ megakaryocyte size and proliferation activity (endoreduplicative capacity) in ET as determined by proliferating cell nuclear antigen labeling during anagrelide therapy compared with previously reported states of ploidy classes

CD61⁺ megakaryopoiesis in false ET (early-stage CIMF with thrombocythemia): effect of anagrelide therapy on size and endoreduplicative activity as determined by proliferating cell nuclear antigen labeling. Ploidy classes were indicated in keeping with previously performed studies

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 hypercellularfalse ET (early prefibrotic stage chronic idiopathic myelofibrosis, right). Before anagrelide therapy (platelets 9.7 × 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 × 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	Low risk	High risk	High risk
Completely asymptomatic	Microvascular disturbances only[1]	Major thrombosis, and/or bleeding	> 1000 × 10^9/L and minor thrombosis/bleeding = high
No vascular risk	No vascular risk	Vascular risk	No vascular risk
No bleeding risk	No bleeding risk		
Age < 65 yr[2]	Age < 65 yr except[2]	Age > 65 yr except[3]	All ages
Aspirin uncertain	Low dose aspirin	Platelet reduction to normal or near normal	Platelet reduction to < 1000 × 10^9/L
Wait and see?	50 to 100 mg/d		
	Intermediate risk		
Aspirin primary prevention?	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
ET patients and their physician usually prefer the use of low dose aspirin			

[1]At platelet counts in excess of 1000 × 10^9/L, aspirin will usually elicit bleeding symptoms, which disappear after reduction of platelet counts to below 1000 × 10^9/L with continuation of aspirin (Figure 6). If side effects of aspirin (gastritis, gastrointestinal bleeding) occur, reduce platelets to normal and consider indomethacine 25 mg/*bid*; [2]Age over 65 plus one of the following: platelets > 1000 × 10^9/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; [3]Age over 65, platelets < 1000 × 10^9/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 21th 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[65] 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 × 10^9/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 × 10^9/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 × 10^9/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 × 10^9/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 × 10^9/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 × 10^9/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 × 10^9/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 × 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 × 10^9/L, and platelet count in excess of 1000 × 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 × 10^9/L) but may remain slightly increased (400-600 × 10^9/L) or even moderately increased (600-800 × 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 × 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 (× 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 × 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

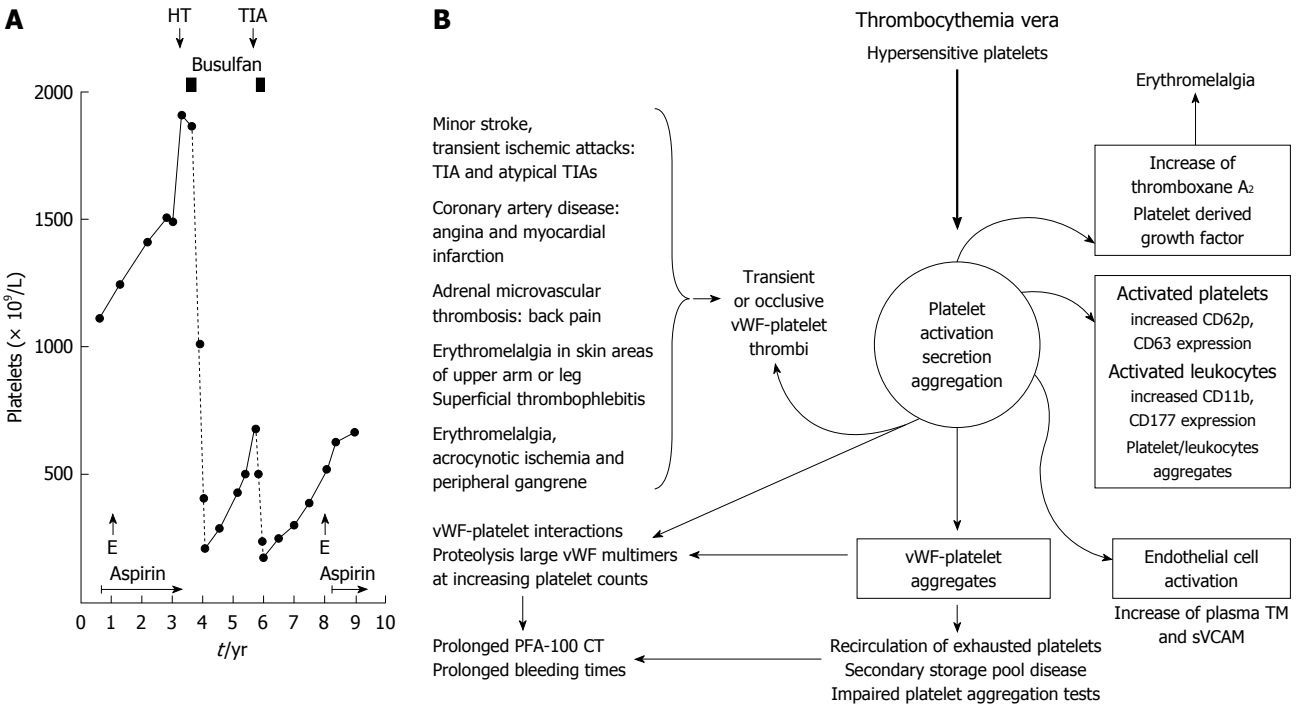


Figure 6 JAK2^{V617F} or MPL^{515} constitutively activated (hypersensitive) platelets in thrombocythemia spontaneously aggregate and secrete products (platelet derived growth factor et) at high shear stress in the end-arterial cerebral, ocular, coronary and peripheral circulation. This is associated with a broad spectrum of microvascular ischemic circulation disturbances (B) caused by transient von Willebrand factor (vWF)-rich platelet agregates 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 thrombomoduline (TM) and soluble vascular adhesion molecule (sVCAM) plasma levels (right). Increase of platelet counts from below to far above 1000 × 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 × 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 × 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 × 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) × 10^9/L, indicating the need for platelet reductive therapy. In patients receiving platelet lowering agents, either busulphan or hydroxyurea and no aspirin, 10 thrombotic complications occurred during follow-up of 40 patient/years (25%) at platelet counts of 624 ± 255 × 10^9/L, indicating inadequate control of platelet-mediated thrombotic 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 prefibrotic 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 × 10^9/L, which are relieved by reduction of platelet counts to normal (< 359 × 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 × 10^9/L is frequently associated with the paradoxical occurrence of ETT and HT. At platelet counts between 1000 to 2000 × 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 × 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^{515} 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 × 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-

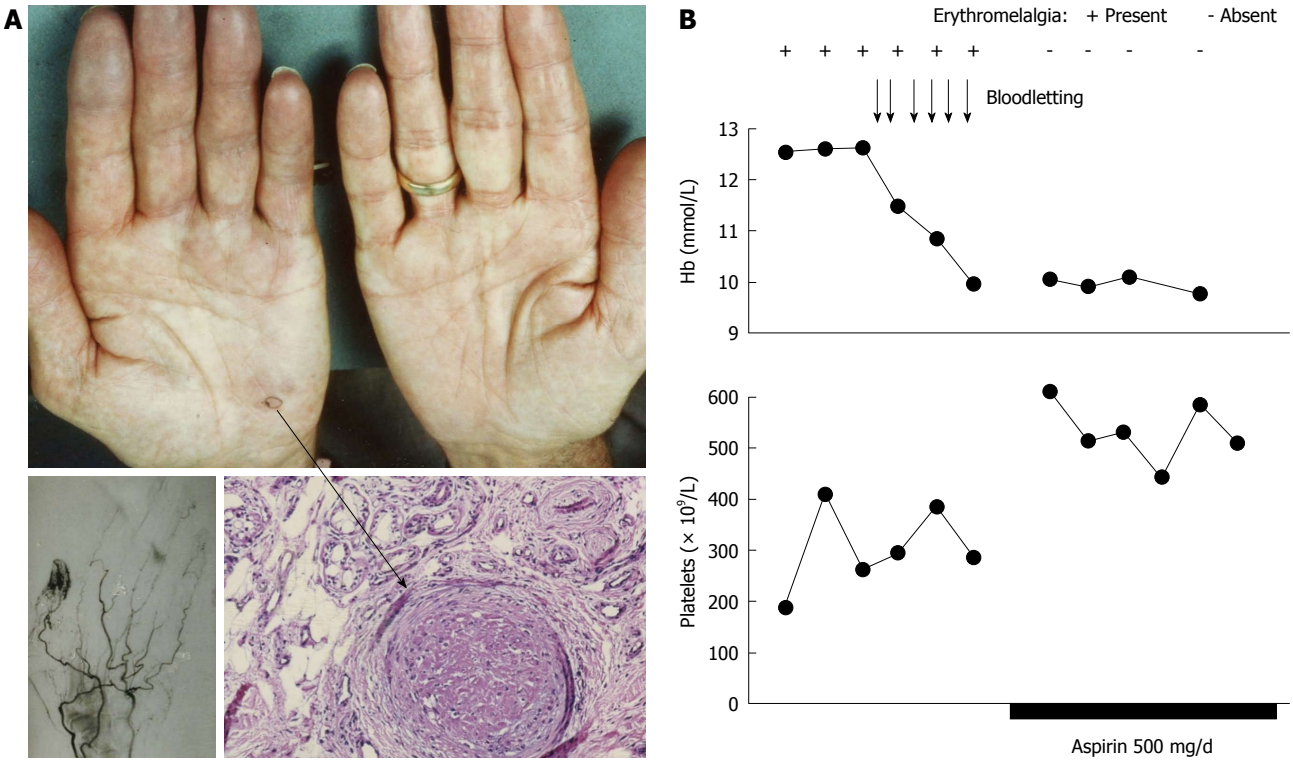


Figure 7 Acrocyanotic ischemic progression in a case of polycythemia vera 6 mo after presentation of erythromelalgia in the fingers of the right hand caused by endthrombotic occlusions of the superficial arterial arcade of the digital arteries. A: A skin biopsy taken from the blue ischemic spot of the handpalm (arrow) showed an occluded arteriole consistent with thrombo-angiitis obliterans; B: The erythromelalgic acrocyanotic congested fingers persited 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 painfull blue swelling, which was associated with a significant increase of circulating platelets counts from below to above 400 × 10^9/L. Four weeks of phlebomotomy 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 painfull acrocyantic erythromelalic sign and symptoms and a significant rise in circulating platelet counts of about 200 × 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 (× 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 × 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 *vs* placebo[75]: *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 × 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 myelosuppressive 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 × 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 phleboto-

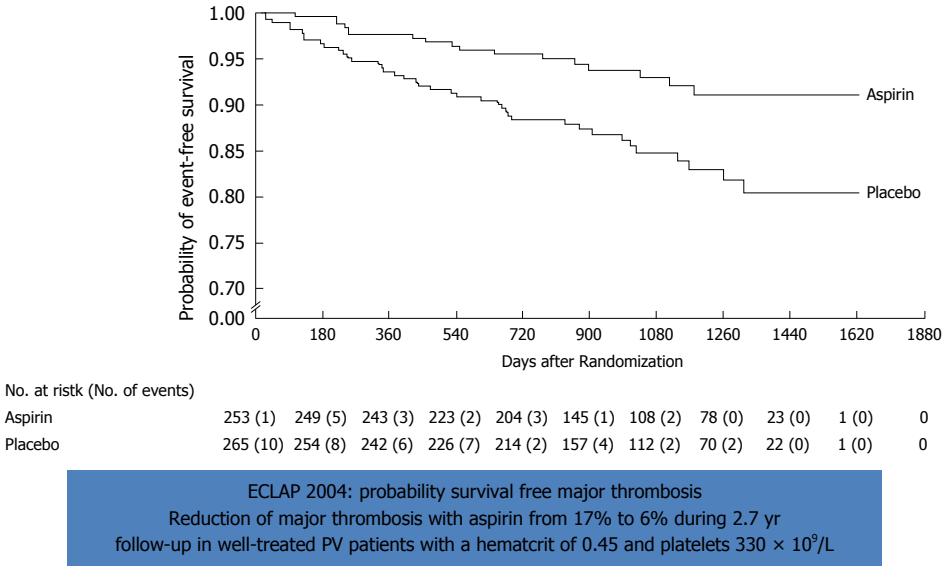


Figure 8 Effect of low dose aspirin on major thrombosis in treated polycythemia vera with bloodletting (40%) or hydrea (60%).

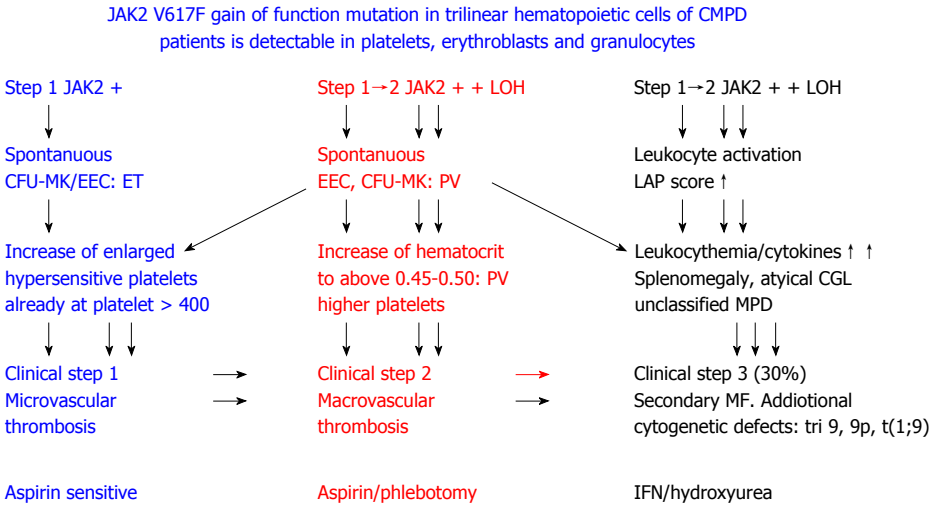


Figure 9 2005 ECMP concept on the molecular etiology of essential thrombocythemia, prodromal and overt polycythemia vera, leukocythemia, platelet-mediated microvascular thrombosis, increased blood volume and secondary myelofibrosis in JAK2 muated classical myeloproliferative disorders. ET: Essential 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 × 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 rational 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

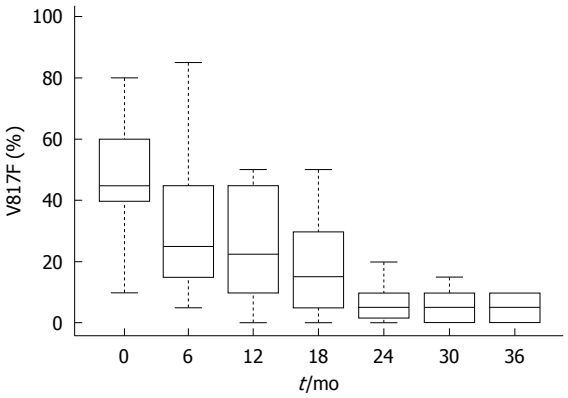


Figure 10 JAK2^{V617F} molecular responses in 38 patients with polycythemia vera during the first three years of treatment with interferon α-2a (Pegasys R) Courtesy of Jean Jacques Kiladjian, France.

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]. Peglyated 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 peglyated 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 responses at the bone marrow and molecular level remained elusive. It is known to hematologists that IFN may be rather effective in prefibrotic primary myelofibrosis (PMFMF 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-

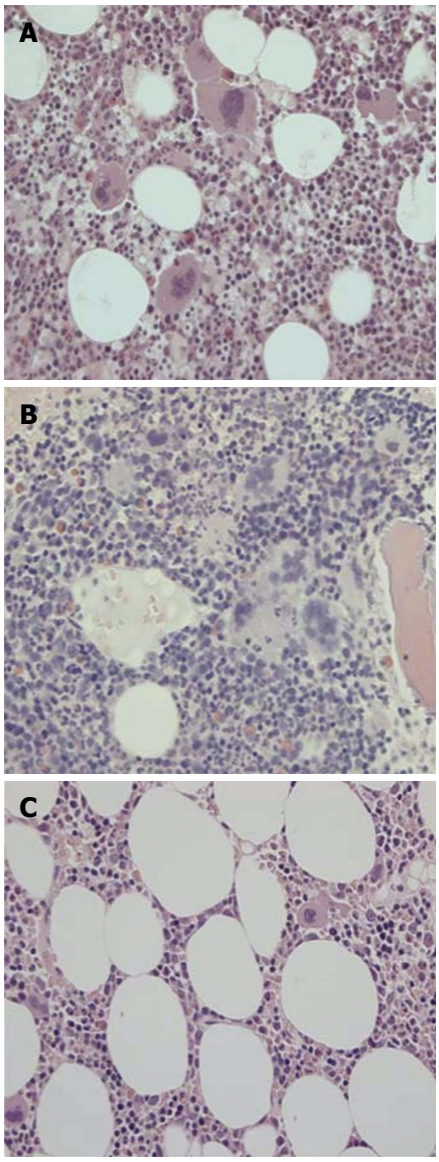


Figure 11 Bone marrow histomorphology from a patient with early prefibrotic polycythemia vera at diagnosis in 1996 (A) (hemoglobin 19.7 g/dL, hematocit 0.60. leukocytes normal, platelets 750 × 10^9/L, modest splenomegaly, JAKV617F > 54%) just prior to interferon α-2b treatment (B) showing typical hypercellular 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].

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 peglyated 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 GMSCF. The JAK2^{V617F} mutation makes the mutated hematopoietic progenitor cells hypersensitive for TPO, EPO, IL3, G-SCF 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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REVIEW

Another look at the life of a neutrophil

Siroon Bekkering, Ruurd Torensma

Siroon Bekkering, Ruurd Torensma, Department of Tumor Immunology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, 6500HB Nijmegen, The Netherlands
Author contributions: Bekkering S and Torensma R designed the research and performed the research; Bekkering S wrote the paper and designed the figures; Torensma R edited the paper.
Correspondence to: Dr. Ruurd Torensma, Department of Tumor Immunology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, PO Box 9101, NCMLS 278, 6500HB Nijmegen,
The Netherlands. r.torensma@ncmls.ru.nl
Telephone: +31-24-3610544 Fax: +31-24-3540339
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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.

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

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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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-

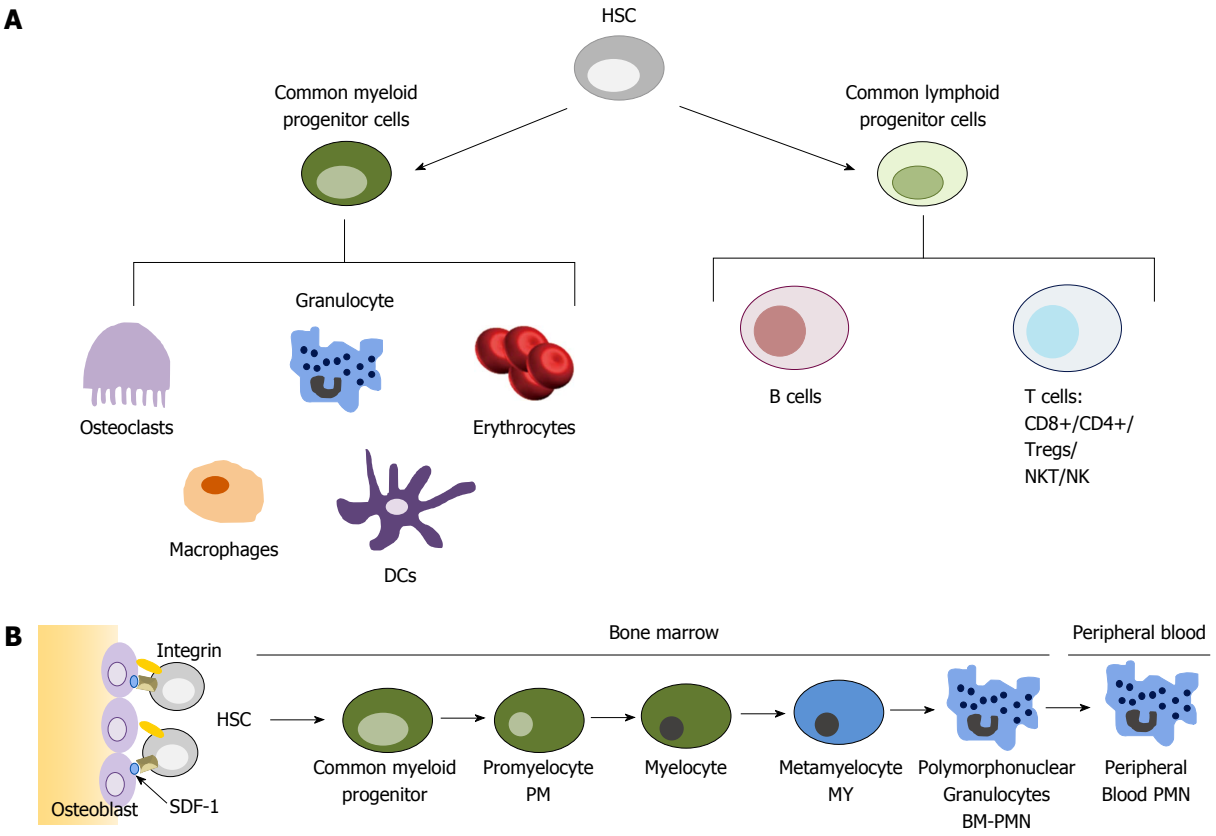

Figure 1 Granulopoiesis process. A: The production of blood cells from a hematopoietic stem cell. Modified from[131]; B: Neutrophil maturation from the hematopoietic stem cell to mature neutrophils. Modified from[1]. HSC: Hematopoietic stem cell; NK: Natural killer; DCs: Dendritic cells; PMN: Polymorphonuclear 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-1β[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 recruitmen 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, LTB_4, 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-1α and pathogen associated molecular patterns like endotoxin, as well as the earlier mentioned late-phase chemoattractants as IL-8, LTB_4 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 H_2O_2, a marker of primed or activated neutrophils. Mixed venous blood (blood before the pulmonary circulation) has higher H_2O_2 compared with arterial blood (blood after the pulmonary circulation), suggesting the lung to be the de-priming compartment[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].

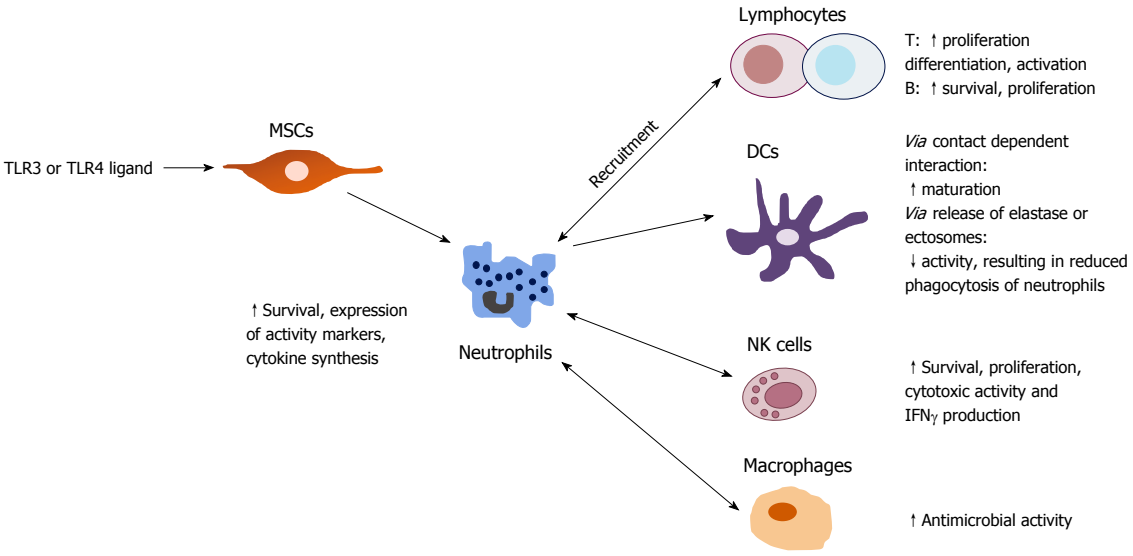

Figure 2 Cellular crosstalk of neutrophils in the tissues and in the lymph nodes. Modified from[78]. TLR: Toll-like receptor; NK: Natural killer; DCs: Dendritic cells; MSCs: Mesenchymal stem cells; IFN: Interferon.

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 maturate into macrophages and subsequently phagocytose apoptotic neutrophils. The macrophages in turn release anti-inflammatory mediators such as IL-10, further 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)-β1[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 peripherial development of NK cells[83]. They can modulate the survival, proliferation, cytotoxic activity and interferon γ (IFNγ) 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 IFNγ expression by NK cells. IFNγ 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, γδ 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-1β. 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-1α, 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,

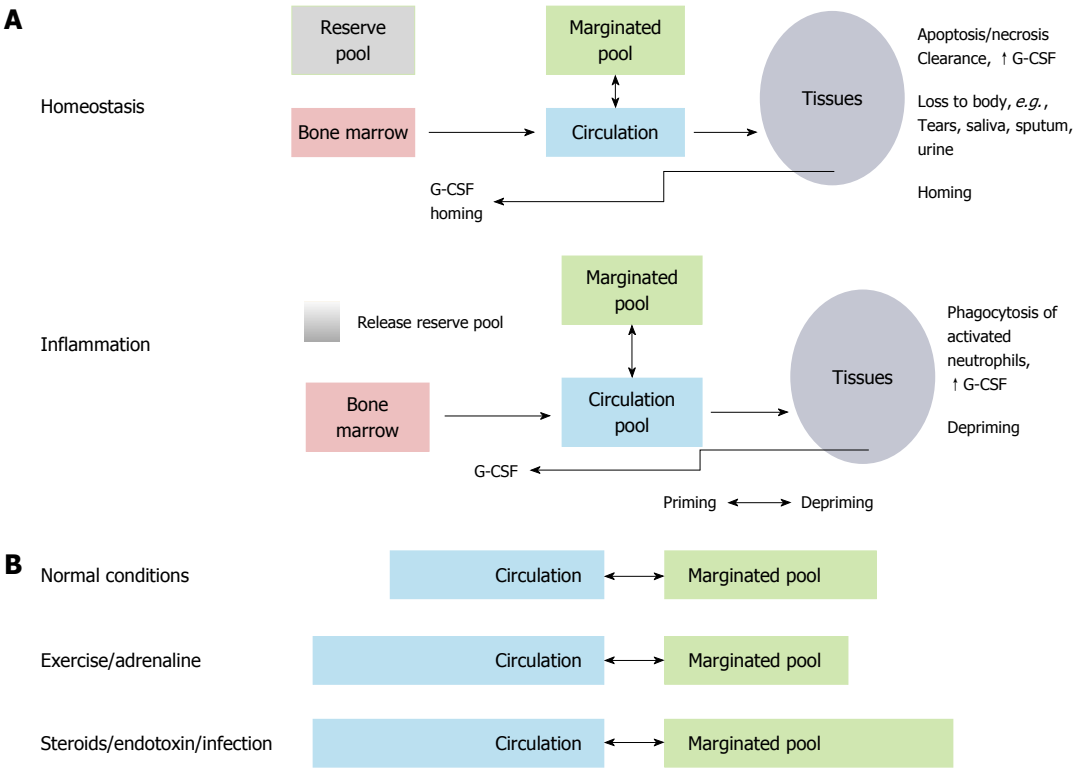

Figure 3 The marginated pool. A: Neutrophil pools under homeostatic and inflammatory conditions. All pools remain present, but the pool sizes change significantly; B: The size of the marginated and circulating granulocyte pool can be affected due to exercise or endotoxins or steroids. Modified from[88]. 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 marrow 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 [^{32}P] 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 ^{3}H-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.

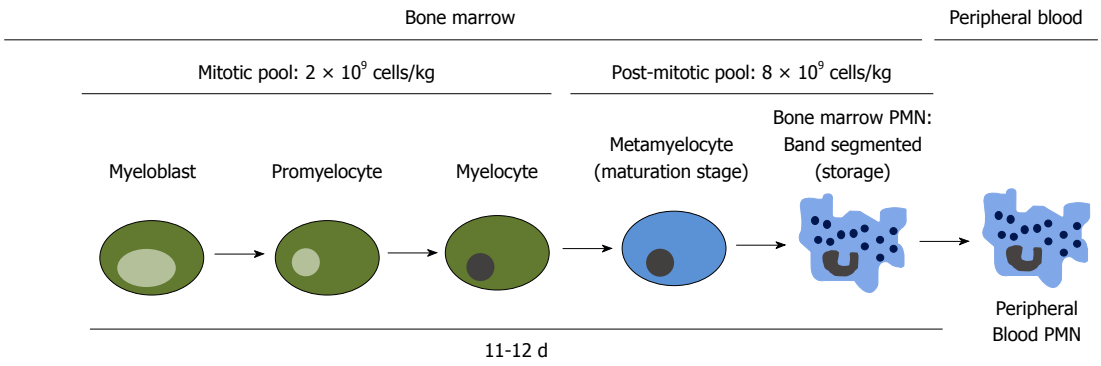

Figure 4 Neutrophil kinetics of the differentiation towards neutrophils in the bone marrow. Modified from[106]. PMN: Polymorphonuclear leukocyte.

However, when cells were labeled with di-isopropylfluorophosphate-^{32}P, 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 2×10^9 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 10^{11} 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 maturated[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 clearance 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 then 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 extend 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. ^{32}*P*-diisopropylfluorophospate (DFP^{32}) 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 $Na_2{}^{51}CrO_4$

or $SnCl_2$-reduced $^{99m}TcO_4^-$. 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 $Na_2{}^{51}CrO_4$ and $SnCl_2$-reduced $^{99m}TcO_4^-$, which showed less optimal results in the chemotactic responsiveness studies. Other labels are still used, for example ^{32}DFP or ^{3}H-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 an 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*[16] used ^2H_2O, 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.*, ^{32}DFP, H^3-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[18]. 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, ^{3}H-thymidine, ^{32}P-labeled sodium phosphate and ^{32}DFP, in their kinetics regarding the blood granulocyte radioactivity curves measured after administration[103]. ^{3}H-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. ^{32}P-labeled phosphate was found in the same subsets of neutrophils, as it is also incorporated in DNA[103]. ^{32}DFP 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: ^{32}DFP levels start high, where after the labeled neutrophils disappear in marginated pools and the level of ^{32}DFP declines. ^{3}H-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,

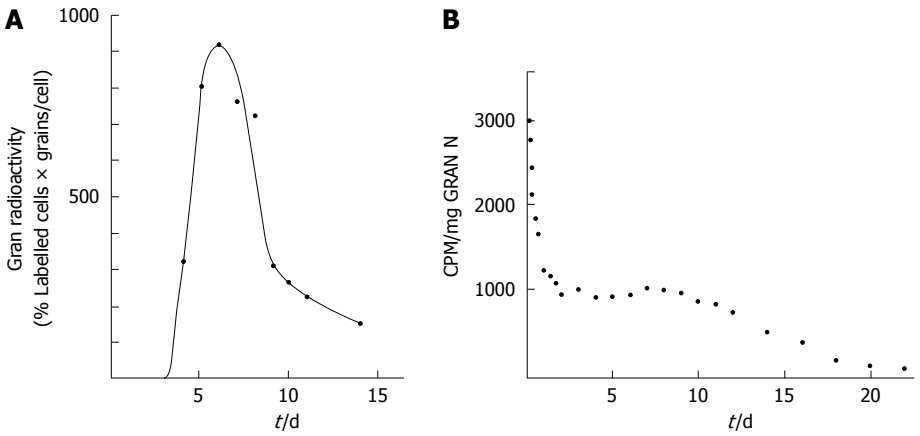

Figure 5 The kinetics of neutrophil production, the amount of cells that are produced each day, is measured as a rate of turnover of neutrophils in the blood. A: Decay of radioactivity of blood granulocytes after intravenous injection of ^{3}H-thymidine; B: Decay of radioactivity of blood granulocytes 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 potently 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%[2]. 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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CASE REPORT

Autologous lymphocytes infusion

Luis F Porrata, Svetomir N Markovic

Luis F Porrata, Svetomir N Markovic, Division of Hematology, Department of Medicine, Mayo Clinic, Rochester, MN 55905, United States
Author contributions: Porrata LF and Markovic SN were attending doctors for the patients, organized the report and wrote the paper.
Correspondence to: Luis F Porrata, MD, Assistance Professor, Division of Hematology, Department of Medicine, Mayo Clinic, 200 First St. SW, Rochester, MN 55905, United States. porrata.luis@mayo.edu
Telephone: +1-507-2843158 Fax: +1-507-2843158
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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.

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

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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)

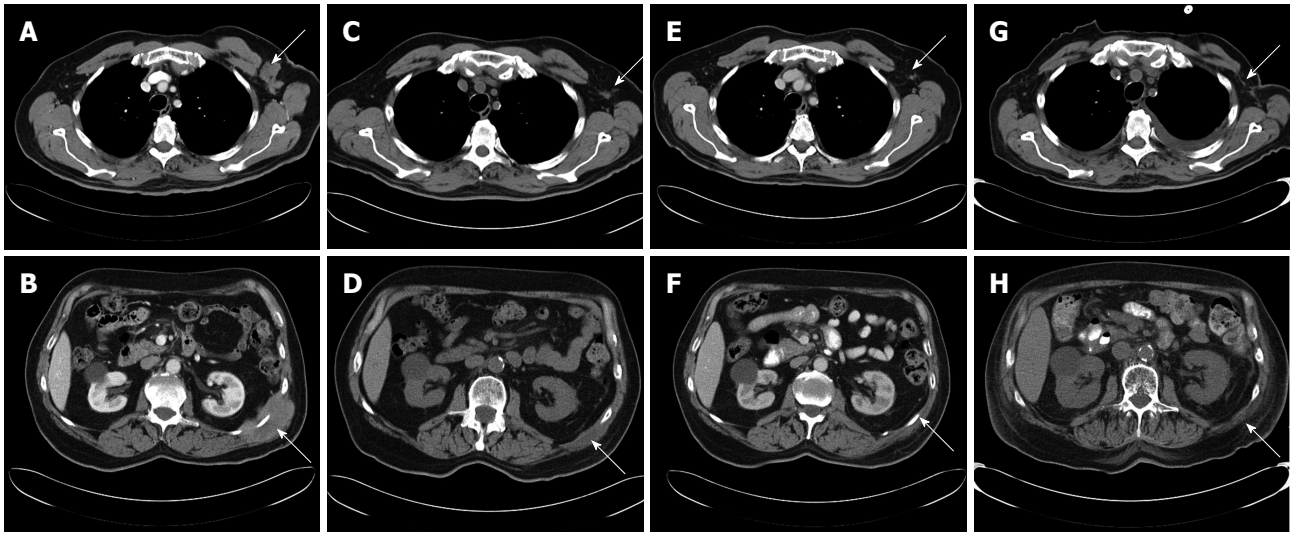

Figure 1 Computer tomography scans before and after autologous lymphocyte infusion (arrow). A, B: In June 2005 he developed new flank pain and a computer tomography scan; C, D: Follow-up computer tomography scan in August 2005 showed improvement of the left axillary adenopathy (1.4 cm × 0.5 cm) and the left flank mass (4.3 cm × 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 × 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 × 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 × 2.6 cm and left flank mass of 8.4 cm × 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 0.24×10^9/L. After the infusion, the absolute lymphocyte count improved to 0.44×10^9/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 × 0.5 cm) and the left flank mass (4.3 cm × 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 verus 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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Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
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