Contribution of cellular microparticles to pre-thrombotic states

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ISBN: 978-90-6464-710-9

Design cover: Rob Boeije, based on a work by Yves Klein
Layout: Ferdinand van Nispen, Citroenvlinder-dtp.nl DTP&Vormgeving, Bilthoven, The Netherlands
Print: GVO drukkers en vormgevers | Ponsen&Looijen, Ede, The Netherlands

Printing of this thesis was made possible by a genereus grant from Teaching Hospital OLVG which is gratefully acknowledged.

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PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht
op gezag van de Rector Magnificus, Prof. dr. L.L. G. Soete
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op woensdag 4 december 2013 om 14.00 uur

door

Marijke Clara Trappenburg

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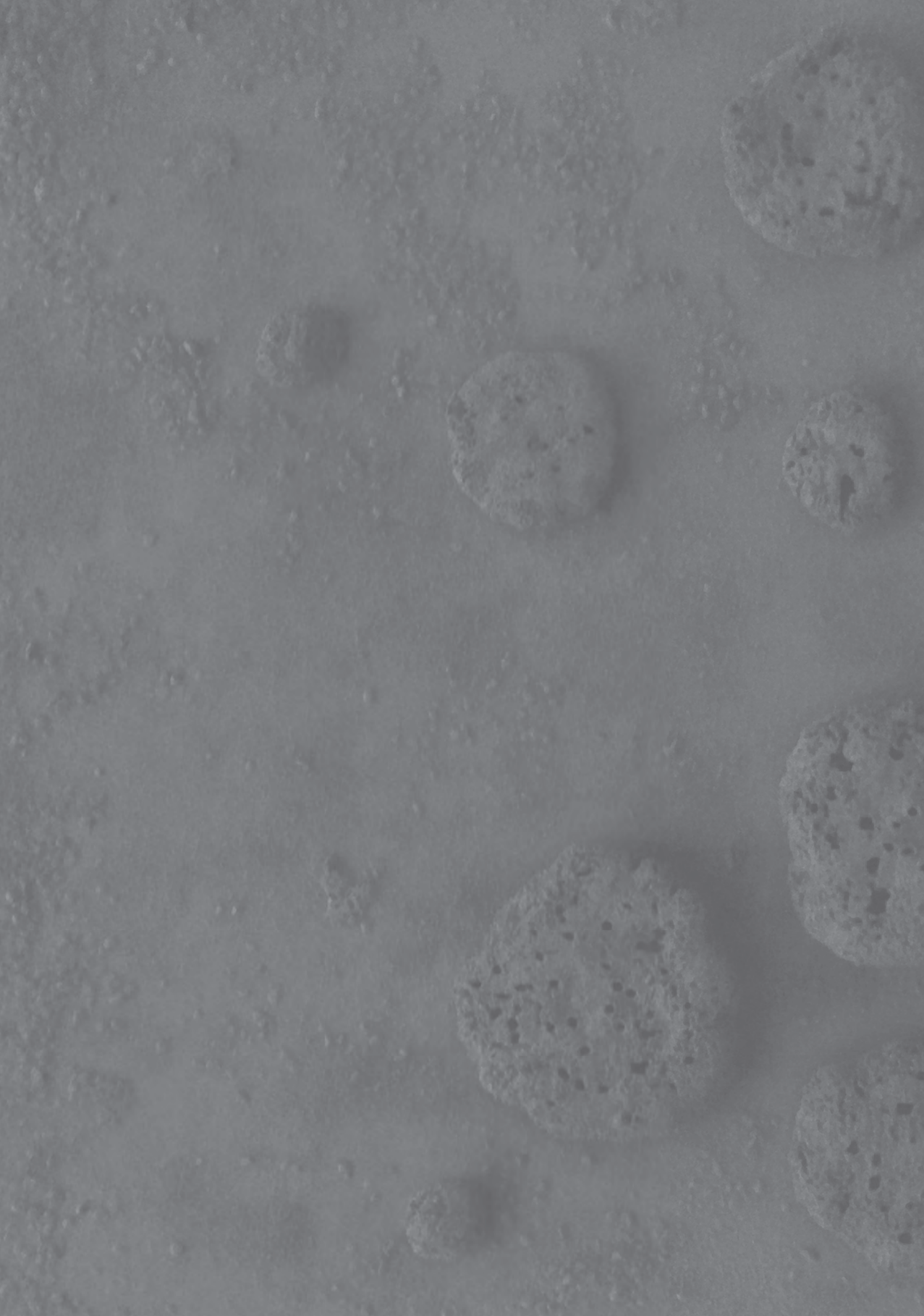
Financial support for the work described in this thesis by the Annadal Foundation, the E.C. Noyons Stichting, Teaching Hospital and Stichting onderzoek interne geneeskunde of the Onze Lieve Vrouwe Gasthuis is gratefully acknowledged.

Voor mijn ouders, Tom, Janna en Lena

Contents



Chapter 1

General Introduction

Abstract

Cell-derived microparticles (MP) are receiving increased attention in recent years, and the number of publications is rapidly increasing. MP are involved in thrombosis, inflammation, angiogenesis, cellular interactions and signal transmission. We focus on their role in thrombosis. A wide variety of methodologies to study MP have been employed and results can therefore be contradicting. Flowcytometry and solid phase capture are most commonly used nowadays. MP may be procoagulant by expressing phosphatidylserine and tissue factor, and this procoagulant potential can be measured in different ways. Thrombin generation is often used, but more MP-specific measurements are also possible. As tissue factor is so important in thrombin generation we pay special attention to the role of tissue factor in MP and the impact on thrombin generation.

What are microparticles

Microparticles (MP) were first described by Wolf in 1967 [1] and termed platelet dust, a sort of cellular debris, as it has been considered for many years. The past two decades MP research has been booming, and more and more biological functions of MP are being revealed. Cellular MP are small irregularly shaped vesicles that arise from the plasma membrane of blood – and vascular- cells during cell activation, or apoptosis. A precise definition of MP is lacking. They are usually defined as a heterogeneous population of vesicles ranging in diameter from 0,1 -1 μm characterized by differences in size, as well as phospholipid and protein composition. Exosomes are another kind of extracellular circulating vesicles. MP differ from exosomes in size; exosomes are smaller, ranging in diameter from 30 to 100 nm [2] and exosomes do not derive from the plasma membrane but from intracellular membranes.

Process of microvesiculation

A plasma membrane consists of two leaflets; the inner and the outer leaflet. The inner leaflet contains phosphatidylserine and phosphatidyl-enthanolamine, which are negatively charged proteins, while phosphatidylcholine and sphingomyelin are located on the outer leaflet. The preservation of this asymmetry is essential and is maintained through a complex transmembrane enzymatic balance [3]. Apoptosis or cell activation results in the transfer of phosphatidylserine to the outer leaflet, and thereby in a loss of this asymmetry (Figure 1). As a result, phosphatidylserine exposing MP may be released from the cells [4]. Several stimuli trigger microvesiculation in blood cells; chemical stimuli such as cytokines, thrombin and endotoxin, and physical stimuli such as shear stress or hypoxemia. The mechanism of microvesiculation involves five enzymes; aminophospholipid translocase, floppase, scramblase, gelsolin and calpain. In quiescent cells aminophospholipid translocase is responsible for the transport of phosphatidylserine and phosphatidyl-enthanolamine from the outer leaflet to the inner leaflet, thereby maintaining the asymmetry of the membrane [5]. Floppase, on the other hand, moves the same phospholipids from the inner to the outer leaflet when the cell is activated or undergoing apoptosis [6]. Scramblase has a similar effect as it facilitates bidirectional

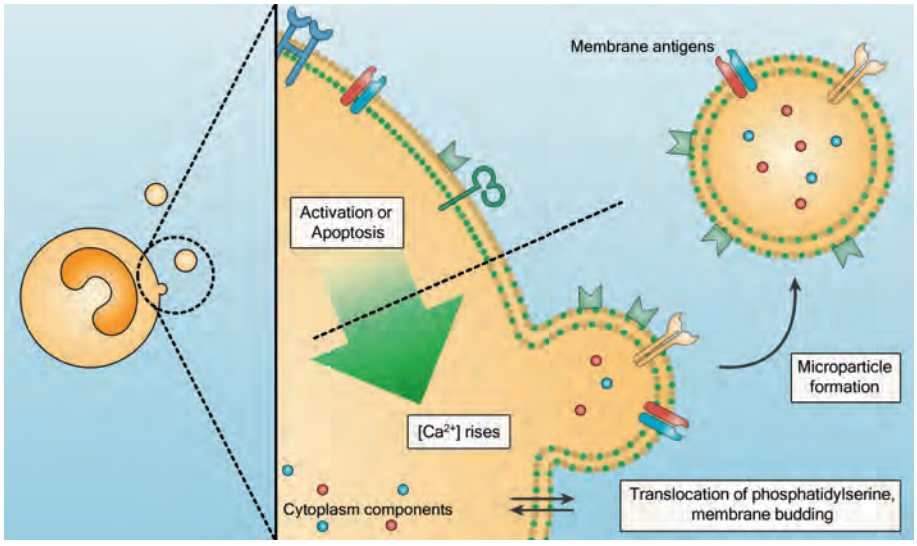


Figure 1 Process of microvesiculation. Due to activation or apoptosis the intracellular calcium concentration increases and this results in translocation of phosphatidylserine from the inner leaflet to the outer leaflet resulting in membrane budding and microparticle formation. Figure by Bob Smit, printed with permission.

Clearance of microparticles

Little is known of the mechanisms by which MP are cleared from the bloodstream. The presence of phosphatidylserine on MP activates the macrophage scavenger system [8]. MP and apoptotic bodies are cleared by phagocytes [9]. Phagocytes recognize phosphatidylserine containing cell fragments in two ways, either directly or indirectly through receptors that bind proteins that have opsonized MP, such as protein S, lactadherin, GAS6 and complement [10]. The estimated half-life of MP in the blood stream varies from five minutes to three hours [9,11]. Recently we estimated a half-life of one hour in healthy male volunteers after performing a bungee jump (Chapter 7). The differences in clearance between individuals as well as the factors that affect clearance have however not been identified yet.

Functions of microparticles

Many different functions have been attributed to MP, including a role in thrombosis and haemostasis, inflammation, angiogenesis, cell-cell

communication [12] and signal transmission [13]. MP can interact with different cells through proteins on their surfaces. Many proteins are described to be involved in binding MP to other cells. Platelet-derived MP interact with endothelial cells and monocytes by up-regulating cell adhesion molecules ICAM-1, CD 11a, CD11b [14]. Glycoprotein 1b and P-selectin are involved in interaction of MP with endothelial cells [15]. Moreover, MP can fuse with target cells or other MP, delivering proteins [15,16,17]. Mack *et al.* showed that MP containing chemokine receptor CCR5, the receptor for HIV-1 virus, could transfer this receptor to CCR5 negative cells and thus exacerbate infection [18]. MP contain micro-RNA and this can also be transferred to other cells [16,19]. Because MP have so many different functions they have been studied in many different diseases. In this chapter we further focus on the role of MP in thrombotic disease.

Microparticles and thrombosis

Several lines of evidence indicate that MP are indeed procoagulant. Firstly, increased numbers of MP are present in diseases associated with higher risk of thromboembolic diseases. Secondly, when these MP are isolated they support coagulation in vitro and thirdly, when platelets have a decreased capacity to generate MP such as in Scott syndrome, this results in a bleeding tendency. It is however unknown whether variations in MP production contribute to variations in bleeding diathesis in patients with congenital or acquired disorder [20].

We give an overview of the literature on MP in diseases that are known to have an increased risk of thrombosis, such as cardiovascular disease, cancer and haematological disorders. Thrombosis can be divided in arterial and venous thrombosis, which both have a different pathogenesis. Arterial thrombosis is related to atherosclerosis and endothelial dysfunction, whereas venous thrombosis is related to hypercoagulability (congenital or acquired such as in cancer), inflammation, immobilisation and/or endothelial dysfunction. Both in arterial and venous thrombosis MP are thought to play a role.

Cardiovascular and metabolic diseases

Elevated levels of platelet and endothelial derived MP are described in cardiovascular and metabolic diseases such as acute coronary syndrome [21-24], hypertension [25], renal disease [26-30], diabetes [31-33] and stroke [34]. In acute coronary syndromes higher levels of procoagulant MP were detected [23,24,35] and their levels at day 1 of myocardial infarction were indicative of a poor outcome [23], suggesting a prognostic potential for MP. Endothelial-derived MP (EMP) in acute coronary syndromes appear suitable for assessing endothelial cell injury as these MP were higher in myocardial infarction compared with stable and unstable angina [22]. With regard to platelet-derived MP (PMP), these are increased in acute coronary syndromes [22,24,35]. PMP subpopulations reflecting platelet activation by exposing CD63 and P-selectin were increased in peripheral arterial disease and myocardial infarction [21]. PMP could discriminate myocardial infarction from unstable angina [22]. In hypertensive patients EMP and PMP correlate with blood pressure, EMP and PMP may therefore be mediators of, as well as markers for, endothelial and platelet activation and hypertensive target organ injury [36]. In diabetes the level of EMP is closely associated with vascular dysfunction, as CD31+/CD42b- and CD51+ levels correlated with brachial ankle pulse wave velocity and endothelium dependent flow mediated dilatation of the brachial artery [31]. In well regulated type 2 diabetes, tissue factor-positive MP were increased and correlated with fasting glucose and insulin. However, no correlation was observed between tissue factor-positive MP and markers of coagulation, which suggests that tissue factor-positive MP in diabetes may be involved in other processes than coagulation, including transcellular signalling or angiogenesis. Differences in MP profile between type 1 and 2 diabetes are described, both in MP subsets (diabetes type 1 more PMP and EMP) and MP-associated procoagulant activity (higher in diabetes type 1) measured with a prothrombinase assay [33]. An increase in CD105+ EMP was observed in patients with acute ischemic stroke [34].

Solid tumors

Bouillaud was the first to recognize the association between cancer and thrombosis in the 19th century [37]. Indeed, an increased rate of venous thrombosis is observed in cancer, and cancer associated thrombosis is an important cause of mortality in affected patients [38]. In cancer patients venous thrombosis is associated with tissue factor-positive MP levels [39]. Although MP

levels of patients without metastasis do not always differ from controls [17,40], patients with disseminated adenocarcinoma have higher MP levels, as observed for breast [17,40,41], colorectal [42], gastric [43], and pancreatic [17] cancer. In colorectal cancer TF- bearing MP correlated with d-dimer levels, an indicator of hemostatic system activation [42]. In cancer patients MP contribute to higher thrombin generation [40]. Thrombin can result in tumor growth and angiogenesis [44]. Hence, a vicious circle can be formed in which thrombin generation can result in tumor progression with an increase in both tumor- and endothelial-derived MP, and tumor progression and MP can result in increased thrombin generation. Cancer cells can shed MP, which can be measured in flowcytometry as they express epithelial mucins. Tesselaar *et al*. [17] showed that patients with both high MP-associated tissue factor activity and MP-associated epithelial mucin had a lower survival rate at 3-9 months follow up. Highly interesting is the role of MP in tumor progression, including angiogenesis, tumor growth and metastasis. MP can activate tumor environment [45], what might result in survival of cancer cells and resistance to chemotherapy. In angiogenesis MP can promote proliferation, stimulate the forming of a capillary network and stimulate the expression of pro-angiogenic factors by tumor cells [46]. There is evidence that MP may enhance metastasis. Janowska *et al.* [47] observed that MP derived from activated platelets interacted with lung cancer cells and enhanced their metastatic potential.

Haematological disorders

In 1985 Carr described membrane vesicles that shared antigens with leukemic cells, and related these membrane vesicles to coagulation disorders [48]. However, these vesicles were observed only in leukemic patients with clinical coagulopathies, of which the severity did not show a direct correlation with vesicle numbers. Since then MP have been studied in many haematological disorders, both in malignant and non-malignant diseases. In lymphoma, B-cell chronic lymphocytic leukemia and acute myeloid leukemia MP derived from tumor cells are described [45,49]. In the latter these MP were shown to be more procoagulant at the onset of the disease compared to time at remission. MP numbers declined during chemotherapy. In a recent study by Ghosh *et al.* in CLL patients a phenotypic shift was observed from predominantly platelet-derived MP in early stage of disease to leukemic B-cell-derived MP in the advanced stage [45]. Furthermore these leukemic B-cell-derived MP were capable of activating

human bone marrow stromal cells by activating and modulating signalling pathways. Interaction between CLL MP and bone marrow stromal cells induced increased VEGF production, which may modulate the CLL microenvironment in favor of CLL survival and resistance to chemotherapy.

In myeloproliferative neoplasms such as Essential Thrombocythemia and Polycythemia Vera elevated numbers of both platelet- and endothelial-derived MP are found [50]. MP in myeloproliferative neoplasms were shown to be more procoagulant [51], although correlations with thrombosis were not observed.
In sickle cell disease higher numbers of erythrocyte-MP were found during a sickle crisis [52,53]. In patients in steady state these MP were still increased. In addition, Shet *et al.* [53] described elevated numbers of endothelial-derived MP that did not bind to phosphatidylserine, but co-expressed tissue factor, resulting in a shortened plasma clotting time compared with control MP. In paroxysmal noctural haemoglobinuria [54] and thrombotic thrombocytopenic purpura [55] increased levels of endothelial-derived MP are described. In conclusion, in hematological disorders MP have been shown to be involved in the risk of thrombosis and might be involved in disease progression.

How to measure MP

MP analysis is widely applied to investigate the potential involvement of MP in various diseases. Although still considered investigational, it may be poised to enter the mainstream of clinical testing [56]. However, a wide variation in methodologies is responsible for the problem that results from different studies cannot be always directly compared and that results can even be inconsistent or conflicting. Furthermore, variation in pre-analytical methods also account for differences in results. Pre-analytical variables such as blood collection, sample processing, transportation and centrifugation conditions may have a major impact on MP measurements, and have not been adequately addressed in literature [57]. Also the process of freezing as storage method may influence endothelial-derived MP numbers depending on the phenotype studied, for example increase in CD31+CD42b- and CD62E+ MP and a decrease in CD144+ MP [58]. Many different technique principles are in use, of which flowcytometry and solid phase capture are the most common ones. All techniques have their own specific advantages and limitations.

Flowcytometry

Flowcytometry is well known for counting and separating MP in fluids at a rate of thousands per second. MP are labelled using antibodies with fluorophores and/or phosphatidylserine binding protein. Flowcytometry is the most commonly applied optical method to detect MP, approximately 75% of laboratories use flow cytometry to enumerate MP in clinical samples [59]. An advantage of flowcytometry is that thousands of MP can be analysed in one sample, and that multiple markers can be simultaneously determined. Even small subpopulations can be identified and analysed. All MP can be measured, and not only those positive for the surface binding marker as is the case in capture assays. A limitation is that in flowcytometry the smallest MP may escape detection due to the detection limit of most commercial flowcytometers [56].

Solid phase capture

In solid phase capture assays Annexin V or biotinylated antibodies are insolubilized onto streptavidin coated microtiter plates to which platelet-free plasma is added, incubated and washed. A prothrombinase assay is used for the determination of the amount of captured MP. The phosphatidylserine content of MP is measured through its ability to promote the activation of prothrombin to thrombin. In this prothrombinase assay the blood clotting factor concentrations have been determined to ensure that the phosphatidylserine content is the rate-limiting parameter of the reaction. With this assay most of the MP can be captured also allowing functional characterization of the procoagulant potential of MP. However, possible interference of soluble antigens may lead to underestimation of MP levels by antigenic capture [60]. Moreover, due to the washing steps prior to analysis this technique can fail to immobilize platelet-derived MP > 100nm of diameter, which result in an underestimation of this group of MP [61].

Other (impedance based flowcytometry, atomic force microscopy)

Both impedance based flowcytometry and atomic force microscopy are new and promising techniques for the measurement of MP. The Coulter principle is employed in an impedance- based flowcytometer to count and measure the size of MP in a fluid [62]. In atomic force microscopy MP must bind to an extremely flat surface such as mica. The surface binding might affect the morphology of MP, and the efficiency of MP binding to a surface is unknown [63,64]. In atomic force microscopy smaller MP can be detected than with flowcytometry [63].

In both techniques the observed concentrations of MP were 1000-fold higher than those counted by standard light scatter flowcytometry.

Functional tests for microparticles

The procoagulant activity of MP may result from their phospholipid content and/or the bearing of tissue factor. Therefore functional MP testing is aimed at phospholipid content, tissue factor activity, or both. Three types of tests to assess the procoagulant activity of MP are available. Procoagulant phospholipid activity assays reflect the ability of the phospholipid content of MP to generate thrombin. MP-associated TF activity tests are used to investigate the functional capacity of TF on the membrane of MP. In thrombin generation tests results are influenced by both the phospholid content of MP as well as the presence of TF on MP.

Procoagulant phospholipid activity assays

Anionic phospholipids present in MP membranes act as catalytic sites for factor Xa and thrombin formation, giving MP a procoagulant activity *in vivo* [65]. In this procoagulant phospholipid activity (PPA) assays phosphatidylserine is measured through its ability to promote activation of prothrombin to thrombin [60]. Two different methods are used; chromogenic and clot-based. In the chromogenic method, plasma is added to a microplate well coated with Annexin V, which binds MP with exposed phosphatidylserine on their surface. The plate is washed with buffer, followed by addition of factors Va and Xa and calcium to generate thrombin, which is measured using a chromogenic substrate [60]. Thrombin generation is proportional to the anionic phospholipid content. In the clot-based method patient plasma is added to human phospholipid-depleted plasma, which provides human factor V, prothrombin, and fibrinogen, followed by addition of bovine factor Xa and calcium. This complex reaction involves multiple steps ending with clotting. The clotting time is dependent on the amount of anionic phospholipid present. A shorter clotting time represents higher amounts of anionic phospholipids. This method can detect procoagulant phospholipid activity arising from phosphatidylserine on the MP surface, however, other factors bound to the surface of MP may also contribute to enhanced thrombin generation detected by this assays. Connor

et al. observed that MP were capable of higher degrees of thrombin generation than equivalent concentrations of phosphatidylserine. This was not due to the presence of TF on MP as this test was insensitive to TF, but may be due to the presence of other coagulation factors [66].

Functionality of TF on MP

Tissue factor (TF) is a small 47 kd transmembrane glycoprotein, which initiates blood coagulation by binding coagulation factor VII in its activated form (factor VIIa) [67]. The factor VIIa/TF complex activates factor IX and X, triggering the coagulation pathway [67]. The ultimate results of this cascade are thrombin generation and thrombin-catalyzed events, as well as activation of factors V and VIII, formation of fibrin and activation of factor XIII, which lead to thrombus formation and stabilization. Tissue factor can exist in a latent (or "encrypted") form that lacks coagulant activity or in an active form that initiates blood coagulation [68]. The activity of TF is regulated by its physiological inhibitor; tissue factor pathway inhibitor (TFPI), which not only inhibits the factor VIIa/TF complex but also exerts a direct inhibition of factor Xa [67]. Giesen *et al.* were the first to describe that normal blood contains circulating tissue factor in the form of MP [69], detectable with flowcytometry. Subsequently, Zwicker *et al.* used imdepance based flowcytometry to detect TF-bearing MP [39]. Next it was demonstrated that blood-borne TF, in the form of MP, is indeed required for thrombus extension in a laser-induced microvascular injury model in mice [70]. MP-associated TF activity is measured by determining factor VII-dependent factor Xa generation in the presence of excess negatively charged phospholipids under conditions that the TF concentration is rate limiting [17].

MP-linked TF activity was increased in ST elevation myocardial infarction, and correlated with failure of fibrinolysis [71]. MP-linked TF activity in myocardial infarction correlated with survival [72]. Circulating MP were identified as the prevailing reservoir of plasma TF activity. Increased TF activity was observed by Tesselaar *et al.* [17] in patients with disseminated adenocarcinoma, and correlated with mortality.

Thrombin generation; definition

Thrombin is one of the central enzymes in blood coagulation and both the rate of thrombin formation and the total amount of thrombin formed can be

considered a reflection of the potential coagulation activity in plasma [73]. This thrombin potential in plasma can be assessed by several commercial methods, of which the Calibrated Automated Thrombogram (CAT; Thrombinoscope BV, Maastricht, The Netherlands) is the most commonly used application. In this method a calibration factor is measured in a plasma sample identical to that in which thrombin generation is being determined and the course of the calibration factor is assessed during the entire experiment [73].
The initial concept of a thrombin generation test was described in 1953 by two British groups. However, this method was manual, time-consuming and the requirement for clot removal during the procedure was a source of error [74]. In the 1980s a new computer-assisted method to obtain a thrombin generation curve using a chromogenic substrate of thrombin was developed by Hemker and Beguin [74]. In 2000 automated measurement of thrombin generation was possible in plasma. This made the method more practical and suitable for performance in clinical laboratories and significantly improved the accuracy. In the CAT, coagulation is initiated by adding phospholipids and TF to platelet poor plasma or platelet rich plasma, and then one or more of the following variables of thrombin generation are measured: lag time, peak height, time to peak, time to tail, velocity and endogenous thrombin potential (area under the curve).

Thrombin generation assay for MP

A thrombin generation assay can be used to test the procoagulant ability of MP. MP can be a source for phospholipids and TF in thrombin generation. Berckmans et al. [75] discovered that MP in blood from healthy individuals supported thrombin generation. In the thrombin generation assay a correlation between phospholipid content and peak height as well as a correlation between MP number and peak height are described, which indicates that phospholipids on the membrane of MP account for thrombin generation [76,77]. Changes in lag time on the other hand are dependent of TF activity [77]. A higher TF activity results in a shorter lag time. Hellum *et al.* described that changes in the lag time in the CAT assay reflect levels of MP-associated TF activity in a more sensitive manner than TF activity measured by the Zymuphen MP-TF kit [77]. Thrombin generation is not only performed in MP rich plasma. Isolated MP can be added to human MP-free pooled plasma to make the assay more sensitive for MP, and to exclude any effects of plasma. A higher MP dependent thrombin generation was found in plasma of patients with recurrent thrombosis [78].

With regard to the procoagulant potential of MP many questions remain, which will be partly addressed in this thesis. Firstly, it is of importance to elucidate whether the ancestry of MP results in differences in procoagulant ability. For example, are monocyte-derived MP more procoagulant than platelet-derived MP. Monocyte-derived MP expres TF, and shorten lag time and increase peak height in a TF-dependent manner [79,80]. Aleman *et al*. described that platelet-derived MP did not support plasma thrombin generation in the absence of a procoagulant trigger (contact or TF). However, after initiation, platelet-derived MP significantly increased thrombin generation [79]. Van der Meijden *et al.* observed that platelet-derived MP, as well as erythrocyte-derived MP, initiated thrombin generation indepedently of TF in a Factor XII-dependent manner [80]. Platelet-derived MP and monocyte-derived MP differentially modulate thrombosis, the physiological relevance of this finding, especially in various disease states, requires further investigation. Secondly, it is of interest whether (tromboembolic) diseases influences the procoagulant potential of MP or mainly result in an increase of MP. Patients with recurrent venous thromboembolism and with myeloproliferative disease have been shown to have increased MP-associated thrombin generation [51,78]. Lastly, it is of great interest whether therapy can result in a change in the procoagulant ability of MP. The relation between therapy and procoagulant ability of MP have not yet been firmly established.

Outline of the thesis

The aim of this thesis is to investigate the phenotypic profile and procoagulant potential of MP in pre-thrombotic states. In this pre-thrombotic state patients have an increased risk of developing thrombosis due to their disease and/or medication used. We report a number of studies on the characterization of MP in association with different pathophysiological conditions. **Chapter 1** is the General Introduction to the work described in this thesis. We describe the definition of MP, their formation and the technical difficulties involving their analysis. In addition, we provide an overview of the literature on MP in thromboembolic disease. In **Chapter 2 and 3** MP in myeloproliferative diseases are described. Myeloproliferative diseases are clonal disorders: Essential Thrombocythemia (ET) patients demonstrate a large increase in platelet numbers and Polycytemia Vera (PV) patients show an increased erythrocyte

and leukocyte count. Patients with either disorder have an increased risk of thrombosis, and patients with the JAK2V167 mutation and high leukocyte numbers are at an even higher risk. In **Chapter 2** we focus on MP in Essential Thrombocythemia and investigate whether there is an association between MP and established risk factors for thrombosis. The study described in **Chapter 3** compares the phenotypic profiles of MP and their procoagulant potential in Essential Thrombocythemia and Polycythemia Vera. In **Chapter 4** we determined MP levels and their procoagulant potential in patients with chronic renal failure. The ratio between mature von Willebrand factor antigen and its propeptide was used as a measure for acute or chronic endothelial activation to study endothelial activation in these patients. In Chapter 5 and 6 we look further into the effect of anti-cancer therapy on MP numbers and MP-dependent thrombin generation. In **Chapter 5** we describe a cross-sectional study in 40 breast cancer patients using endocrine therapy. Half of the patients had had curative therapy and were considered disease free, and the other half of the patients had metastatic disease. All patients used either an aromatose inhibitor or an anti-estrogen. The effect of endocrine therapy on MP levels and MP-assosciated thrombin generation is investigated in this study. We used the same approach to assess MP numbers in acute myeloid leukemia (AML) patients in **Chapter 6**. In three patients we measured MP levels and their procoagulant potential in subsequent samples obtained before and during chemotherapy and in remission. Changes in MP levels due to chemotherapy were determined, as well as changes in procoagulant potential of MP during this therapy. Finally, we determined MP levels in a bungee jump study that was designed to investigate the effect of stress on the immune system. Twenty healthy volunteers performed a bungee jump from a 60 meter high crane, half of them were pre-treated with propranolol three days prior to the jump. With this design, described in **Chapter 7**, we were able to gain more insight into biological parameters of MP such as their formation and clearance. Moreover we could investigate the effect of stress on the numbers and phenotypic profiles of MP. Finally, in **Chapter 8** the principal findings of this thesis and relevance into understanding the role of microparticles in pre-thrombotic states are discussed in relation to literature.

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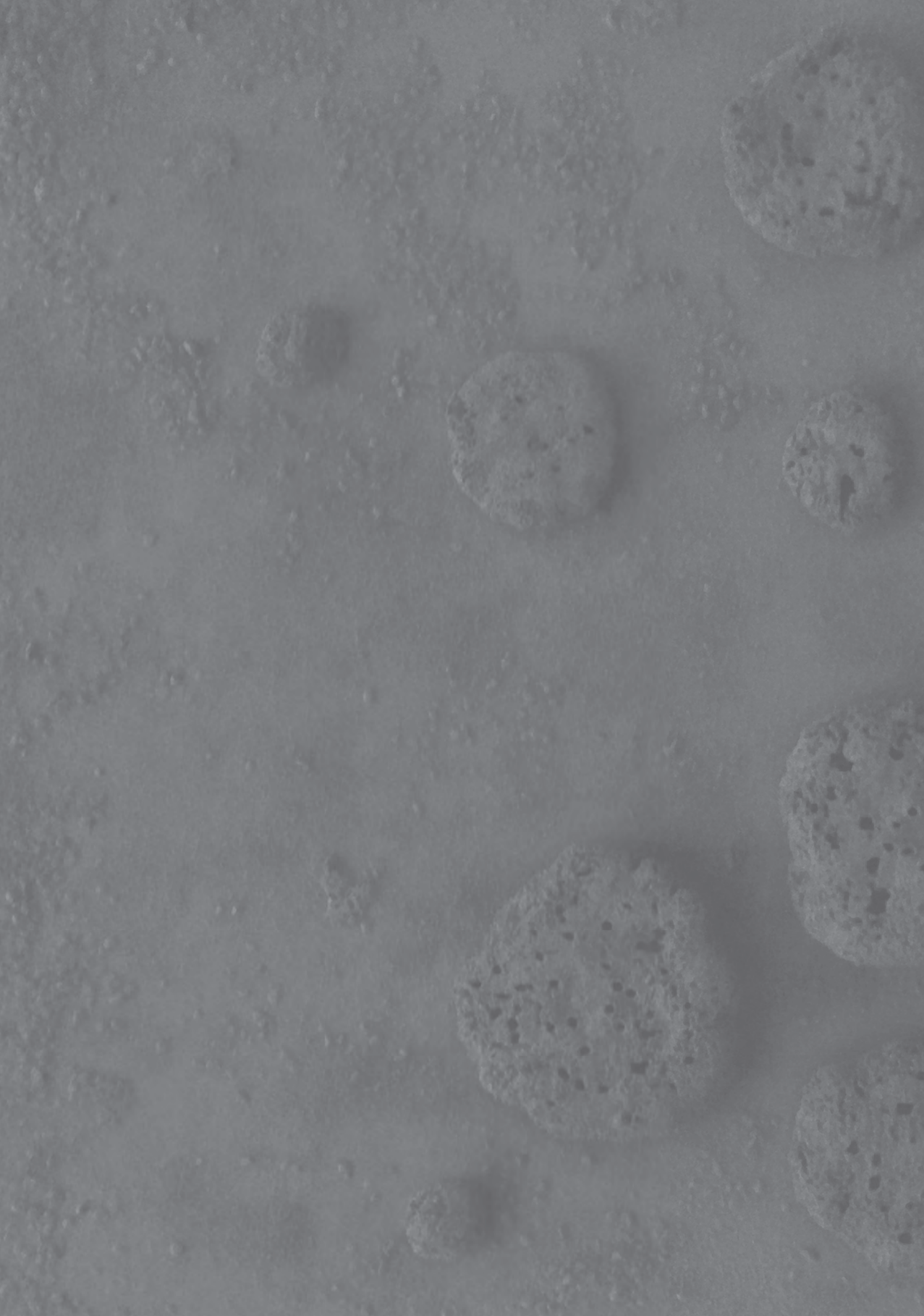

Chapter 2

Elevated procoagulant microparticles expressing endothelial and platelet markers in essential thrombocythemia

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Haematologica 2009; 94: 911-918

Abstract

Background: Most cell types, including blood - and vascular cells, produce microparticles (MP) upon activation. Since cellular MP are known to be elevated in thromboembolic diseases, we hypothesized a role for MP in the pathogenesis of thrombosis in essential thrombocythemia.

Design and methods: In plasma samples from 21 essential thrombocythemia patients and ten healthy subjects, the levels and the cellular origin of MP were determined by flowcytometric analysis, while the MP-associated procoagulant activity was measured using a thrombin generation assay.

Results: Patients with essential thrombocythemia had significantly higher numbers of circulating AnnexinV-positive MP than controls (median 4500 vs. $2500x10^6$ events/L; p=0.039), including significantly higher number of MP positive for the platelet marker CD61 (p=0.043), the endothelial markers CD62E (p=0.009) and CD144 (p=0.021), and for tissue factor (p=0.036). CD62E was co-expressed with the platelet marker CD41 on MP, suggesting a bilineal origin of such MP, which were observed only in patients with risk factors for thrombosis. Patients with essential thrombocythemia had higher plasma levels of mature von Willebrand factor (p=0.045) but similar propeptide levels compared to controls. In thrombin generation analyses, MP-rich plasma from patients with essential thrombocythemia had a shorter lag time (p=0.001) and higher peak height (p=0.038) than plasma from controls. Peak height correlated significantly with the total number of MP (R=0.634, p<0.001).

Conclusions: Patients with essential thrombocythemia had higher number of circulating MP with platelet and endothelial markers, suggesting ongoing platelet and endothelial activation. This was confirmed by an increased level of mature von Willebrand factor, an abnormal mature von Willebrand factor/propeptide ratio, and a hypercoagulable state reflected in thrombin generation. These findings suggest a role for microparticles in thrombosis in essential thrombocythemia.

Introduction

Cellular microparticles (MP) are plasma membrane vesicles of <1,5 μm in diameter, mainly composed of lipids and proteins, which are released into the circulation by blood cells and vascular cells during cellular activation or apoptosis [1]. MP are heterogeneous, differing in size, as well as in phospholipid and protein composition. In addition, MP display some specific cell surface proteins that indicate their cellular origin. Depending on the cellular process and the cellular origin triggering their formation, the outer surfaces of cellular MP may contain phosphatidylserine, which provides a suitable anionic phospholipid surface for assembly of the tenase and prothrombinase complexes, and they may express tissue factor (TF), the primary initiator of coagulation [2]. Such phosphatidylserine- and/or TF-bearing MP may contribute to the pathogenesis of thrombosis in different diseases, including cancer–associated thrombosis and sepsis [3].

Indeed, the numbers and characteristics of circulating MP have been found to be altered in many vascular diseases associated with an increased risk of both arterial and venous thrombosis. In particular, elevated numbers of platelet-derived MP have been described in diabetes mellitus [4], acute coronary syndrome [5], myocardial infarction [6] and disseminated intravascular coagulation [7]. Elevated levels of endothelial-derived MP have been found in venous thromboembolism [8] and in the antiphosholipid syndrome [9]. The majority (more than 90%) of MP in healthy controls are of platelet origin, whereas less than 10% originate from granulocytes and less than 5% from endothelial cells, red blood cells and monocytes [10].

Essential thrombocythemia (ET) is a chronic myeloproliferative disease characterized by an increased risk of both arterial and venous thrombosis. At the time of diagnosis approximately 20% of the patients with ET have had a major thrombotic event and approximately another 20% will subsequently have an event [11]. This makes venous and (more often) arterial thrombosis the leading causes of morbidity and mortality in ET. Established risk factors for thrombosis in ET are older age (over 60 years) and previous thrombotic events [12]. Recently, leukocytosis has been identified as an additional risk factor [11,13,14]. Numerous mechanisms, including blood hyperviscosity and quantitative/qualitative abnormalities of blood cells, have been advocated to be at the origin of the hypercoagulable state in these patients [15]. An increased

number of platelets [16], abnormal function of platelets [12], activation of platelets and leukocytes [17,18], their interaction to form platelet-leukocyte aggregates [17], and endothelial activation may all contribute to the increased thrombotic state in ET, which is still not completely understood.
In the present study, we investigated the numbers and the procoagulant potential of circulating MP in a group of patients with ET, using flowcytometry and a thrombin generation assay, respectively. In addition, levels of mature von Willebrand factor (vWF) and propeptide, and soluble E-selectin were determined in the same plasma samples as a measure of ongoing endothelial activation.

Design and methods

Study subjects

Twenty-one consecutive ET patients (8 males and 13 females, median age: 58, range: 34-83 years) were enrolled at the Haematology Department of Bergamo Hospital (Italy), after giving informed consent. Patients were diagnosed as having ET according to the Polycythemia Vera Study Group (PVSG) criteria [19]. All investigations were approved by the local ethical committee (Comitato di Bioetica, Ospedali Riuniti, Bergamo, Italy). The patients' characteristics are shown in Table 1. Nine patients were heterozygous carriers for the $JAK2^{V617F}$ mutation. At the time of the sample collection 18 patients were receiving treatment with aspirin, eight with hydroxyurea and three patients were not receiving any treatment. The sample size was chosen on a power calculation using data of a preceding unpublished pilot study in which the mean number of microparticles in control subjects was 3400 $x10^6$/L and that in ET patients was 7500 $x10^6$/L. With an α of 0.05 this calculation showed that nine controls and 18 ET patients would provide the analysis with sufficient power. In addition, we investigated ten healthy controls (6 males and 4 females, median age 46, range 19-59). None of the healthy controls was on antiplatelet medication at the time of blood collection. Controls were significantly younger than ET patients, p=0.015.

Blood collection and isolation of microparticles

Blood samples were drawn early in the morning, before any therapy, with a 21-gauge needle after applying a light tourniquet. After discarding the first 3 mL, blood was collected into a 5 mL tube containing 3.2 % citrate (BD, Plymouth,

UK). Plasma was prepared within 30 min after blood collection by centrifugation for 20 minutes at 1,550 x g at room temperature, without brake. Aliquots of plasma were snap-frozen in liquid nitrogen, and then stored at –75^{0}C until use. In order to isolate the MP, 250 µL of plasma was thawed on ice for 60 min and then centrifuged for 30 min at 17,570 x g at 20 °C. Subsequently, 225 µL of supernatant (i.e. microparticle-free plasma) were removed. The remaining 25 µL containing the MP pellet was resuspended in 225 µL of phosphate-buffered saline (PBS; 154 mM NaCl, 1.4 mM phosphate, pH 7.4), containing 10.9 mM trisodium citrate to prevent coagulation activation. Samples were centrifuged for 30 min at 17,570 x g at 20 °C; thereafter, 225 µL of supernatant were removed and the MP pellet was resuspended in 125 µL of PBS.

Table 1. Characteristics of study subjects at enrollment into the study. Data are presented as median (range) or number (%)

	Controls	ET patients (n=21)
Male/Female	6 /4	13/8
Age (years)	46 (19-59)	58 (34-83)
Platelets (x10^9/L)	ND*	510 (315-1340)
White blood cells (x10^9/L)	ND	7.2 (3.13-14.7)
Polymorphonuclear cells	ND	4.46 (1.71-10.2)
Hematocrit (%)	ND	40.5 (31.5-47.6)
Patients' therapy	-	18 (85%)
Aspirin	-	18 (85%)
Hydroxyurea	-	7 (38%)
History of thrombosis	-	6 (28%)

*Not determined

Phenotypic analysis of plasma-derived microparticles

Flowcytometric analysis was used to quantify and characterize plasma-derived MP, as previously described [20]. Briefly, 5 µL of MP sample were diluted in 35 µL PBS containing 2.5 mM $CaCl_2$ (pH 7.4). The samples were then incubated for 30 min at room temperature in the dark with 5 µL Annexin V-allophycocyanin (Caltag Laboratories, Burlingame, CA, USA) and/or 5 µl fluoresceinisotyocianate (FITC)-, phycoerythrin (PE)-, peridinin-chlorophyll-protein complex (PerCP)-labelled anti-human monoclonal antibodies, or 5 µL isotype-matched control monoclonal antibodies.

For the phenotypic characterization of microparticles the following cell specific monoclonal antibodies were used: anti-CD8-PE (SK1, IgG_1), anti-CD14-PE (MΦP9,

IgG2b), anti-CD15-PE (HI98, IgM), anti-CD20-PE (L27, IgG_1), anti-CD45-PerCP (H130, IgG1, k), anti-CD61-FITC (VI-PL2, IgG_1), anti-CD63-FITC (H5C6, IgG_1), anti-CD146-PE (P1H12, IgG1), labeled isotype controls IgG_1 (X40) and IgG_{2a} (X39), all from Becton Dickinson (San Jose, USA); IgG_{2b}-PE, anti-glycophorin A-FITC (JC159, IgG_1) and anti-CD41-FITC (5B12, IgG_1) from DAKO (Glostrup, Denmark); anti-CD144-FITC (BMS158FI, IgG1) from Bender MedSystems (Vienna, Austria); anti-CD106-FITC (1.G11B1, IgG1) from Calbiochem (Darmstadt, Germany); anti-CD54-PE (K562, IgG_1), anti-CD66b-FITC (80H3, $IgG_1\kappa$) and anti-CD62P-PE (CLB-Thromb/6, IgG1) from Immunotech (Marseille, France); anti-CD62E-PE (HAE-1f, IgG_1) from Kordia (Leiden, the Netherlands); anti-CD4-PE (CLB-T4/2, IgG_1) and anti-CD66acde-PE (CLB-gran/10, IH4Fc, IgG1) from Sanquin (Amsterdam, the Netherlands).

For TF measurement on MP, anti-TF-FITC from American Diagnostics (VD8, IgG_1, Stamford, CT, USA) was used in the same experimental conditions.

After incubation, 760 μL PBS/calcium buffer was added and the samples were analysed on a FACS Calibur using Worklist Manager (BD) for 1 min during which the flowcytometer analyzed approximately 55 μL of the suspension. Forward scatter (FSC) and sideward scatter (SSC) were set at logarithmic gain. To distinguish MP from events due to noise, MP were identified on the basis of their specific FSC and SSC characteristics, with gates set using *in vitro* platelet activation and microparticle generation data (results not shown), and by annexin V positivity (Figure 1A and 1B) [21]. To identify annexin V-positive events, a threshold was set in a MP sample prepared without calcium. The number of MP per liter of plasma was calculated as previously described $\#\ MP_{per\ minute} * (Volume(V)_{tube}/V_{minute}) * (V_{end}/V_{start}) * (1000/V_{labeled}) = \#$ events/mL [7]. Data were analyzed with CellQuest-pro software (Becton Dickinson).

Thrombin generation measurements

Thrombin generation in platelet-poor but MP-rich plasma as prepared for MP isolation (described above) was measured with the calibrated automated thrombogram method (Thrombinoscope BV, Maastricht, the Netherlands) [22,23]. Thrombin generation was triggered in 80 μL plasma by different conditions; 1 pM of TF and 4 μM of phospholipids, 4 μM of phospholipids alone, and buffer (no exogenous TF or phospholipids added), using reagents obtained from Thrombinoscope BV. Thrombin generation was measured as fluorescence, read in a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland) equipped

with a 390/460 filter set and thrombin generation curves were calculated with the Thrombinoscope software (Thrombinoscope BV). Three parameters were derived from the thrombin generation curves: lag time (initiation phase of coagulation), endogenous thrombin potential, and peak height. Lag time was defined as the time to reach 1/6 of the peak height. Validation of the calibrated automated thrombogram method showed normalization of non-time-dependent parameters to be mandatory to obtain acceptable inter-assay variations [23]. Intra-assay variations for normalized parameters are typically below 6%, and inter-assay variations below 8% [23]. Therefore, each thrombin generation measurement includes normal pooled plasma and both the endogenous thrombin potential and peak height values are expressed as the ratio of patients' value to the value in normal pooled plasma, expressed in percentages.

Plasma markers of endothelial activation

Plasma concentrations of soluble E-selectin were determined by a commercially available enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions (Diaclone, Cedex, France). Mature vWF and propeptide plasma levels were measured by ELISA as described previously [24]. The half-life of mature vWF is four times that of the propeptide half-life, and due to this difference in half-life their relative concentration is a distinctive indicator for ongoing chronic as opposed to acute endothelial activation [25].

Statistical analysis

We established the statistical significance of differences in MP numbers between groups with the non-parametric Mann Whitney U test and Kruskal-Wallis tests, where appropriate. Bivariate correlations were estimated by Spearman's rank correlation (R). All tests for statistical significance were two-tailed and P values of <0.05 were considered statistically significant. Analyses were performed using SPSS 15.0 for windows (SPSS Inc., Chicago, USA).

Results

Number and phenotypic characterization of microparticles

The total number of MP is significantly higher in ET patients (median: 4500 x 10^6/L) than in controls (2500x10^6/L, p=0.039). For most study subjects, more than 90% of circulating MP bound to annexin-V, indicating the presence of phosphatidylserine on their membrane (Figure 1A and 1B).

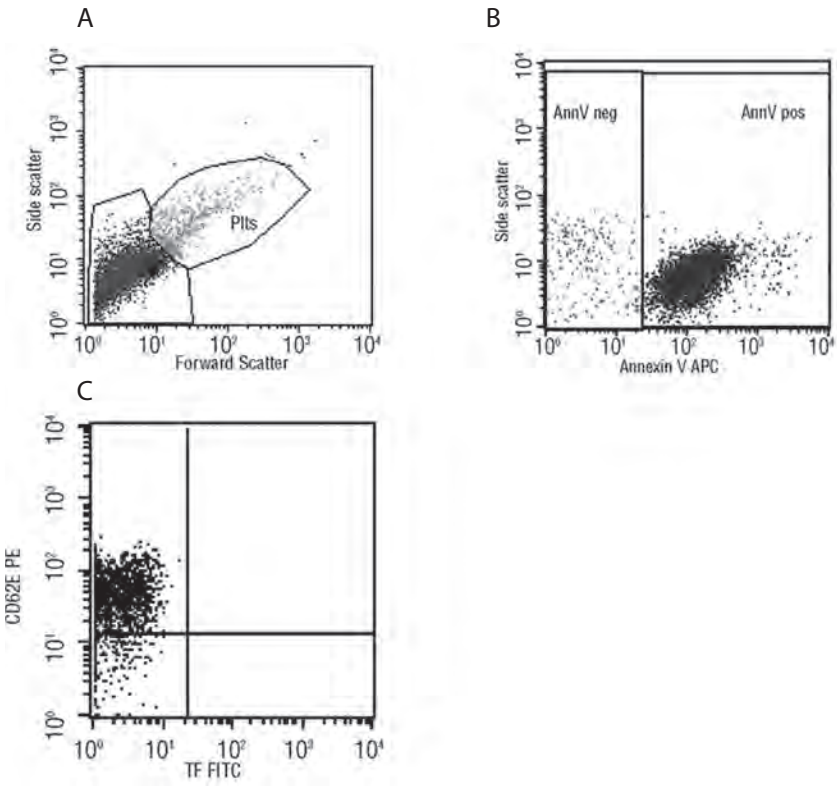

Figure 1: Flowcytometric analysis of MP. A representative set of scattergrams from flowcytometric MP analysis in a sample from an ET patient is shown to illustrate MP and subpopulation definition. Panel A: Forward and side scatter were used to define the MP and platelet (Plts) gates as shown. Panel B: Events defined as MP were then selected for their annexin V binding, determined by positivity for AnnexinV-allophycocyanin fluorescence (on the x-axis). Panel C: annexin V-positive MP were further examined for expression of other antigens by co-labelling with PE - and FITC- labelled antibodies as is shown here for CD62E-PE and TF-FITC binding.

The phenotypic characterization of MP depicted in Figure 2 showed that the subset composition of the MP population (i.e. of platelet -, leukocyte -, endothelial cell or erythrocyte origin) is similar in ET patients and controls. The large majority of MP is of platelet origin in both groups of subjects, as determined by positivity for the platelet markers CD41 and/or CD61 (CD41 ET vs. controls: median±SD 95±0,34% vs. 95,7±0,7%; p=0,15). Accordingly, the number of platelet-derived MP was greater in ET patients than in controls (CD61 median 4000 vs. 2400x10^6/L; p=0.043). The levels of the two platelet activation markers CD62P and CD63 were lower on MP from ET patients than on those from controls, and this difference was statistically significant for CD63 (median 5,5 vs. 40x10^6/L, p<0.001).

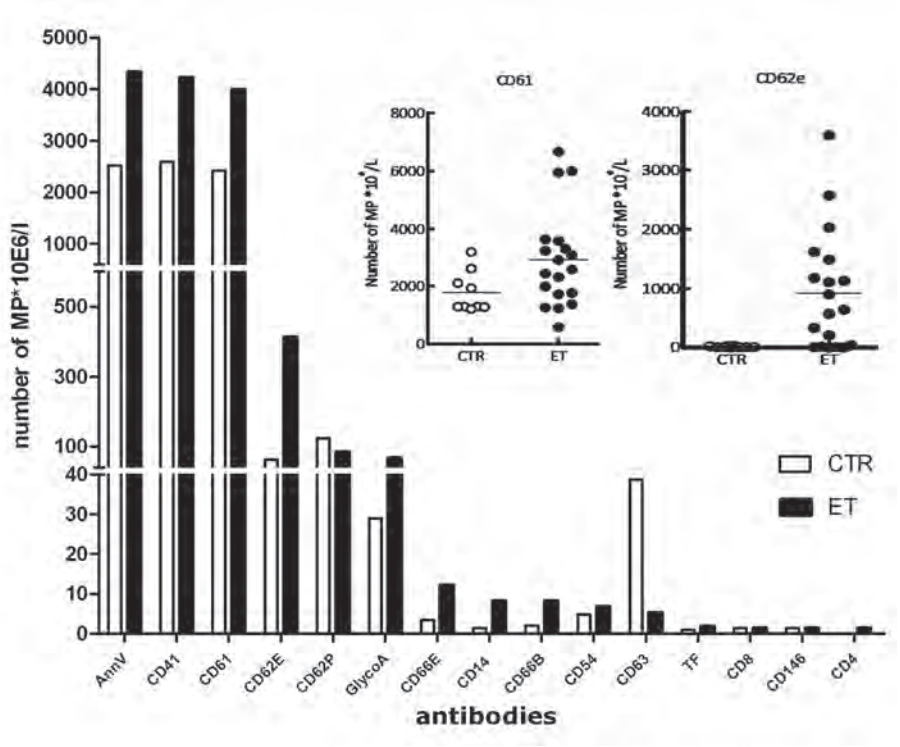

Figure 2 Size of circulating MP subpopulations in ET patients and controls. Bar graph: Number of MP from specific cellular origin as defined by marker positivity in plasma from ET patients (ET) and controls (CTR). Data are presented as medians. Numbers of MP positive for CD15, CD20, CD45, CD106 and CD144 were too low to adequately show in this graph. Inset: dotplot of the same data presented individually for the platelet marker CD61 and the endothelial marker CD62E.

With regard to endothelial-derived MP, the number of MP expressing the endothelial marker CD62E was significantly higher in ET patients than in controls (median 875 vs. $14x10^6$/L; p=0.007) (Figure 1C) as was the number expressing CD144 (p=0.021).

MP of granulocyte (CD66b and CD66acde) and of monocyte (CD14) origin were present in low but significantly higher numbers in ET patients. MP derived from T-cells (CD4 or CD8), B-cells (CD20), intracellular adhesion molecule (ICAM)-positive cells (CD54) and vascular cell adhesion molecule (VCAM)-positive cells (CD106) account for less than 1% of all MP and their numbers were not different between ET patients and controls (data not shown).

Relation of MP with the presence of JAK2^{V617F} mutation or pharmaceutical treatment

Neither JAK2^{V617F} mutation status nor the treatment given (hydroxyurea or aspirin) affected the number or the cellular origin of MP in ET patients, including TF-positive MP in ET patients (data not shown).

CD41- and CD62E- positive MP

Since more than 95% of MP were positive for CD41 and CD61, we suspected that the CD62E-positive MP, constituting 27% of all MP in ET patients, but 1% in controls, could also co-express a platelet marker. We, therefore, analysed the MP with a combination of CD41-FITC/CD62E-PE monoclonal antibodies. Indeed, in both groups 90% of the CD62E-positive MP also expressed CD41. The CD62E-positive MP constituted 24% of the CD41-positive MP in ET patients and 1% in controls. When patients with ET were classified according to a risk score, allocating one point for a history of thrombosis, age over 60 years, platelet count over 1000x10^9/L, and the presence of a cardiovascular risk factor (for example hypertension or diabetes) [26], MP with combined expression of CD41/CD62E were increased only in patients with one or more risk factors (Figure 3, p=0.045). This correlation was not observed for MP originating from other cells.

Plasma markers of endothelial cell activation

Since ET patients had higher levels of CD41/CD62E-positive MP, which suggests endothelial activation, we investigated the activation status of endothelium by measuring plasma levels of soluble E-Selectin, mature vWF and propeptide. No significant differences in the plasma levels of E-selectin were observed between ET patients (20 ng/mL, range, 5-40) and controls (14 ng/mL, range, 5-41, p=0.52). The removal of MP from plasma by centrifugation did not affect the levels of E-Selectin (data not shown), indicating that most of the soluble E-Selectin was not bound to these MP. Furthermore, no correlation was found between soluble E-selectin levels and the number of CD41/CD62E-positive MP (data not shown).

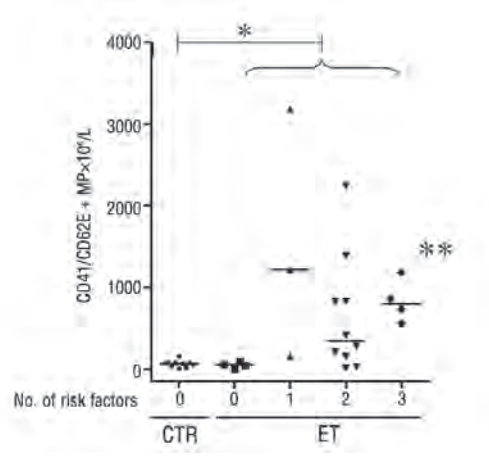

Figure 3: CD41/CD62E-positive MP and risk factors for thrombosis. Dotplot of the number and the median of CD41/CD62E-positive MP in controls and in ET patients categorized for number of risk factors (age older than 60, previous thrombotic event, platelets > 1000x10^9/l, presence of a cardiovascular risk factor [26]: 0=no risk factor, 1=one risk factor, 2= two risk factors etc.) * ET patients have significantly more CD41/CD62E-positive MP than controls (p=0.01), and ** ET patients with one or more risk factors have higher number of CD41/ CD62E-positive MP compared to ET patients without risk factors (p=0.034).

ET patients had significantly higher concentrations of mature vWF in plasma than did controls (median 50 vs. 35 nM, p=0.045) but similar concentrations of propeptide (7 vs. 5 nM, p=0,07). The mature vWF and propeptide pattern was, therefore, significantly different in patients and controls, resulting in a higher mature vWF: propeptide ratio, a pattern previously shown to indicate a state of chronic endothelial activation [25] (Figure 4). No correlation was found between mature vWF, propeptide and CD41/CD62E-positive MP.

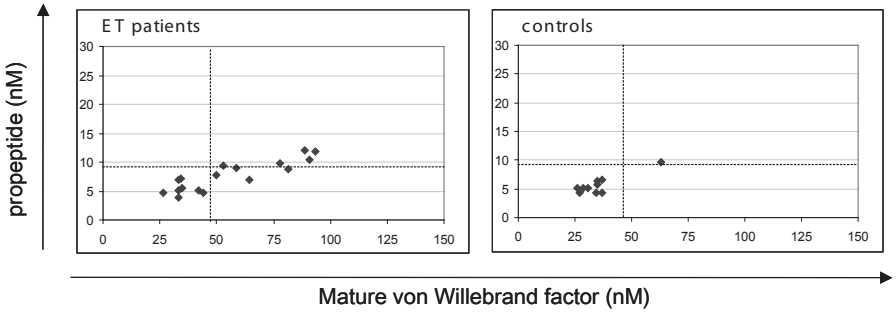

Figure 4: Relation between mature vWF and propeptide levels in patients with ET and controls. Dotted lines represent the upper limit of the 95% confidence interval of mature vWF and propeptide levels of the control group. ET patients have higher levels of mature vWF (p=0.045), and similar levels of propeptide as compared to levels in controls.

Tissue factor- positive MP

TF-positive MP accounted for less than 1% of all MP in both patients and controls. TF was expressed on MP also expressing platelet markers and CD62E. The number of MP carrying TF was significantly higher (p=0.036) in ET patients (median $1.8x10^6$/L) than in controls (median $0.9x10^6$ /L). No correlation was found between a history of thromboembolic events or positive risk score for thrombosis and TF-positive MP.

Thrombin generation

Thrombin generation triggered with 1 pM of TF and 4 μM of phospholipids was increased in MP -rich plasma from ET patients as indicated by increased peak height [ET vs. controls: 411 nM (95%CI: 358-465) and 279 nM (95%CI: 196-361), respectively, p=0.01]. No differences were found in lag time and endogenous thrombin potential, suggesting attenuated inhibition rather then altered activation or stimulation under these conditions. Since plasma-, possibly MP-, derived TF may contribute to the initiation of coagulation, thrombin generation was repeated in the absence of additional TF and only 4 μM of phospholipids were added. Again, the peak height was higher for ET patients (325 nM 95%CI: 289-360) than for controls (171 nM 95%CI: 94-249, p=0.001). Furthermore, the lag times were on average 4 min shorter for ET patients (12.0 min 95%CI: 10.7-13.4) than for controls (15.8 min 95%CI: 12.0-19.7, p=0.04). This latter observation is suggestive of the presence of more TF in plasma, and indeed maybe on MP, from ET patients than from controls. To further characterize the procoagulant potential of plasma and MP from patients with ET, the thrombin generation assay was performed in the absence of both TF and phospholipids (Figure 5). Using these conditions, ET patients had a shorter lag time (9.7 min; 95%CI: 8.7-10.7 vs. 15.9 min; 95%CI: 10.9-20.9, p=0.001), and an increased peak height (215 nM; 95%CI: 189-241 vs. 142 nM; 95%CI: 87-189, p=0.038), indicating endogenous presence of procoagulant phospholipids and TF, possibly provided by MP. Indeed, a negative correlation was found between the total number of MP and lag time (R= -0.379, p=0.039) and a positive correlation between the total number of MP and peak height (R=0.634, p<0.001) (Figure 6). Finally, after the removal of MP by centrifugation there was no activation of thrombin generation (data not shown), which is compatible with the absence of MP-derived TF and phospholipids.

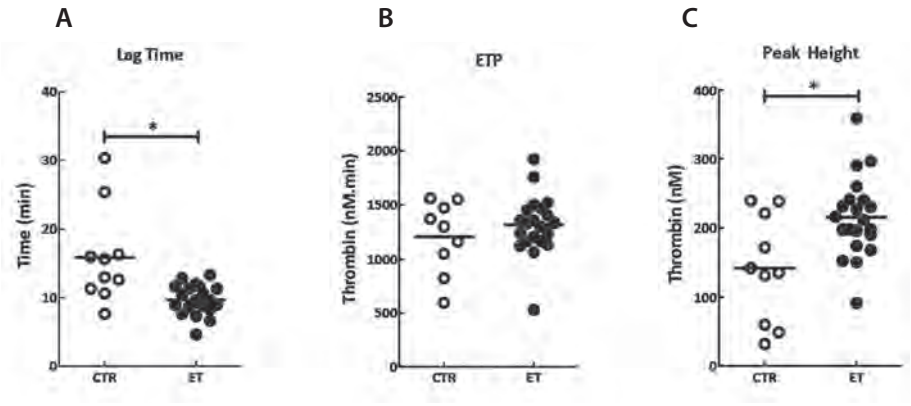

Figure 5: Thrombin generation in the absence of exogenous TF and phospholipids in platelet-poor, MP -rich plasma

The results of the parameters lag time (A), endogenous thrombin potential (ETP) (B) and peak height (C) are shown for ET patients (•) and controls (CTR) (◦). Lines represent the median value. ET patients have a significantly shorter lag time and a higher peak height compared to controls. *p<0.05.

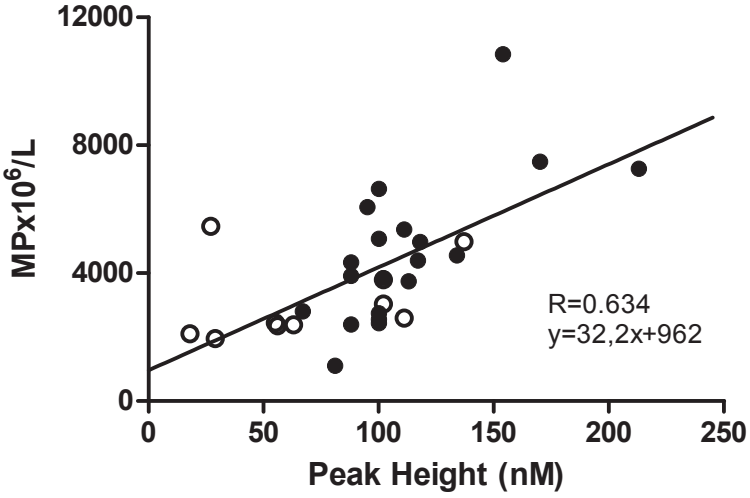

Figure 6: Correlation between the peak height of thrombin generation in the absence of exogenous TF and phospholipids, and the total number of MP.

Data are shown from MP-rich plasma of ET patients (•) and controls (◦). R=0.634, p<0.001.

Discussion

This is the first extensive analysis of MP and their cellular origin in patients with ET. Like patients with other thromboembolic diseases, ET patients show higher levels of platelet-derived MP than healthy subjects [4,5,27]. This is not

necessarily attributable to higher numbers of platelets because neither in ET patients nor in controls did platelet-derived MP numbers correlate with the platelet numbers. This lack of correlation was also observed in an earlier study [28], suggesting that MP formation may be a regulated rather than a constitutive process. In spite of the large proportion of platelet-derived microparticles in ET patients, the actual number of these MP with markers of platelet activation (CD62P and CD63) was not increased. This could be because most ET patients were on anti-thrombotic drugs at the time of blood sampling, which may have affected markers of platelet activation; aspirin inhibits the expression of CD62P and CD63 on platelets [29].

Remarkably, half of the ET patients showed a large increase in CD62E-positive MP. These CD62E-positive MP are not normal endothelial MP since they co-expressed CD41, a platelet marker, a finding that we did not observe in other conditions characterized by endothelial perturbation, including diabetes mellitus [20] and renal failure (data not shown). Chirinos *et al*. [8] described marked elevations of CD62E-positive MP in patients with venous thromboembolism, but co-expression with platelet markers was not investigated. CD62E, or E-selectin, is an adhesion molecule that mediates contact between endothelial cells and other cells, including platelets. Normal resting endothelial cells do not express E-selectin, but a soluble form of this molecule is released from activated cells [30]. The presence of CD62E-positive MP suggests endothelial activation, a finding substantiated by the higher levels of mature vWF in ET. The observation that this was not accompanied by a rise in propeptide levels suggests that the endothelial activation is chronic rather than acute in nature [25].

However, these CD62E-positive MP co-express CD41, a platelet marker. CD62E was not observed on platelets from controls or ET patients (data not shown). An explanation for this double positivity could be interaction between platelets (or platelet fragments) and endothelial cells resulting in cellular activation and generation of MP of bilineal origin. Circulating MP with characteristics of two distinct cell populations have been described, and substantiated by confocal immunofluorescence microscopy [10]. Membrane transfer from MP to cells resulting in expression of cell lineage-unrelated receptors is also a recognized phenomenon [31,32]. In these cases MP express antibodies of both original cell types, thereby showing that they are the result of direct or indirect cell-cell contact, in this case endothelial cell/platelet activation. It is also conceivable that MP acquire CD62E during their formation via expression by the MP source, the

activated platelet. Passive adherence of CD62E was considered as an alternative explanation. However, we consider this unlikely since there was no relation between MP CD62E expression and soluble E-selectin levels and removal of the MP from plasma by centrifugation did not affect soluble E-selectin levels, indicating a very low quantity of CD62E on MP compared to soluble E-selectin in the plasma of patients as well as controls. A low quantity of CD62E on MP compared to soluble E-selectin in the plasma was shown previously for septic patients [33].

Increased numbers of CD41/CD62E-positive MP may be of pathophysiological significance since they appear to be related to risk factors for thrombosis in ET. A relation between these MP and actual thrombosis was not apparent in our present study that was not designed to detect such a relation, and had a limited sample size and short follow-up.

Higher numbers of CD66acde and CD66b-positive MP in ET are likely to be related to granulocyte activation in this condition [34]. The level of TF- positive MP was increased for ET patients, but such MP accounted for less than 1% of all MP in this study, and it is unclear whether this difference is clinically relevant. In this respect, the absence of a correlation between TF-positive MP and clinical parameters, such as a history of thromboembolic events or a positive risk score for thrombosis, may be primarily due to a lack of power. TF expression on platelets is related with the presence of a *JAK2*^{V617F} mutation [34], but a correlation was not observed in our study between TF-positive MP and the presence of a *JAK2*^{V617F} mutation.

A limitation of this study is that the ages of the control subjects and patients differed significantly. However, we did not find a correlation between older age and MP in our study, and such a correlation has not been described in other studies [35].

The thrombin generation measurements showed an increased peak height for assays with 1 pM of TF and 4 μM of phospholipids, which is compatible with a hypercoagulable state in ET patients [36]. The shorter lag times observed for ET patients whose assays were conducted without additional TF and phospholipids provide evidence for the presence of a procoagulant factor, possibly on the membrane of MP, in the plasmas of these patients. We suggest that the differences found in thrombin generation are due to MP, since removal of phospholipids abolished thrombin generation. This result is compatible with the observation from Pereira *et al.* [37] that thrombin generation is largely dependent on the

number of MP in plasma, the only available phospholipid source in such a system. We found a correlation between peak height and the total number of MP, as well as for lag time and MP under these conditions, suggesting that phospholipids from MP and intrinsic or extrinsic (TF) coagulation activators in plasma and/or on the membrane of MP do indeed account for the observed differences that were abolished after removal of MP by centrifugation. In the light of the previously described evidence of the presence of non- MP bound functional TF in human plasma [38], future studies should address the question of whether a particular form of plasma TF contributes to the observed differences between MP-rich ET plasma and normal plasma.

In conclusion, ET patients have higher numbers of MP expressing platelet and endothelial markers, suggesting ongoing endothelial activation. This is confirmed by a signature of chronic endothelial activation given by an elevated level of mature vWF in the presence of a relatively low propeptide. MP from patients with ET are associated with increased thrombin generation, shortened lag time and increased peak height. CD41/CD62E-positive MP are elevated only in ET patients with risk factors for thrombosis. These findings suggest a role for MP in thrombosis in ET and this deserves further prospective studies.

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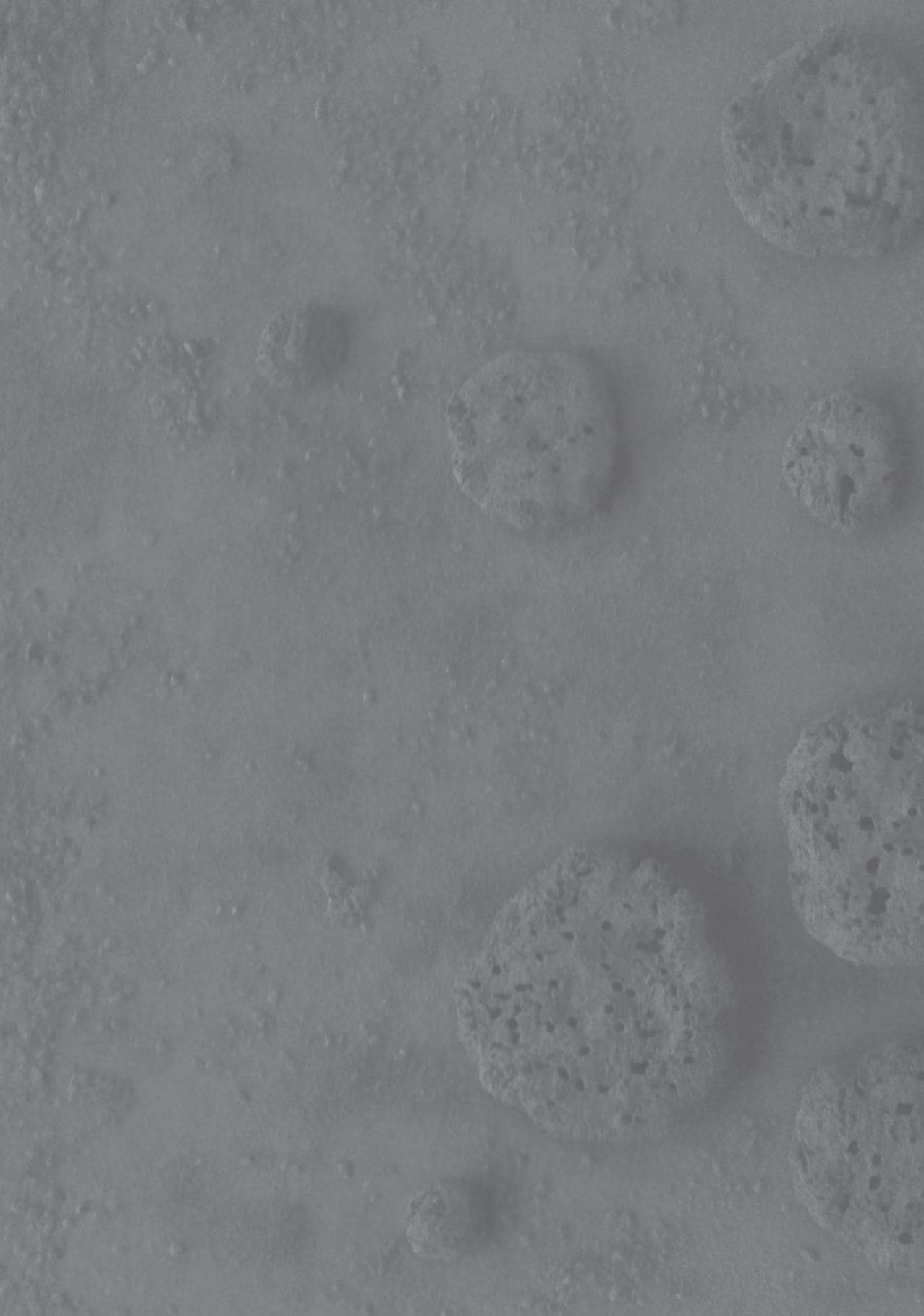

Chapter 3

Increased numbers of microparticles, with the ability to induce thrombin generation, in essential thrombocythemia and polycythemia vera

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Submitted

Abstract

Background: Essential thrombocythemia (ET) and polycythemia vera (PV) are complicated by thrombotic events. In this study we compare microparticle (MP) numbers, phenotypic profiles and MP-dependent thrombin generation between ET and PV patients, and with healthy controls.

Materials and methods: In plasma samples from 18 ET patients, 24 PV patients and 20 controls, the levels and cellular origin of MP were determined by flowcytometry and MP-dependent thrombin generation by an adapted ETP assay.

Results: ET patients, but not PV patients, had higher numbers of platelet-derived MP (CD41+) than controls (median: ET 9000, PV 5970, controls 4100 $x10^6$/L; $p<0.001$), not clearly related to the thrombocyte count. Numbers of leukocyte-derived MP (CD45, CD11b, PSGL-1+) were higher in PV patients than in ET patients and controls, and correlated with the leukocyte count ($p<0.001$) and $JAK2^{V617F}$status. MP expressing the endothelial marker CD62E were highly abundant in ET and moderately increased in PV compared with controls (median: ET 2975, PV 324, controls $80x10^6$/L; $p<0.001$ and $p=0.02$ respectively). Related to their MP numbers, ET patients had a higher MP-dependent endogenous thrombin potential (ETP) than controls (median: ET 377, PV 340, controls 282 milliAbsorbance; $p<0.01$).

Conclusions: ET and PV patients had elevated numbers of MP with phenotypic profiles reflecting different degrees of platelet, endothelium and leukocyte ancestry. MP attributed to hypercoagulability in patients as the MP-dependent thrombin generation was highest for ET patients. The MP-specific endogenous thrombin potential, however, was equally proportional to ET, PV and controls suggesting similar procoagulant properties.

Introduction

Essential thrombocythemia (ET) and polycythemia vera (PV) are both myeloproliferative neoplasms (MPN). MPN are associated with a high risk of thromboembolic events, 20 % of patients had a major arterial or venous thrombosis at time of diagnosis, and another 20 % will have an event during follow-up [1]. The pathogenesis of thrombosis in MPN is ill understood. Currently accepted risk factors for thrombosis are age >60 years and previous thrombotic events [2,3]. Other suggested risk factors are leukocytosis and $JAK2^{V617F}$ mutational status [4,5]. Leukocytosis has been related to thrombosis in several retrospective analyses [1,6], although its value in predicting future thrombosis remains under debate [7]. In addition, a positive $JAK2^{V617F}$ mutation status correlated with an increased risk of thrombosis [8]. Patients with the $JAK2^{V617F}$ mutation [9] have elevated levels of activated polymorphonuclear cells [10] and neutrophil-platelet aggregates. Such aggregates form through interaction of membrane adhesion molecules such as CD11b and PSGL-1 on neutrophils and GP1b (CD42b) and P-selectin (CD62P) on platelets and have been related to the risk of thrombosis [11-13]. Furthermore, several alterations in expression of platelet adhesive molecules such as CD42b, GpIIb/IIIa (CD41/CD61) and CD62P have been described in these diseases, also possibly contributing to altered haemostasis [14,15].

These activation markers involved in coagulation in MPN can be expressed by blood cells, but by microparticles (MP) as well. MP are small (0.1-1.0 μm) membrane vesicles shed by cells upon activation or apoptosis. They mainly mirror their parent cell's membrane and cytoplasmic composition. Most MP are platelet-derived, defined by CD41-positivity. Especially interesting in patients with ET is the observation made by Flaumenhaft *et al*. that these CD41-positive MP could be derived from megakaryocytes as well [16]. MP are active in coagulation through several mechanisms and are not just a passive by-product. MP expressing phosphatidylserine, tissue factor (TF), P-selectin and PSGL-1 were shown to be involved in several mechanisms resulting in coagulation [17].

MP are increased in thrombotic diseases and involved in thrombosis [18,19]. In addition, MP induce thrombin generation and in previous studies, MPN patients had increased thrombin generation [20]. Earlier we observed elevated numbers

of MP in patients with ET and MP-dependent thrombin generation was shown to closely correlate to the numbers of MP [19]. In PV however, MP have not yet been assessed.

ET is characterized by an overproduction of mature cells, predominantly of the megakaryocytic lineage and PV by cells of the erythroid lineage. Therefore we determined whether the spectrum of origin of MP (red blood cells, platelets, leukocytes and polymorphonuclear or endothelial cells) differed between patients with ET and patients with PV and to compare with the spectrum in healthy controls. In this study we used flowcytometry to assess the number and phenotypic characteristics of MP in patients with ET and PV. In addition, levels of mature von Willebrand factor (vWF) and propeptide were determined as a measure of ongoing endothelial activation. To gain insight into the interplay between MP phenotypic characteristics and direct MP procoagulant properties, we measured the endogenous thrombin generation potential of MP in these groups, using an adapted MP-dependent thrombin generation assay.

Materials and Methods

Study subjects

After giving written informed consent, 18 consecutive patients with ET (8 males and 10 females, median age: 63, range 36-85 years) and 24 consecutive patients with PV (14 males and 10 females, median age: 60, range 39-92 years) were enrolled at the haematology department of the Onze Lieve Vrouwe Gasthuis in Amsterdam (The Netherlands). Patients were diagnosed as having ET or PV according to the revisited WHO criteria [21,22]. All investigations were approved by the local medical ethical committee (Medisch Ethische Commissie, Onze Lieve Vrouwe Gasthuis, Amsterdam, The Netherlands). Patients' characteristics are shown in Table 1. Eight patients with ET (44%) and 21 patients with PV (88%) were diagnosed with the $JAK2^{V617F}$ mutation. Seven patients with ET (39%) and 6 patients with PV (25%) had a history of vascular thrombotic events. At the time of the sample collection, all patients with ET and 22 patients with PV were treated with aspirin, nine ET and seven PV patients were treated with hydroxyurea, and one patient with PV did not receive any medical treatment. All patients with PV were treated with phlebotomy. In addition we investigated

20 healthy controls (13 males and 7 females, median age: 51, range 22-69 years). None of the healthy controls were on antiplatelet medication shortly before or at the time of blood collection. Controls were significantly younger than ET and PV patients ($p<0.05$ for both). Differences in the male to female ratios were not significant between patients and controls.

Isolation and phenotypic analysis of MP

Blood sampling and MP isolation were performed as previously described [19]. Flowcytometric analysis was used to quantify and characterize plasma-derived MP. Briefly, 5 µL of MP sample was diluted in 30 µL phosphate-buffered saline (PBS; 154 mM NaCl, 1.4 mM phosphate, pH 7.4). The samples were then incubated at room temperature in the dark for 30 min with 5 µL of diluted Annexin-V-allophycocyanin (Caltag Laboratories, Burlingame, CA, USA), 5 µL of diluted Mouse IgG1 Pure (BD Biosciences, San Jose, CA, USA) and 5 µL fluoresceinisothiocyanate (FITC)- and phycoerythrin (PE)-labeled anti-human monoclonal antibodies.

Table 1: Characteristics of study subjects. Data are presented as median (range) or number(%)

	Controls (n=20)	PV patients (n=24)	ET patients (n=18)
Male/Female ratio	13/7	14/10	8/10
Age	51 (22-69)	60 (39-92)	63 (36-85)
Presence of $JAK2^{V617F}$ mutation	nd	21 (88%)	8 (44%)
Platelets ($x10^9$/L)	237 (147-377)	369 (119-1495)*	519 (198-1945)*#
Leukocytes ($x10^9$/L)	5.3 (4.3-9.4)	11.8 (2.4-26.9)*§	6.5 (1.8-23.8)
Hematocrit (%)	0.43 (0.34-0.49)	0.44 (0.32-0.53) §	0.38 (0.19-0.47)
F1F2 (pmol/L)	128 (71-300)	180 (32-304)	255 (119-746)*
TAT (µg/L)	4.4 (1.5-14.6)	4.7 (2-15)	5.5 (2.4-43.5)
Mature vWF antigen (nM)	46 (27-102)	49 (32-65)	52 (33-198)
Propeptide (nM)	4.5 (2.7-8.9)	4.8 (3.1-7.7)	4.4 (3.0-12.4)
Aspirin	-	22 (92%)	18 (100%)
Hydroxyurea	-	7 (29%)	9 (50%)
Phlebotomy	-	24 (100%)	-
History of thrombosis	-	7 (29%)	6 (33%)

Nd= not determined *$p<0.05$ vs. controls, §$p<0.05$ vs. ET patients, #$p<0.05$ vs. PV patients

For the phenotypic characterization of MP the following cell specific monoclonal antibodies were used: anti-glycophorin-A-PE (JC159, IgG_1) and anti-CD41-FITC (5B12, IgG_1) from Dako (Glostrup, Denmark); mouse IgG_1-PE (X40); mouse IgG_1-FITC (X40), anti-CD11b-PE (ICRF44, $IgG_{1\,\kappa}$), anti-CD31-PE (WM-59, IgG_1), anti-CD36-PE (CB38, IgM κ), anti-CD41-FITC (MWReg30, $IgG_{1\,\kappa}$), anti-CD42b-PE (HIP1, IgG_1), anti-CD45-FITC (HI30, IgG_1), anti-CD71-FITC (M-A712, IgG_{2a}) and anti-CD162-PE (KPL-1, Mouse IgG_1) from BD (San Jose, USA); anti-VEGF R2-PE (89106, IgG_1) from R&D systems (Abingdon, UK); anti-CD54-PE (84H10, IgG_1), anti-CD62P-PE (CLB-Thromb/6, IgG_1) and anti-CD66b-PE (80H3, IgG_1) from Immunotech (Marseille, France); anti-CD62E-PE (HAE-1f, IgG_1) from Ancell (Bayport, USA); anti-CD144-FITC (BMS158FI, IgG_1) from Bender MedSystems (Vienna, Austria); anti-CD142-FITC (VD8, IgG_1) from American Diagnostics (Stamford, USA); anti-CD105-FITC (MEM-226, IgG_{2a}) from antibodies-online.com (Aachen, Germany); anti-CD239-FITC (BRIC221, IgG_{2b}) from Santa Cruz Biotechnologies (Heidelberg, Germany); anti-CD66a,c,d and e-PE (CD66e, CLB-gran/10, IH4Fc, IgG_1) from Sanquin reagents (Amsterdam, The Netherlands).

After incubation, 360 µL PBS/calcium buffer was added and the samples were analysed on a FACS Calibur using Worklist Manager (BD) for 30 seconds during which the flowcytometer analysed approximately 30 µL of the suspension. Forward scatter (FSC) and sideward scatter (SSC) were set at logarithmic gain. To distinguish MP from events due to noise, MP were identified on the basis of their specific FSC and SSC characteristics, with gates set using in vitro platelet activation and MP generation data (results not shown), and Annexin V-positivity. Variations in FACS Calibur sensitivity due to maintenance and altered settings of the FACS may explain the differences in numbers of MP in patients with ET and controls in this study and in the previous study [19]. To identify Annexin V-positive events, a threshold was placed in a MP sample prepared without calcium. The number of MP/L plasma was calculated as previously described [23]. Data were analysed with CellQuest-pro software (BD).

Markers of coagulation and endothelial activation

Mature von Willebrand Factor and propeptide plasma levels were measured by ELISA as described previously [24]. Coagulation activation was assayed by measuring the concentration of prothrombin fragments 1+2 (F1+2) and thrombin antithrombin complex (TAT). F1+2 levels were determined using

the Enzygnost F1+2 (monoclonal) ELISA kit (Siemens Healthcare Diagnostics Deerfield, USA). TAT was determined using the Enzygnost TAT micro testkit.

Analysis of MP-dependent thrombin generation

To assess MP-dependent thrombin generation we adapted the Siemens endogenous thrombin potential (ETP) assay on a BCS-XP analyser (Siemens Healthcare Diagnostics, Marburg, Germany). Measurements lasted 30 min and the read interval was 0,5 seconds. The reaction was initiated using 30 times diluted Actin FS (Siemens) instead of Innovin (Siemens), thus reducing the added amount of phospholipids and not providing reagent-derived TF in the ETP reaction mixture. To exclude the role of soluble plasma-derived factors, thrombin generation was determined after addition of washed MP to MP-poor normal human pooled plasma. MP were isolated as described for FACS analysis [19]. To obtain MP-poor pooled plasma, fresh MP-plasma from healthy controls was submitted to a second centrifugation step at 1,550 x g for 20 min at 20˚C and the top layer of the plasma was centrifuged through a 0,2 µm filter at 3,000 x g for 5 min in a Vivaspin 20 tube (Sartorius Stedim, Goettingen, Germany) after which the plasma was stored at -80˚C until use. The thrombin generation curves were calculated with Curves (v 1.0 spec. 3.2, Dade Behring). Three parameters from the thrombin generation curves were analysed: ETP (Endogenous Thrombin Potential, or area under the curve, that represents the total amount of thrombin generation), lag time (time from onset of the reaction to start of thrombin generation) and peak height (represents the maximum speed of thrombin generation). Linear dose-response relations between MP numbers derived from normal pooled plasma and the ETP-parameters were observed within certain limits of numbers of MP added (data not shown). Therefore, ETP was determined in three different MP dilutions (2x, 5x and 20x diluted) in MP-poor pooled plasma for each subject.

Analysis of MP-specific endogenous thrombin potential

In order to determine the procoagulant properties of the MP population, we calculated ETP per MP (MP-specific ETP). Since the ETP assay depends on a minimum number of MP for thrombin generation to occur, and ETP values reached a maximum (plateau phase) when large amounts of MP were added to the reaction, we determined test limits for these calculations. Within these limits ETP values showed a linear correlation with the number of MPs of normal

pooled plasma added. The linear phase of the ETP values for this particular normal MP-poor pooled plasma was 170-350 milliAbsorbance (mA) (data not shown). MP-specific ETP was calculated by calculating (ETP of dilution/number of MP in dilution) x 1000 = ETP per 1000/MP. During each analytical session ETP values were corrected for the background ETP of the MP-poor pooled plasma, which in general was negligible.

Results

Number and phenotypic characterization of MP

The total number of MP was highest in patients with ET (median: ET 9050, PV 6125 and controls 4240x10^6/L), and differed significantly from controls, $p<0.001$. The mean fluorescence intensity of Annexin V did not differ between the groups. The phenotypic characterization of MP is shown in Table 2. The large majority (>95%) of MP was platelet-derived in all groups, as determined by positivity for platelet marker CD41 (GpIIb). Patients with ET, but not patients with PV, had significantly higher numbers of CD41-positive MP than controls (median: ET 9000, PV 5970 and controls 4100x10^6/L, $p<0.001$).

With regard to the platelet activation marker CD62P, patients with ET showed the highest absolute numbers of MP expressing CD62P (median ET 291, PV 252 and controls 146x10^6MP/L) without reaching significance. When looking at the relative numbers, patients with PV had the highest percentage of CD62P-positive MP compared with both ET patients and controls (median ET 3.4, PV 4.8 and controls 3.7%).
Patients with ET had the highest number of CD42b-positive MP (ET 1700, PV 1100 and controls 1000x10^6/L) without reaching significant differences.

Numbers of leukocyte-derived MP (CD45-positive) were higher in both ET and PV patients compared with controls, being highest in patients with PV (median: ET 77, PV 112 and controls 21x10^6/L; $p<0.001$; $p<0.001$). MP expressing the granulocyte-marker CD66e were observed in higher numbers in ET and PV patients (median ET 65, PV 145 and controls 5x10^6/l; $p<0.001$; $p<0.001$) compared with controls.

Table 2: Number of MP from specific cellular origin as defined by marker positivity in plasma from ET and PV patients and controls. Data are presented as median (interquartile range) x 10^6/L and median percentage.

	Controls	*PV patients*	*ET*
AnnexinV+ MP	4240 (2630-6480)	6125 (3050-11030)	9050 (5420-11760)*
CD41+ MP	4100 (2470-6340)	5970 (2920-10940)	9000 (5370-11690)*
	97.8	98.2	99.2
CD62P+ MP	146 (60-249)	252 (109-436)	291 (140-466)
	3.7	4.8	3.4
CD42b + MP	1000 (620-2500)	1100 (540-1900)	1700 (1100-3000)
	23.3	16.9	20.8
CD45+ MP	21 (14-54)	112 (64-174)*	77 (40-174)*
	0.7	2.2	1.0
CD11b + MP	30 (16-48)	77 (77-187)* #	32 (25-72)
	0.8	1.9	0.4
CD66a,c,de, + MP	8 (5-16)	145 (26-478)*	65 (26-211)*
	<1	3.6	<1
PSGL-1+ MP	131 (98-176)	235 (163-361)* #	167 (76-220)
	3.1	4.6	2.0
CD62E	80 (34-211)	324 (64-2680)*	2970 (146-7300)*
	2.8	21	51
glycoA	96 (66-145)	273 (87-972)*	508 (50-1150)
	2.1	6.6	8.4
CD71	0 (0-1)	21 (10-57)* #	5 (0-29)*
	<1	<1	<1
tissue factor	2 (0-10)	6 (2-9)	6 (4-21)
	<1	<1	<1

*p<0.05 compared with controls, #p<0.05 compared with ET patients

MP expressing the myeloid adhesion molecule CD11b were found in higher numbers in PV patients than in ET patients and controls (median: ET 30, PV 77 and controls 32x10^6/L, p=0.02 and p<0.001, Figure 1, Figure 2C). In addition, the number of MP expressing PSGL-1 was increased in PV patients as well (median: ET 167, PV 235 and controls 131x10^6/L; p=0.01; p=0.002)(Figure 1).

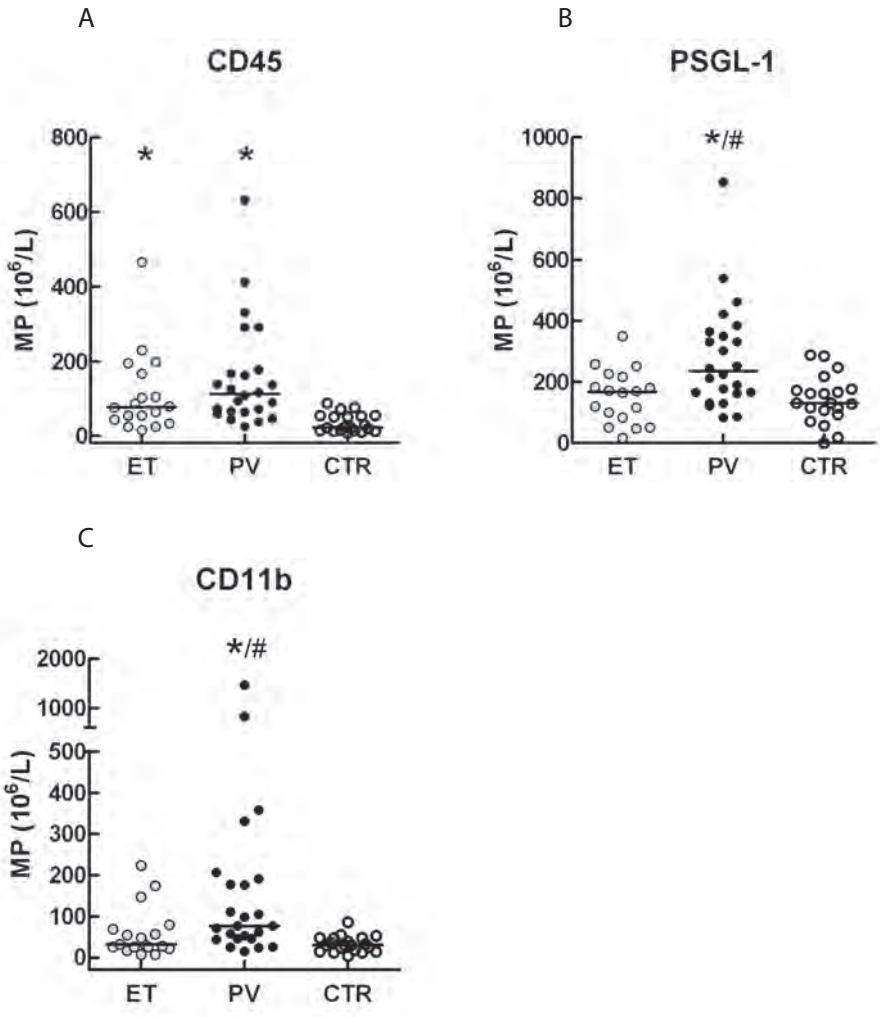

Figure 1: Number of MP expressing leukocyte markers CD45, CD11b and PSGL-1 in ET and PV patients and controls (CTR). Data are expressed individually, lines represents the medians. Both patients with ET and PV have more CD45-positive MP than controls. With regard to leukocyte-activation markers PSGL-1 and CD11b, these markers were expressed in higher numbers in PV patients compared with ET patients and controls.*$p<0.05$ compared with controls. #$p<0.05$ compared with ET patients.

Overall the expression of TF on MP was low. In each group the percentage of TF- positive MP was less than 1%. Despite the low numbers, ET patients had significantly more TF-positive MP than controls, $p<0.05$. TF was co-expressed with PSGL-1, with PSGL-1 positive MP co-expressing TF in up to 32% (Figure 2D).

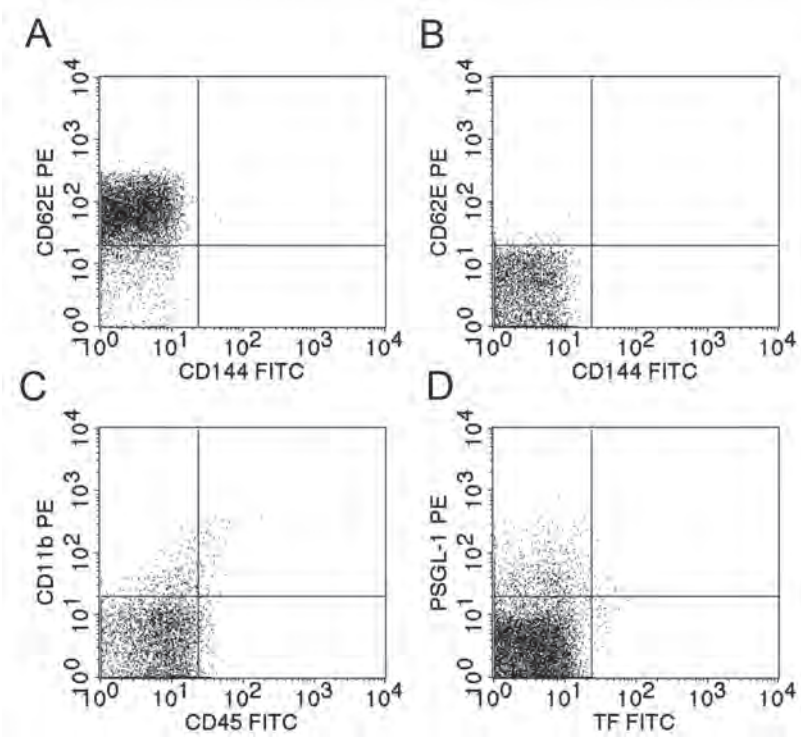

Figure 2: Scattergrams of MP expressing different antibodies. CD62E-positivity was often present in patients as is shown on the y-axis (A) however, in some patients completely absent(B), as it was in controls, expression of CD11b (C) and PSGL-1 (D) was clearly visible, however, TF-positivity was weak (D, x-axis).

With regard to endothelial-derived MP, a significant increase of MP expressing the endothelial marker CD62E (median: ET 2975, PV 324, controls 80x10^6/L) was observed in ET and PV patients compared with controls ($p<0.001$ and $p=0.02$ respectively, expression shown in figure 2A and 2B). Other endothelial cell markers CD144, CD105 and CD54 were expressed on <1% of MP and did not differ between the groups.

With regard to the erythrocyte-derived MP, these were higher in patients with PV than in controls (median: ET 508, PV 273, controls 96x10^6/L, $p=0.01$). ET patients had the highest median, however probably due to the wide range of values, this did not result in a significant increase compared with controls. Expression of erythrocyte transferring receptor CD71, which could be used as a marker for reticulocytes [25], was higher in PV patients compared with ET

patients and controls (median ET 5, PV 21 and controls 0x10^6/L, p=0.02 and p<0.001, respectively)

There was a weak correlation between leukocyte count and leukocyte-derived MP (CD45+) in patients with PV and ET (R= 0.45, p=0.03 and R=0.49, p=0.04 respectively). This correlation was absent in controls (R=-0.21, p=0.37). In addition, platelet count correlated strongly with platelet-derived MP in PV patients (R= 0.59, p=0.005), this correlation was weaker for ET patients (R=0.51, p=0.03) and again was absent in controls (R= 0.26, p=0.26).

Relation of MP with presence of JAK2^{V617F} mutation or pharmaceutical treatment

Compared with patients with a negative JAK2^{V617F}mutational status (n=13), patients with a positive JAK2^{V617F} mutation status (n=29) not only had a higher number of leukocytes (median: 11.8 vs. 6.5x10^9/L, p<0.001) but also an increased number of MP expressing leukocyte makers CD45 (median: 116 vs. 43x10^6/L, p=0.001, Figure 3) and CD11b (median 78 vs. 26x10^6/l, p=0.002). The mutational status did not correlate with number of CD62E-positive MP or thrombin generation results. Treatment with hydroxyurea did not result in differences in number of leukocytes at the time of blood sampling, numbers of MP, or in thrombin generation results. The impact of aspirin on the above mentioned parameters is unknown as all but two patients used aspirin.

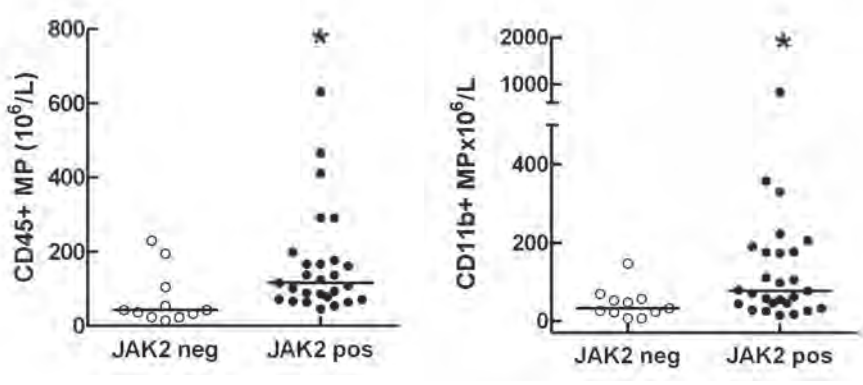

Figure 3: Dotplots of leukocyte-derived MP (CD45 and CD11b) and JAK2 mutational status. Lines represent the median value. JAK2 positive patients have more leukocyte-derived MP than those with a JAK2 negative status. *p<0.05

Markers of coagulation and endothelial activation

No differences were observed in levels of mature von Willebrand Factor and its propeptide between groups (Table 1). The ratio in all groups was compatible with chronic as opposed to acute endothelial activation [19,26]. With regard to markers of coagulation activation, results of thrombin-antithrombin complexes (TAT) did not differ between patients and controls. F1F2 was higher in ET patients compared with controls (median: ET 255, PV 180 and controls 128 pmol/L; p<0.001, Table1).

MP- dependent thrombin generation

One PV patient was excluded from ETP analysis because the ETP values of MP-dilutions showed that the MP pellet could not be properly resuspended. As observed before [19], MP-dependent thrombin generation of ET patients was higher than controls since higher ETP and peak height and lower lag times were observed (p=0,001, p=0,001 and p=0,12 (NS) respectively, Table 3). In contrast, ETP of MP-dependent thrombin generation of PV patients was slightly increased and therefore values were intermediate between ET patients and control values, without significant differences (Figure 4A). Median (IQR) ETP values were: ET 377 (312-392), PV 340 (250-376) and controls 282 (213-347) mA, Table 3).

Table 3: Thrombin generation (TG) results in 2x dilution. Data presented in median (IQR)

	controls	PV patients	ET patients
MP-dependent TG			
Lag time (sec)	693 (660-768)	680 (635-760)	665 (608-710)
Peak Height (mA/min)	37 (29-48)	46 (35-57)	52 (43-58)*
ETP (mA)	282 (213-347)	340 (250-376)	377 (312-392)*
MP-specific TG			
ETP/1000MP	1.54	1.80	1.41

*p<0.05 versus controls

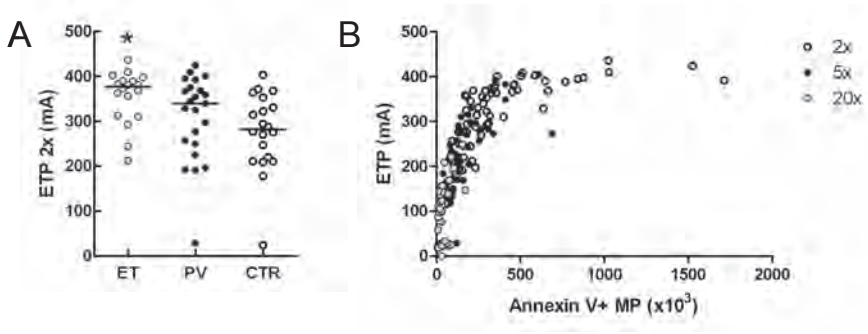

Figure 4 A: endogenous thrombin potential (ETP) results of isolated MP (2 times diluted) of patients and controls (CTR) added to human pooled plasma. Lines represent the median value. ETP was higher in patients, especially ET patients had a higher ETP than controls, *p<0.05. B: Correlation between total numbers of MP and ETP in different dilutions (2x, 5x and 20x diluted).

MP-specific thrombin generation

In general, ETP values (figure 4B), as well as peak height, correlated with the total number of MP (R^2=0.6, p<0.001 and R^2=0.6, p<0.001, respectively). To further investigate qualitative functional differences in MP, MP-specific thrombin generation (i.e. thrombin generation per MP) was determined. In MP-specific thrombin generation the ETP has been corrected for MP numbers. No differences were observed between the groups concerning MP-specific thrombin generation (median: ET 1.41, PV 1.80 and controls 1.54 ETP per 1000 MP), indicating that no overall qualitative functional MP differences were present in patients compared with controls. However only in patients, mostly PV patients, an ETP/MP ratio above 2.5 was detected.

MP and thrombin generation in high risk patients for thrombosis

No correlations were observed between ETP values and established thrombosis risk factors (age >60 and previous thrombosis), MP numbers or MP phenotypic characteristics.

Discussion

Patients with ET and PV had elevated numbers of MP with phenotypic profiles reflecting different proportions of platelet, endothelium or leukocyte ancestry. PV patients differed from ET patients only regarding numbers of leukocyte-derived MP, being higher in PV. Furthermore thrombin generation was increased in ET patients, which was dependent on the increased number of MP in these patients and could not be related to a functional difference in MP properties. A high clinical risk of thrombosis by established risk factors did not correlate with the afore mentioned parameters.

ET patients had more platelet-derived MP than controls. In concordance with our previous results the vast majority of MP was derived from platelets [19]. Indeed, CD41-positive MP could in part be megakaryocyte-derived as well [16,27] which would especially be important in ET, and might explain why the correlation between platelets and platelet-derived MP in ET was weaker than in PV. However, this correlation was absent in controls.

Similar to our previous results [19], abundant CD62E expression was again found on MP in patients with ET, and moderately increased on MP in patients with PV. Again, CD62E was co-expressed with CD41. These MP of bilineal origin were described in our previous study and their origin can only be speculated upon, but most probably resulted of membrane transfer when platelets and endothelial cells interact. As patients with PV had less platelets than patients with ET this might contribute to their difference in expressing CD62E. In our previous study we showed that patients with ET with high numbers of CD62E-positive MP had more risk factors for thrombosis [19]. This was not observed in the current study, probably because the included patients had less risk factors for thrombosis. CD62E-positive MP suggest endothelial activation, however, in contrast to our former study, no higher levels of mature vWF were observed in these patients compared with controls, due to higher values of mature vWF antigen in the control group. This is not well understood, but could be related to increased age of the control group, as vWF antigen is known to increase with age [28]. As described before the ratio between mature vWF and its propeptide in the patients groups was compatible with chronic endothelial activation. The increased F1+2 fragment, values in the patient group provided evidence for ongoing coagulation activation.

With regard to leukocyte-derived MP, especially in patients with PV higher numbers were observed, and their numbers correlated with the leukocyte counts. Moreover, patients with PV had higher numbers of MP carrying markers of neutrophil activation CD66b, CD11b and PSGL-1. Neutrophil activation is important in the formation of leukocyte-platelet aggregates in PV and ET patients [11], which have been related to thrombosis [13]. These aggregates are formed through the interaction of platelet and leukocyte adhesive molecules, such as between platelet CD62P and leukocyte PSGL-1, inducing the formation of leukocyte-derived MP, which can express TF and so enhance clot formation [17] but also between platelet CD42b and leukocyte CD11b [11]. MP expressing these activation markers might contribute to the forming of leukocyte-platelets aggregates as well, or influence adhesion to the vessel wall. Patients with ET showed a trend to increased numbers of CD42b-positive MP, and patients with PV had the highest numbers of MP expressing CD11b and PSGL-1.

We observed a correlation between the presence of the $JAK2^{V617F}$ mutation and both leukocyte count and numbers of leukocyte-derived MP. A correlation between $JAK2^{V617F}$ mutational status and leukocytosis has been described previously [9].

The procoagulant potential of MP was confirmed in MP-dependent thrombin generation experiments. Patients with ET had higher ETP compared with controls. The higher phospholipid load as provided by the higher number of MP in ET patients is most likely responsible for this result. Moreover, both ET and PV patients had a shorter lag time than controls, which suggest that they had more tissue factor. Tissue factor could be carried by leukocyte-derived MP. Differences in lag time are mostly influenced by the amount of tissue factor in the reaction [29], and no exogenous tissue factor was added. Numbers of tissue factor-bearing MP did not differ between groups, and were relatively low (<1%). As observed before using the CAT assay, the ETP assays are probably more sensitive for the presence of tissue factor than the FACS analysis [19].

Qualitative differences of MP in thrombin generation experiments were investigated by calculating MP-specific thrombin generation, which is the amount of thrombin that is generated per MP. This MP-specific thrombin generation did not differ between the groups. This suggests that the increased

total MP-dependent ETP is a reflection of the increased numbers of MP, and probably of the increased surface of negatively charged membrane phospholipids, rather than a result of differing phenotypic characteristics. A recently published study by Duchemin *et al.* [20] on circulating procoagulant activity in ET and PV patients, also reported on increased procoagulance in patients due to increased circulating phospholipid content. Our MP-specific ETP did not involve the isolation of MP from one specific lineage and therefore represents the average ETP taken over a group of MP derived from several cell lines. As previously described higher percentages of leukocyte-derived MP could influence ETP [30]. However, as leukocyte-derived MP were present at very low numbers, it is possible that minor effects escaped detection, or that their main effects are mediated through interaction with the vessel wall.

In conclusion, MP in patients with ET and PV showed differences with regard to both phenotypic characteristics and thrombin generation potential compared with controls. Patients had higher numbers of MP and patient groups differed in the finding that PV patients showed higher numbers of MP expressing markers of leukocyte activation. Interestingly, leukocytosis as well as the number of leukocyte-derived MP correlated to the presence of the $JAK2^{V617F}$ mutation. ET patients showed an impressive, and PV patients a moderate, increase of MP expressing CD62E, a selectin involved in leukocyte-endothelium interactions. This was entirely absent in controls and may reflect a novel mechanism involved in the increased risk on thrombosis in MPN patients.
With regard to the direct procoagulant properties of MP in these patients, MP in patients with ET showed a particularly high ETP. As no differences were found in thrombin generation per MP, this effect seems to rely on numbers of MP, and more specifically on their phospholipid content. Therefore MP in myeloproliferative disease could partake in the thrombotic diathesis through both indirect mechanisms (mediation of intercellular adhesion), and direct mechanisms (MP-induced thrombin generation).

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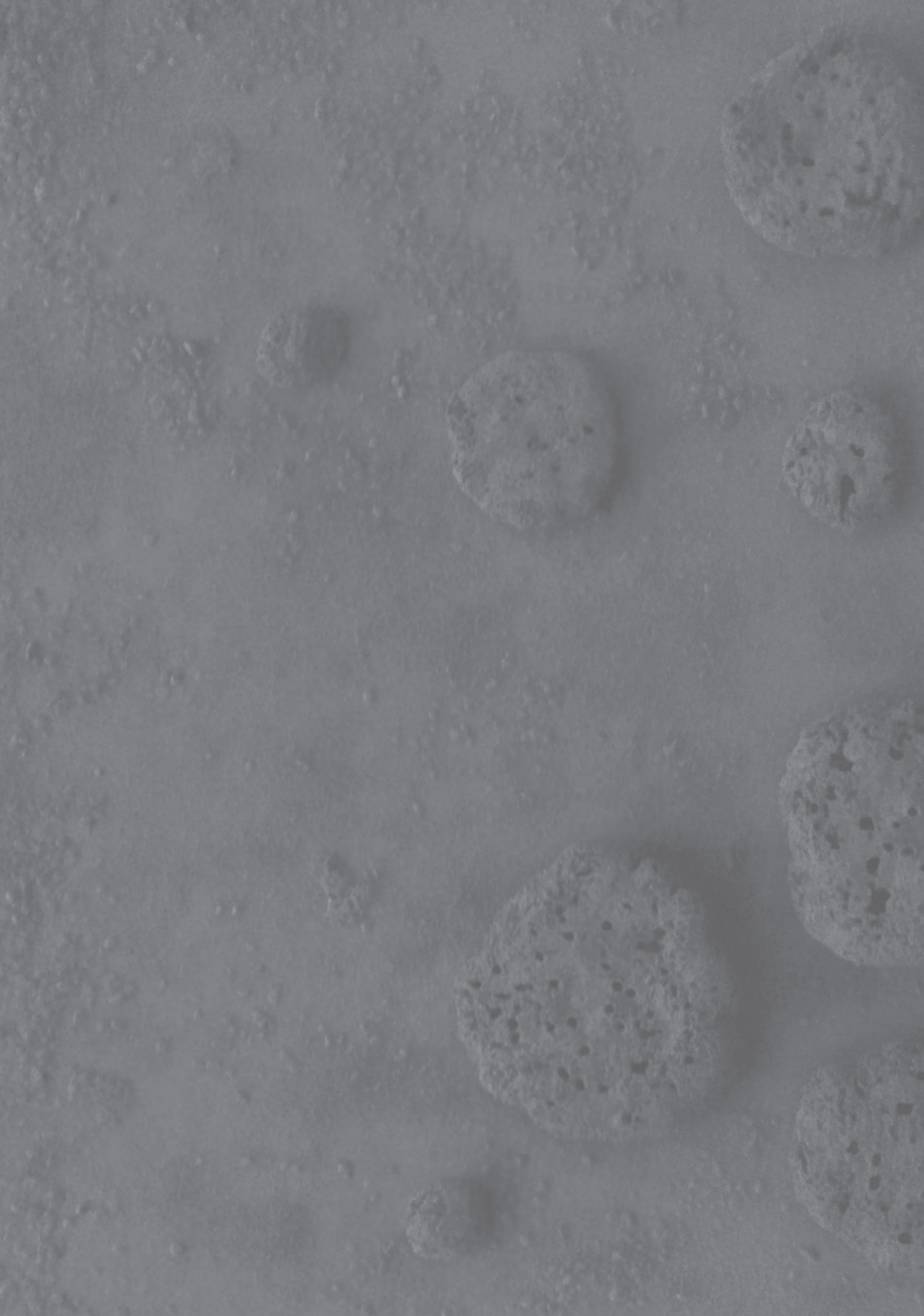

Chapter 4

Chronic renal failure is accompanied by endothelial activation and a large increase in microparticle numbers with reduced procoagulant capacity

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Nephrology dialysis and transplantation 2012; 27: 1446-1453

Abstract:

Background: In patients with chronic renal failure (CRF), cardiovascular disease is the leading cause of increased morbidity and mortality. We hypothesized a role for endothelial activation and microparticle (MP) numbers and procoagulant activity in the pre-thrombotic state of these patients.

Methods: We analysed blood samples of 27 patients with CRF (8 chronic kidney disease Stage 4 (CKD4), 9 peritoneal dialysis (PD) and 10 haemodialysis (HD), samples taken before and after haemodialysis, and 10 controls. Degree and nature of endothelial activation were assessed by measuring mature von Willebrand factor (vWF) and vWF propeptide levels. Cellular MP were characterized by flowcytometry and MP-specific thrombin generation (TG) measurements.

Results: CRF was accompanied by chronic (CKD4 and PD) or acute (HD) endothelial activation. Patients with CRF had substantially higher MP numbers than controls (median 9400 vs. $4350*10^6$/L, p=0.001), without significant differences between the treatment subgroups, or between pre- and post-HD. The vast majority of MP was platelet derived. Of the minor populations, endothelial MP and tissue factor-bearing MP were more abundant in CRF. MP were procoagulant, but the increase in numbers was not reflected in a proportional increase in MP-specific TG.

Conclusions: Renal failure is accompanied by endothelial activation of a different nature in CKD4 and PD patients compared to HD patients, and results in all subgroups in an increase of mainly platelet derived MP that appear to be less procoagulant than in other disease states, possibly because of the uraemic functional defect of their cellular source.

Introduction

Cardiovascular disease is the leading cause of mortality in patients with chronic renal failure (CRF), regardless of progression to end stage renal disease [1,2]. In fact, patients with chronic kidney disease Stage 3 or 4 (glomerular filtration rate between 30 and 60 ml/min/1.73 m^2) more often die of cardiovascular causes rather than progress to end-stage renal disease [3]. The development of cardiovascular disease in uraemic patients involves atherosclerosis, a complex process accompanied by endothelial dysfunction and inflammation. Moreover patients with CRF have an increased risk of venous thromboembolism [4].

Circulating microparticles (MP) are shed membrane vesicles of <1.0 μm in diameter and are generated as result of cellular apoptosis or activation in response to various stimuli [5]. MP display specific cell surface proteins that indicate their cellular origin, e.g. platelets, leukocytes, endothelial cells or red blood cells. MP expose phosphatidylserine on their outer membrane leaflet, which provides a suitable anionic phospholipid surface for assembly of the tenase and prothrombinase complexes [5,6]. They may express tissue factor (TF) [7], the primary physiological initiator of coagulation. Such phosphatidylserine- and/or TF-bearing MP contribute to thrombosis in different diseases. Moreover, MP contribute to endothelial cell activation and dysfunction leading to vascular inflammation and development of atherosclerosis [8]. Indeed, in patients with cardiovascular disease increased numbers of circulating MP are observed [9,10] and are associated with dysregulation of vascular tone [11,12] and correlate with disease severity and clinical outcome [10].

In uraemic patients, elevated levels of circulating MP have been detected as well [11,13], and two possible mechanisms are described by which chronic renal disease may lead to elevated MP levels. Firstly, *in vitro* high concentrations of uraemic toxins cause a rise in endothelial-derived MP [11], although it is not yet clear whether *in vivo* there is a correlation between the urea level and number of circulating MP. Secondly, patients with uraemia are at a high risk of cardiovascular disease, which may further propagate the production of MP that are also implicated in its cause. When uraemic patients are subjected to haemodialysis (HD), shear stress and the contact between blood and non-human materials could add to the previous two mechanisms in generating MP [14,15].

Because of their established contribution to cardiovascular risk factors, platelet- and endothelium-derived MP are of interest, and an increase in their numbers indicates a poor clinical outcome [16]. Both types of MP have previously been described to be increased in renal disease [11,15,17,18]. Daniel *et al.* [15] described elevated numbers of granulocyte-derived MP as a marker of inflammation in HD. MP carrying membrane-bound markers of coagulation, such as P-selectin and TF, are not described in renal disease yet. P-selectin-positive MP reflect the source platelet activation status and have been observed in increased numbers in myocardial infarction [19]. TF-bearing MP are involved in arterial thrombosis [20] and TF is thought to play a major role in plaque thrombogenicity [21]. In the present study, we have made a first extensive inventory of the cellular and molecular spectrum of MP in renal disease.

To gain more insight into the interplay between endothelial dysfunction and MP numbers and properties in CRF, we have assessed the activation status of the endothelium as reflected by levels of plasma mature von Willebrand Factor (vWF) and propeptide. The half-life of mature vWF measures four times the propeptide half-life, and due to this difference in half-life, their relative concentration is a distinctive indicator for ongoing chronic as opposed to acute endothelial activation [22]. Also, MP procoagulant capacity was assessed in an MP- dependent thrombin generation (TG) assay. Uniquely, this allowed us to eliminate pro- or anticoagulant effects of other patients' plasma components since TG was performed by addition of isolated MP to a normal human pooled plasma background.

Materials and Methods

Patients

A cross-sectional study was performed in 27 patients with CRF (median age 54, range 22-80), including 10 patients with end-stage renal disease on HD, 9 patients with end-stage renal disease on peritoneal dialysis (PD), and 8 patients with CKD stage 4 (CKD4, glomerular filtration rate between 15-30 ml/min/1.73 m^2). Patients' characteristics are listed in Table 1. Patients with diabetes or on coumarin derivatives were excluded. The groups did not differ in age and sex ratio. The average time of HD in the HD group was 2.6 ± 3.1 years. The patients

were dialysed three times a week with a low flux polysulfone membrane (Fresenius Medical Care, Bad Homburg, Germany), and the duration of dialysis was individually adjusted to maintain an eKt/V above 1.3 per dialysis. All patients were dialysed in the same dialysis unit using the same dialysate system (Onze Lieve Vrouwe Gasthuis, Amsterdam, The Netherlands). Heparinization during dialysis session was performed in all patients with infusion of 2500 or 5000 IU of low-molecular weight heparin (LMWH) at the beginning of each HD session. All patients were dialysed through an arteriovenous fistula, and blood samples were obtained from the arterial end of the fistula after two days off dialysis, before and after HD. The PD group consisted of nine patients (three patients on continuous ambulatory PD and six on automated PD) and had an average time on dialysis of 2.2 ± 1.8 years. Blood samples of CKD4 and PD patients were obtained during the routine outpatient visits.

In addition, at the start of the study, we collected blood samples from ten healthy control subjects (median age 46, range 19-59), 6 men and 4 women who were not using oral contraceptives or hormonal therapy. In their blood samples we analysed all parameters discussed here, except TG of isolated MP added to human pooled plasma. As a healthy control group for this MP-specific TG experiment, designed and performed later in time during the course of a study on MP in breast cancer patients (manuscript submitted), we included twenty healthy control subjects (women, median age: 54 ± 12 years), not using oral contraceptives or hormonal therapy. We feel this is valid because the number of Annexin V-positive MP in this control group did not differ significantly from the first control group (median $6400*10^6$/l, p=0.15), with a narrow distribution range. The mean fluorescence intensity (MFI) of MP-bound-labelled Annexin V was very similar in both control groups (MFI 416 in CTR (n=20) and 438 in CTR (n=10), indicating similar MP procoagulant phospholipid exposure. Finally, in thrombin generation, no male/female differences have been found [23]. All patients and controls agreed to participate in the study following informed consent and this study was approved by the local medical ethical committee (Medisch Ethische Commissie, OLVG, Amsterdam, The Netherlands).

Table 1 Patients' characteristics

	controls	CKD4	PD	HD
Number	10	8	9	10
Median age	46	60	50	50
Sex ratio (male/female)	6/4	4/4	8/1	5/5
Mean years on dialysis		N.A.	2.2	2.6
Causes of kidney disease				
Vascular disease		6/8	5/9	4/10
Glomerular disease		1/8	0	2/10
Nephrolithiasis		0	1/9	1/10
IgA nephropathy		0	1/9	1/10
Polycystic Kidney Disease		1/8	1/9	0
Unknown		0	1/9	2/10
Hypertension		8/8	7/9	8/10
Biological parameters (mean)				
Haemoglobin (mmol/L)		7.7	7.7	7.5
Albumin (g/L)		43	37	42
Kreatinin (µmol/L)		284	947	874
Urea (mmol/L)		17	19	27
Ferritin (µg/L)		127	152	573
Treatments				
ARB* or ACE# inhibitor		6/8	4/9	4/10
ß blocker		4/8	8/9	5/10
calcium antagonist		6/8	7/9	5/10
central agents		2/8	3/9	4/10
diuretics		3/8	6/9	7/10
aspirin		0	2/9	4/10
statins		5/8	6/9	3/10
erythropoietin		2/8	6/9	10/10
Ferri oxide saccharate		0	0	9/10
(Venofer®)intravenous				

*Angiotensin II receptor blockers
#Angiotensin converting enzyme

Plasma markers of endothelial activation

Mature von Willebrand Factor and propeptide plasma levels were measured by ELISA as described previously [24].

Blood collection, isolation and flowcytometric analysis of MPs

Blood samples were drawn with a 21-gauge needle after applying a light tourniquet. After discarding the first 4 mL, blood was collected into a 4.5 mL tube containing 3.2 % trisodium citrate [Becton Dickinson (BD), Plymouth, UK]. Plasma was prepared within 20 min after blood collection by centrifugation for

20 min at room temperature at 1,550 x g, without brake. Aliquots of plasma were snap frozen in liquid nitrogen, and then stored at –80^{0}C until use. MP isolation was performed as previously described [25]. For flowcytometric analysis MP (5 µL) were diluted in 35 µL PBS containing 2.5 mmol/L $CaCl_2$ (pH 7.4). Then 5 µL Annexin V-allophycocyanin (APC) from Caltag Laboratories (Burlingame, CA, USA) and 5 µL fluoresceinisotyocianate (FITC)-, phycoerythrin (PE)- and/or peridinin chlorophyll protein complex (PerCP)-labeled cell-specific monoclonal antibodies or isotype-matched control antibody were added. We used anti-TF-FITC (VD8, IgG_1) from American Diagnostics (Stamford, CT, USA); anti-CD8-PE (SK1, IgG_1), anti-CD14-PE (MΦP9, IgG2b), anti-CD15-PE (HI98, IgM), anti-CD20-PE (L27, IgG_1), anti-CD45-PerCP (H130, IgG_1, k), anti-CD61-PE (VI-PL2, IgG_1), anti-CD63-FITC (H5C6, IgG_1), anti-CD146-PE (P1H12, IgG_1) from BD (San Jose, USA); anti-glycophorin A-FITC (JC159, IgG_1) and anti-CD41-FITC (5B12, IgG_1) from DAKO (Glostrup, Denmark), anti-CD144-FITC (BMS158FI, IgG_1) from Bender MedSystems (Vienna, Austria), anti-CD106-FITC (1.G11B1, IgG_1) from Calbiochem (Darmstadt, Germany); anti-CD54-PE (K562, IgG_1), anti-CD66b-FITC (80H3, $IgG_1\kappa$) and anti-CD62P-PE (CLB-Thromb/6, IgG_1) from Immunotech (Marseille, France); anti-CD62E-FITC (HAE-1f, IgG_1) from Kordia (Leiden, The Netherlands); anti-CD4-PE (CLB-T4/2, IgG_1) and anti-CD66acde-PE (CLB-gran/10, IH4Fc, IgG_1) from Sanquin (Amsterdam, The Netherlands). Labelled isotype controls IgG_1 (X40) and IgG_{2a} (X39) were used from BD (San Jose, USA), and IgG_{2b}-PE (MCGb) from IQProducts (Groningen, The Netherlands).

The mixtures were incubated in the dark for 30 min at room temperature. Subsequently, 760 µL PBS/calcium buffer were added. All samples were analysed for 1 min during which the flowcytometer analysed approximately 55 µL of the suspension. The samples were analysed in a FACS Calibur flowcytometer with CellQuest-pro software (BD, San Jose, CA, USA). Forward scatter (FSC) and sideward scatter (SSC) were set at logarithmic gain. To distinguish MP from events due to noise, MP were identified on FSC, SSC, and AnnexinV-positivity. To identify Annexin V-positive events, a threshold was placed in a MP sample prepared without calcium. They were further categorised by binding of a monoclonal antibody directed against a cell-specific antigen. To identify MP that bound cell-specific monoclonal antibodies, MP were incubated with identical concentrations of isotype-matched control antibodies to set the threshold. Some antibodies had higher background fluorescence than the isotype matched control and with these antibodies the threshold was set on

the population MP negative for the antibody. The number of MP per litre plasma was calculated as previously described [25].

Thrombin generation measurements

Thrombin generation (TG) was measured by means of the Calibrated Automated Thrombogram (CAT) method (Thrombinoscope BV, Maastricht, The Netherlands) [23]. TG was determined in the MP rich plasma samples or in the supernatant or pellet after centrifugation for MP isolation (as described above), TG (in 80 μL plasma or after addition of 10 μL MP pellet to 70 μL of normal pooled platelet-poor plasma, obtained from 80 to 90 healthy volunteers) was triggered without the addition of exogenous TF and phospholipids added. All reagents used were obtained from Thrombinoscope BV. TG was measured as fluorescence, read in a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland) equipped with a 390/460 filter set and TG curves were calculated with the Thrombinoscope software (Thrombinoscope BV).

Four parameters may be derived from the TG curves: lag time, endogenous thrombin potential (ETP), time to peak and peak height. For this MP study only the ETP was used. Validation of the CAT method showed normalization of non-time-dependent parameters to be mandatory to obtain acceptable inter-assay variations [23]. Intra-assay variations for normalized parameters are typically below 6%, inter-assay variations below 8% [23].

Statistical analysis

The majority of variables were not normally distributed and therefore, all variables are reported as medians and range, unless otherwise stated. We compared the difference of MP numbers and results of thrombin generation between groups with non-parametric Mann Whitney *U* test or Kruskal Wallis test, where appropriate. Results before and after haemodialysis were compared using a paired student's *t*-test. Bivariate correlations were estimated by Spearman's rank correlation (R). All tests were two-tailed and p values of <0.05 were considered statistically significant. Analyses were performed using SPSS 16.0 for windows (SPSS Inc., Chicago, USA).

Table 2 Levels of vWF antigen and propeptide, presented as medians and IQR

	Controls	*CKD4*	*PD*	*HD $_{before}$*	*HD $_{after}$*
vWF antigen (nM)	34.8	63.6*	71.8*	39.5	46.0*
	28.1-37.0	42.8-78.0	62.3-106.4	30.6-54.8	41.4-61.7
Propeptide (nM)	5.2	8.5*	8.0	4.7	8.0#
	4.4-6.4	5.7-12.9	5.4-11.0	2.9-6.1	5.0-12.8

* p<0.05 compared to controls #p<0.05 compared to before dialysis session

Results

Endothelial activation: mature von Willebrand Factor and propeptide

We investigated the activation of endothelium by measuring mature vWF and its propeptide [22]. Table 2 shows that patients with CKD4 and PD had increased levels of mature vWF [median: CKD4 63.6nM, PD 71.8nM and controls 34.8 nM (ref range 26-49nM); p<0.01 and p<0.01], with similar (PD patients) or slightly elevated (CKD4 patients) propeptide concentrations compared with controls [median: CKD4 8.5nM, PD 8.0nM and controls 5.2 nM (ref range 4.2-9.3nM)], indicating chronic endothelial activation (Figure 1). Mature vWF levels in HD patients were not elevated (median 39.5nM). However, there was a moderate vWF increase during the dialysis process (median HD before 39.5 nM, HD after 46.0 nM, p=0.48) with a disproportional rise in the propeptide levels, indicating acute endothelial activation (median HD before 4.7nM, HD after 8.0 nM, p=0.04).

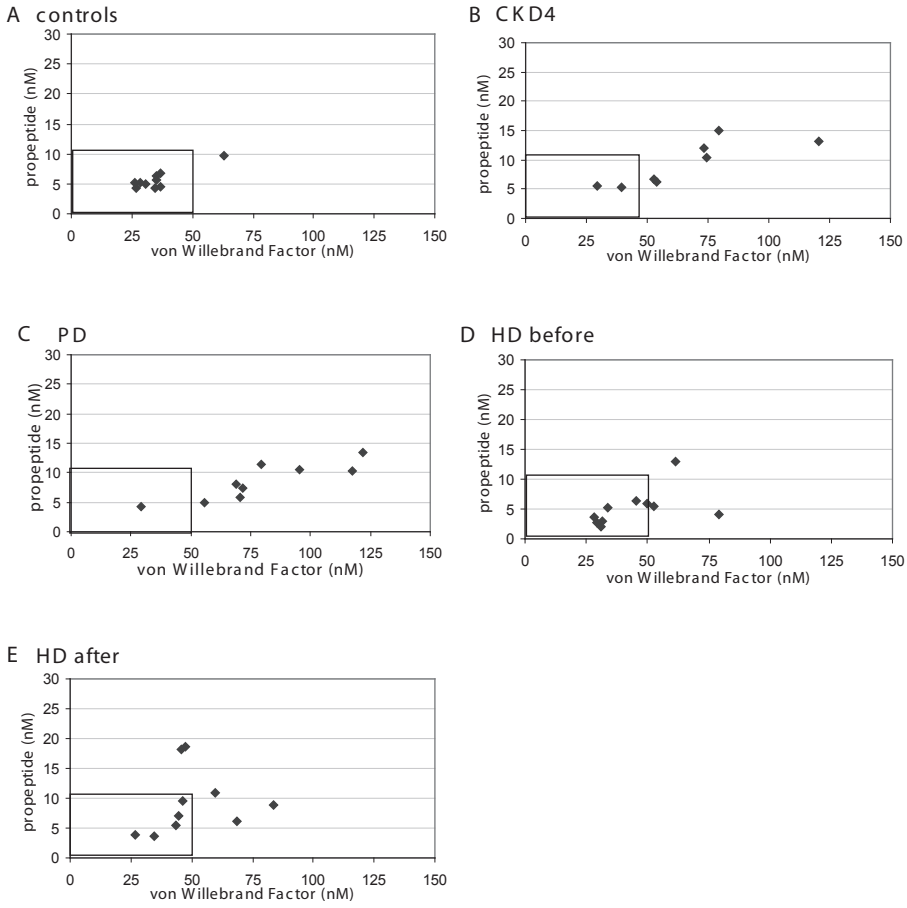

Figure 1: Levels of vWF antigen (x-axis) and propeptide (y-axis) of controls (A), CKD4 (B), PD (C) and HD patients before (D) and after (E) haemodialysis session. The box represents values within the normal range. CKD4 and PD patients have higher levels of vWF antigen compared to controls ($p<0.05$) in a pattern compatible with chronic endothelial activation and HD patients have higher levels of propeptide after haemodialysis compared to before ($p<0.05$) compatible with acute endothelial activation [22].

Table 3 Number, cellular origin and composition of MP in plasma (x10^6/L) in different patient groups and controls. Data presented as medians (range) or % of total (=Annexin V-positive) MP.

	Controls n=10	*All CRF patients n=27*	*CKD4 n=8*	*PD n=9*	*HD $_{before}$ n=10*	*HD $_{after}$ n=10*
AnnexinV+ MP	4350	9400**	9150 *	8500 *	10350 *	13350 *
	(3800-9400)	(4000-25500)	(4000-13100)	(5000-25500)	(4200-22000)	(4000-22200)
CD41+ MP	4100	9200**	9000 *	7700 *	10230 *	11900 *
	(3500-9200)	(3600-21000)	(3600-12000)	(4700-21000)	(3800-19000)	(3250-22200)
	94	95	93	95	95	94
CD62P+ MP	140	320**	340 **	250	410 *	285 *
	(50-270)	(70-720)	(220-720)	(70-650)	(105-710)	(100-690)
	2.0	3.6	4.8	3.0	2.5	2.3
CD63+ MP	8	46**	65 *	50 *	30 *	40 *
	(0-35)	(8-190)	(17-130)	(8-190)	(10-145)	(30-150)
	<1	<1	<1	<1	<1	<1
GlycoA+ MP	62	63	40	65	111	110
	(26-199)	(13-197)	(13-160)	(13-192)	(41-197)	(32-307)
	1.4	0.9	0.4	0.9	1.2	1.0
CD144+ MP	4	8*	8 *	4	9 *	5 *
	(0-13)	(0-150)	(0-30)	(1-70)	(0-150)	(0-175)
	<1	<1	<1	<1	<1	<1
CD66e+ MP	8	14*	12	20*	18*	20*
	(1-19)	(0-44)	(0-16)	(6-44)	(4-39)	(3-49)
	<1	<1	<1	<1	<1	<1
CD14+ MP	7	12	11	10	18 *	26 *
	(0-17)	(1-225)	(1-39)	(4-17)	(7-225)	(6-180)
	<1	<1	<1	<1	<1	<1
TF+ MP	2.5	4.4*	4.6 *	4.1	6.7 *	5.0
	(2-7)	(1-215)	(3-8)	(1-10)	(2-215)	(2-220)
	<1	<1	<1	<1	<1	<1

* p<0.05 compared with controls, **p<0.001 compared with controls.

Number and phenotypic characterization of MP

The total number of MP was substantially increased in CRF patients compared with controls, (9400 vs. 4350 x10^6/L p<0.001, Table 3). No significant differences were observed between patient subgroups, or between pre- and post dialysis samples. The lowest median value was found in PD.

In most study subjects more than 90% of circulating MP bound Annexin-V, indicating the presence of phosphatidylserine on their membrane. The phenotypic characterization of MP depicted in Figure 2 showed that the cellular subset composition of the MP population (i.e. of platelet-, leukocyte-, endothelial cell or erythrocyte origin) is similar between CRF and controls. The large majority of MP in all groups was of platelet origin, as determined by positivity for the platelet marker CD41 (CRF vs. controls: 95 vs. 94%). Reflecting the higher total number of MP, the absolute number of platelet-derived MP (CD41 positive) was significantly higher in CRF than in controls (9200 vs. 4100x10^6/l; p<0.001, Figure 2), again without differences between patient subgroups. Notably, the numbers of platelet derived-MP did not correlate with urea levels.

Co-expression of platelet (CD41) and activated platelet markers (CD62P and CD63) was increased in CRF (CD41, CD62P double positive median 320 vs. 140x10^6/L; p=0.001, CD41, CD63 double positive median 46 vs. 8*10^6/L; p<0.001). For CD62P- positive MP a relative increase was observed as well; in renal failure they accounted for 3.6% of all MP vs. 2.0% in controls (Table 3, Figure 2). Although in PD patients the lowest numbers of MP were observed, differences were not statistically significant in subgroups.

With regard to minor MP fractions, numbers of red blood cell-derived MP (glycophorinA-positive) were a priori hypothesized to be influenced by the HD process. However, similar numbers in all patient subgroups were shown and no significant increase upon HD was observed (Table 3). Endothelium-derived MP however, though relatively low, differed between groups with a higher number of MP expressing CD144 in CKD4 and HD compared to controls, but not in PD. Whereas total numbers of leukocyte-derived MP (CD45-positive) did not differ between CRF and controls, MP positive for the granulocyte marker CD66e indeed were increased in CRF compared with controls (median: 14 vs. 8* 10^6/L; p=0.02), and monocyte-derived MP were observed in higher numbers in HD patients compared with controls (p=0.005). All numbers of MP observed in other subsets did not differ significantly from controls (Figure 2).

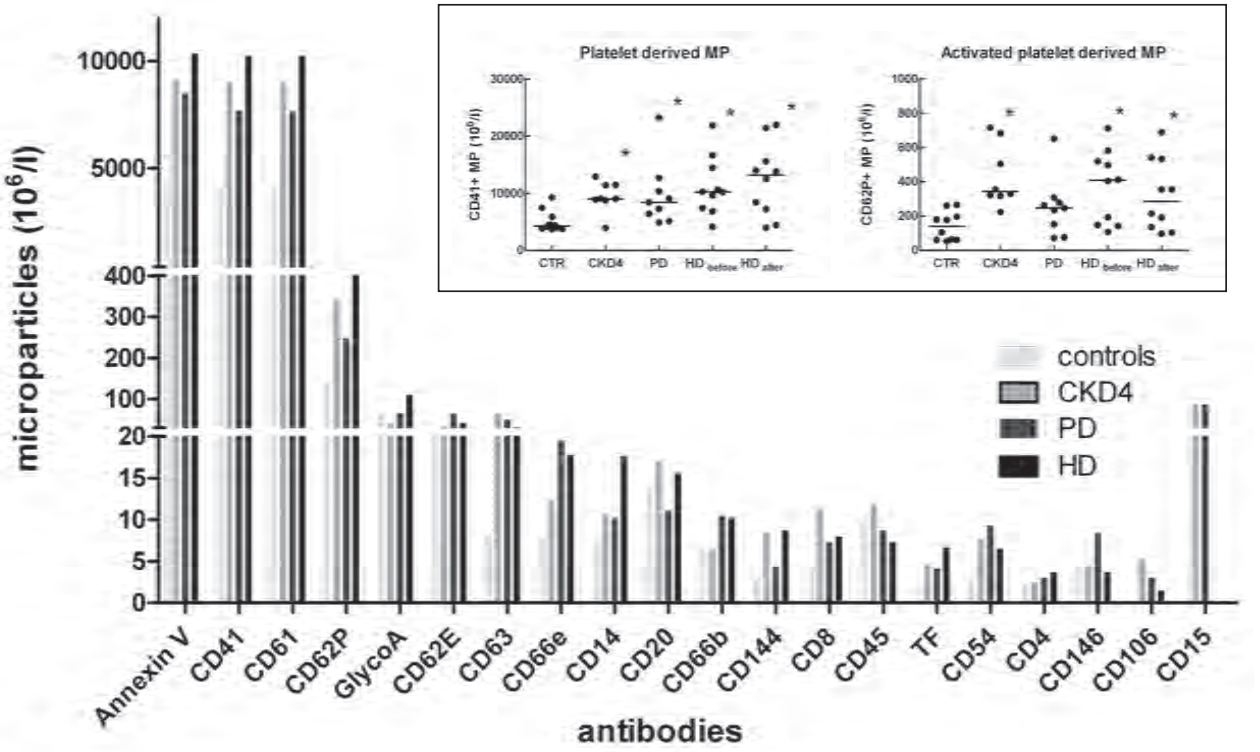

Figure 2: Size of circulating MP subpopulations in CRF patients and controls.
Bar graph: number of MP from specific cellular origin as defined by marker positivity in plasma from patient subgroups: CKD4, PD, HD and controls (CTR). Shown are the number of MP positive for markers of platelets (CD41, CD61), activated platelets (CD62P, CD63), leukocytes (CD66e, CD14, CD20, CD66b, CD8, CD45, CD4, CD15), endothelial cells (CD62E, CD144, CD54, CD106), erythrocytes (GlycoA) and TF. Data presented as medians.
Inset: dotplot of the same data presented individually for the platelet marker CD41 and the marker for platelet activation CD62P. *p<0.05 compared with controls.

Finally, the number of TF-positive MP, accounting for < 1% of total MP, was significantly higher in CRF compared with controls. There were no differences between patient groups (Table 3) but relatively low values were observed in PD without any high outliers. TF was predominantly co-expressed with platelet marker CD41.

Thrombin generation experiments

As a first experiment, TG was performed in MP-rich plasma. The reaction was initiated without exogenous TF and phospholipids added, thus rendering the assay MP sensitive. HD patient samples could not generate thrombin in this manner, probably due to the presence of trace amounts of LMWH before an HD session. Samples of CKD4 and PD patients did not differ from control samples in endogenous thrombin potential (ETP), (median: 1103nM.min (524-1653) vs. 1300 (596-1559) nM.min; p=0.78) (Table 4).

Table 4 TG results for patient subgroups and controls. Results are presented as medians (range). P-value describes differences between the groups by Kruskal Wallis test. Due to heparin remnants in plasma no ETP results in plasma are available for HD patients.

	Controls	CKD4	PD	HD	p-value
ETP plasma (nM.min) (buffer)	1300 (596-1559)	1170 (857-1653)	1031 (524-1624)	N.D.	0.78
ETP microparticles (nM.min) (buffer)	1029 (822-1234)	1263 (1017-1364)	1042 (852-1385)	1115 (932-1163)	0.03

N.D. not determined

In a following experiment, performed to verify the role of MP in TG by excluding the role of the patients' plasma components, MP were isolated and MP and supernatant were added separately to normal pooled plasma and again tested for TG as described above. After removal of MP, the supernatant plasma did not show TG under these conditions (data not shown), indicating that TG is MP-dependent. Indeed, when isolated MP were added to normal human pooled plasma, thrombin was generated and the ETP was higher in patient MP than in control MP (Table 4). Among the various patient subgroups, ETP values were the lowest in PD, without the differences between groups being statistically significant. The total number of Annexin V-positive MP (weakly) correlated with ETP (R=0.43, p=0.03, Figure 3).

When ETP values were expressed relative to MP numbers, MP from CRF patients generated substantially less ETP than those from controls, or in other words, MP in CRF possessed impaired thrombogenicity. MP phospholipid exposure is an important contributor to this property [26]. Interestingly, the mean fluorescence intensity (MFI) of MP-bound-labelled Annexin V was significantly higher in the control group than in patients, which implies that MP in CRF patients expose less procoagulant phospholipids (MFI 416 in CTR (n=20) vs. 305 in CRF; p<0.001).

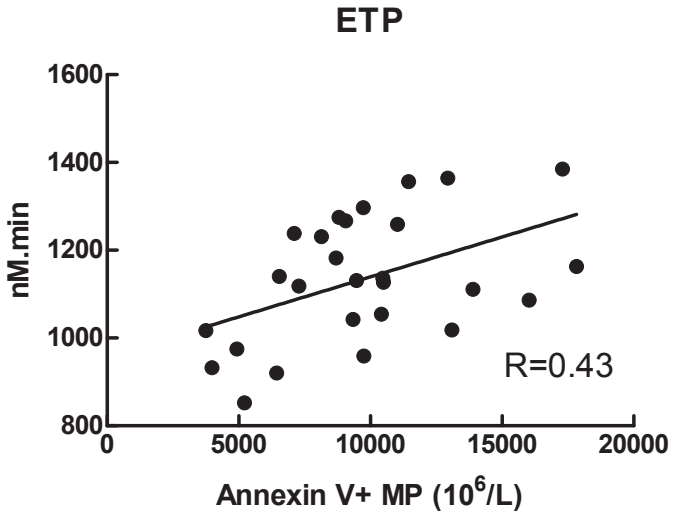

Figure 3: Correlation between number of Annexin V-binding MP and ETP (R= 0.43, p=0.03) performed in human pooled plasma to which MP of CRF patients were added. TG was initiated without exogenous tissue factor and phospholipids.

Discussion

This is the first study that in parallel assesses endothelial activation and characterizes MP with an extensive panel of antibodies in different treatment groups of patients with CRF, and studies MP-specific TG in these patients.

We observed that patients with CRF and an activated endothelial status had elevated levels of platelet, activated platelet and endothelium-derived MP, irrespective of their dialysis modality. Other groups have described an absolute increase of platelet-derived MP in CRF as well [11,17]. This increase in MP, which were procoagulant since they supported TG, did not result in a proportional

increase in MP-dependent TG compared with controls. Apparently, the CRF MP are not as potent thrombin generators as control MP.
The endothelial activation parameters mature vWF and propeptide provided evidence of an important difference between the CKD4 and PD groups, which showed a pattern compatible with chronic activation [22], and the HD group, which showed a pattern compatible with acute activation. This was inferred from the rise in levels of propeptide with an, apparently only moderate, mature vWF increase, observed during the HD session only. Previously, Tomura *et al*. [27] showed that patients on HD suffered from progressive endothelial cell damage, and that the capacity of their endothelial cells for the release of vWF antigen gradually decreases in the months following initiation of HD. This may reflect the duration and intensity of endothelial challenge during HD, putatively leading to a relative vWF secretional exhaustion. However, it is shown here not to coincide with a significantly different MP profile or MP procoagulant capacity.

With further regard to endothelial activation in renal failure, not only plasma vWF levels but also endothelium-derived MP, are considered markers for endothelial dysfunction [11,28] being predominantly produced by endothelial cells. In our study, endothelium-derived MP (CD144+) accounted for approximately 1% of all MP, and as described before were thus a minor fraction [29]. Still, endothelium-derived MP expressing CD144 were increased in CRF patients supporting the concept of ongoing endothelial activation, PD patients formed an exception in this regard: their CD144+ MP numbers did not differ from those in controls. This is in line with a recent study by Merino *et al*. [30] in which an increase was observed in endothelial damage-related CD14+/CD16+ cells in CKD-non dialysis and HD patients but not in PD patients, suggesting a different pathway promoting the endothelial damage in PD.

As stated above, the observed increase in platelet- and endothelium-derived MP in renal failure may be due to either uraemia, or to the chronic vascular dysfunction and atherosclerosis that often accompanies CRF. The actual increase in MP numbers in the present study is substantial, with levels of MP approximately two times higher in patients than in controls. (Pre) analytical conditions being similar, this indicates that metabolic dysregulation indeed plays a major role in the generation of high MP counts in renal failure. In agreement with this conclusion, MP levels have been shown to rapidly decline

after renal transplantation [31], when the renal function improves while the cardiovascular risk factors remain the same. In addition, renal clearance of MP may be speculated to influence MP half-life in blood as urine is known to contain MP [32]. If indeed MP are removed from the circulation by renal clearance, the defective clearance in patients may also cause a rise in MP numbers.

Superimposed on uraemia, the HD process itself was a priori hypothesized to increase MP numbers as described previously [11,15]. Perhaps surprisingly, we did not observe such an increase but counted similar levels of platelet derived MP before and after dialysis. This is in concordance with the results of Ando *et al.* [17] and Boulanger *et al*. [29]. A possible explanation for these findings is the use of different dialysis membranes. We used synthetic membranes, just as Boulanger *et al*. [29], while in the apparently conflicting studies [11,15] cellulose membranes were used. The use of synthetic membranes might result in less platelet activation although attachment of MP to the membrane during dialysis cannot be excluded.

We show an absolute increase of MP carrying markers of platelet activation (CD62P and CD63) [19] in CRF, that may contribute to an increased risk of vascular disease. CD62P, or P-selectin, is an important player in one of the cellular pathways for the initiation of blood coagulation. P-selectin-positive platelets are known to induce leukocyte-derived MP to express TF, which can induce clot formation [33,34]. P-selectin expression by MP is likely to have the same effect. In support of this, we did find higher numbers of leukocyte-derived MP and TF-bearing MP in patients with CRF, although the account of both subsets was low (<1%).

The procoagulant potential of the CRF MP was confirmed in TG experiments. MP-rich plasma samples from CKD4 and PD patients could generate thrombin under MP-dependent (without exogenous TF and phospholipid) conditions. Despite the higher MP numbers in these samples, the resulting ETPs were not different from those in controls. This suggested either less potent procoagulant properties of the CRF MP, and/ or a negative influence of patients' plasma components (inhibition, coagulation factor depletion). TG experiments with isolated control- and CRF MP added to normal pooled MP-free plasma provided evidence for the former mechanism. The higher number of MP in the isolates

of CRF patients resulted in a higher, but not proportionally higher with respect to MP numbers, TG in the normal plasma background. This was not due to an overload of MP since in this MP number range for CRF samples a dose-response relationship with ETP values was apparent (no plateau was reached yet). These data suggest that MP in CRF patients indeed are procoagulant but at the same time points to a qualitative difference in MP properties between CRF patients and controls. Herewith, we provide another example of disease-related MP populations with disease-specific procoagulant potential. In CRF it refers to a decreased potential, whereas in a previous study, we identified myeloblast-derived MP in patients with Acute Myeloid Leukemia (AML) that were more intrinsically procoagulant than control MP [35]. With regard to the relatively poor TG capacity of the CRF MP, it is of interest to note that their main cellular source (platelets) is functionally challenged by the kidney failure (uraemic thrombopathy). For example, uraemic platelets have been reported to show diminished adhesion and aggregation [36]. It may therefore be speculated that thrombopathic platelets in uraemia generate 'thrombopathic' MP being only moderately procoagulant. Indeed, we found that MP in CRF showed a diminished exposure of phosphatidylserine which is expected to result in a lower ETP [37].

It will be of interest to further study the role of plasma components in the net similar MP-driven TG in the control - and patient MP-rich plasma samples. Since apparently the role of MP in cardiovascular complications in CRF does not so much derive from an increase in MP-driven plasmatic coagulability, we speculate that their function is altered regarding the cellular processes leading to endothelial damage and / or the recruitment of procoagulant factors *in vivo*. This is in line with a recent study by Terrisse *et al*. [38] showing that endothelial cells internalised MP, which resulted in enhanced platelet /endothelium interactions, involving vWF and P-selectin. Indeed, in CRF, atherosclerosis is the main vascular problem, not venous thrombosis in which hypercoagulability would be expected.

In conclusion, CRF is accompanied by endothelial activation of a different nature in CKD4 and PD patients versus HD patients but results in all subgroups in an increase (without increment during HD) of mainly platelet-derived MP with minor endothelial marker - and TF-positive fractions. Although presence

of these MP in higher numbers may explain an increased prothrombotic state, these MP appear to be less procoagulant than in other disease states, possibly because of a functional defect of their cellular source due to chronic uraemia. In spite of that, these MP may still be important messengers at the cellular level in the development of atherosclerosis. Further studies are required to investigate these functional properties of MP in CRF.

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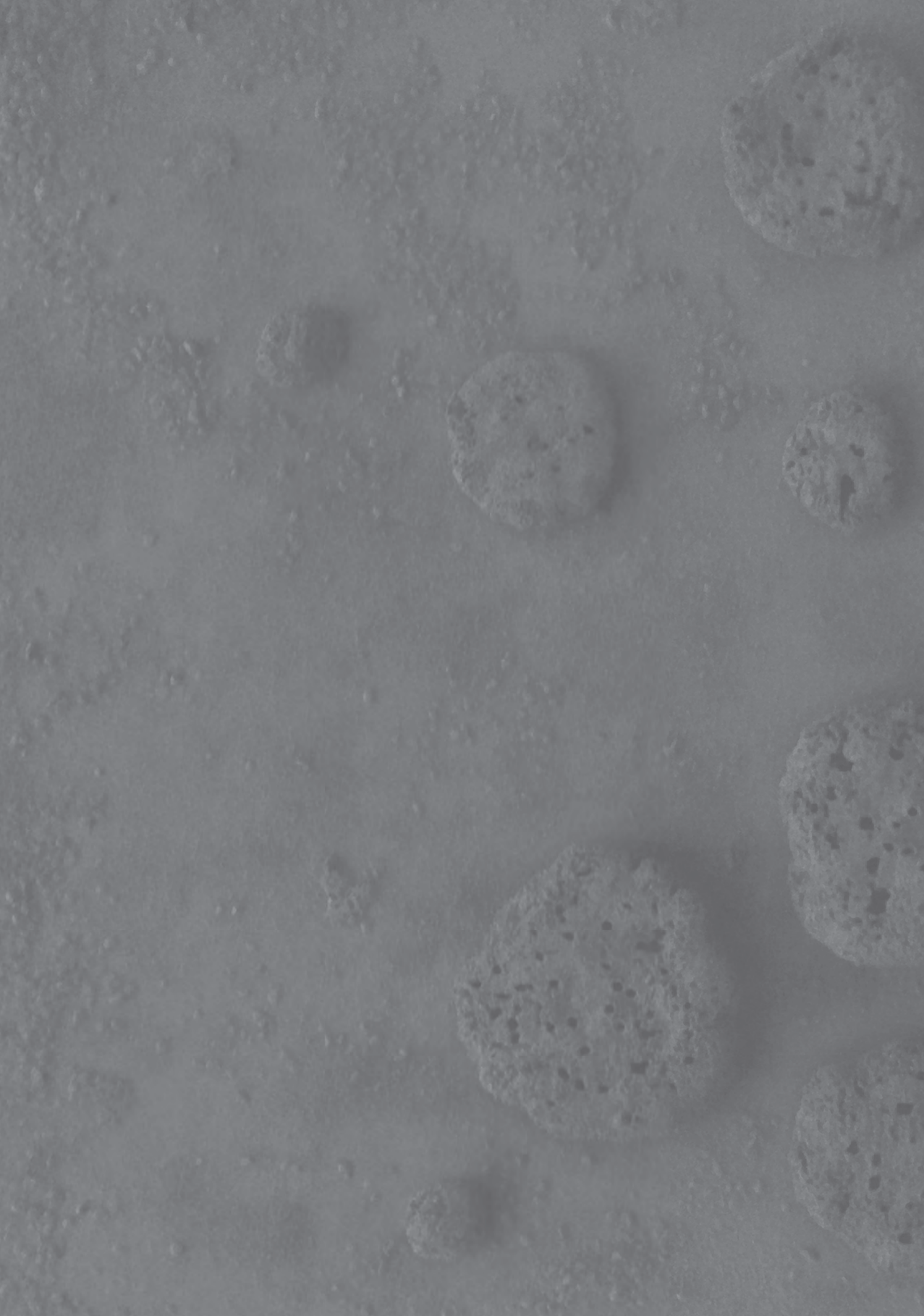

Chapter 5

Elevated numbers and altered subsets of procoagulant microparticles in breast cancer patients using endocrine therapy

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Thrombosis Research 2011; 127: 363-369

Abstract

Introduction: Microparticles (MP) can be elevated in cancer and thromboembolic disease. We hypothesized a role for MP in the hypercoagulable state in breast cancer patients using endocrine therapy, in whom both cancer and the use of endocrine therapy are independent risk factors for the development of thrombosis.

Design and methods: Plasma samples were collected from 40 breast cancer patients using endocrine therapy (20 patients without metastases receiving adjuvant therapy and 20 patients with metastatic disease treated in a palliative setting) and from 20 female healthy controls. The endocrine therapy used was either an anti-estrogen or an aromatase inhibitor. Numbers and cellular origin of MP subsets were analysed by flowcytometry. MP-associated procoagulant activity was measured using a thrombin generation assay using conditions that allow analysis of MP induced thrombin generation.

Results: Breast cancer patients using endocrine therapy had higher levels of MP positive for Annexin V (median 10000 vs. 6500 $x10^6$/L), P-selectin (330 vs. 200 $x10^6$/L), tissue factor (33 vs. 15 $x10^6$/L), and of MP derived from platelets (CD41+) and leukocytes (CD45+). Thrombin generation in plasma was dependent on the presence of MP and thrombin generation performed after addition of isolated MP to normal plasma showed a higher endogenous thrombin potential (1105 vs. 1029 nM.min) in breast cancer patients. No differences were observed in MP levels and thrombin generation parameters between the metastatic and adjuvant group.

Conclusion: Breast cancer patients using endocrine therapy have an increased MP number and a higher MP-dependent thrombin generation, irrespective of the presence of metastatic disease. Altered MP subset characteristics in these patients, especially the higher number of (activated) platelet derived MP and leukocyte derived MP, may in part explain a heightened procoagulant state in breast cancer patients using endocrine therapy.

Introduction

Circulating microparticles (MP) are shed membrane vesicles of <1.0 μm in diameter, resulting from apoptosis or activation of cells in response to various stimuli [1]. MP comprise a heterogeneous population characterized by differences in size, phospholipid- and protein composition [2].

Breast cancer patients and cancer patients in general, are at risk of venous thromboembolic events (VTE) and this risk further increases in advanced stages of cancer and during adjuvant chemotherapy. Traditionally, the hypercoagulability that occurs with cancer and especially in patients with metastatic cancer, has been associated with increased levels of coagulation proteins, e.g. tissue factor (TF) and factor VIIa [3,4]. More specific, tumour associated risk factors include mucinous glycoproteins [5] and selectin-mucin interactions [6].

These hypercoagulable mechanisms may involve the increased shedding of MP, known to play a role in thrombogenic conditions including atherosclerosis, VTE, essential thrombocythemia [7] and other malignant diseases. TF-bearing MP have been associated with thrombosis in cancer [8,9]. Indeed, elevated numbers of platelet-derived MP were demonstrated in metastatic gastric- [10], breast- [8,11] and pancreatic [8] cancer.

In patients with breast cancer Toth *et al.* observed increased numbers of leukocyte-derived MP. The number of leukocyte-derived MP correlated with tumour load [11] and expression of selectins such as P-selectin, which appears to play a role in coagulation by enabling interaction between platelets and leukocytes, mediating the formation of procoagulant TF-bearing MP [12].
Patients with breast cancer are often treated with adjuvant endocrine therapy in the form of either anti-estrogens or aromatase inhibitors. In patients without metastatic disease the intent is to prevent metastases, in patients with known metastatic disease the intent is to reduce tumour load and tumour activity. This treatment and particularly its anti-estrogen component, comprises an independent risk factor for VTE [13]. However, the mechanism by which endocrine therapy contributes to VTE is not clearly understood. Several plasmatic effects have been observed such as tamoxifen-associated reductions

in antithrombin, protein C [14], protein S [15] and tissue factor pathway inhibitor, as well as an increase in activated protein C resistance [16]. The use of aromatase inhibitors also seems to be associated with an increased risk of thromboembolic events, although less so than with tamoxifen [17,18].

The effect of endocrine therapy at a cellular level, specifically platelet and endothelial activation, is largely unknown. It has been demonstrated that tamoxifen increases reactive-oxygen species and raises intraplatelet calcium levels, thus enhancing platelet-mediated thrombosis and platelet activation, respectively [19,20]. The latter may theoretically result in elevated numbers of platelet-derived MP. Similarly, endothelial activation in the course of coagulation, angiogenesis and metastasis may yield endothelial derived MP. In concert with tumour cell derived MP, the shedding of such MP may contribute to the observed cancer and therapy related risk of VTE [21,22].

The main aim of our study was to establish whether MP of breast cancer patients with or without metastatic disease using endocrine therapy have a more prothrombotic profile than those of healthy controls. This prothrombotic profile was defined by the numbers, cellular origin and procoagulant potential of MP.

Design and methods

Study subjects

Forty patients with breast cancer who were treated with endocrine therapy were enrolled (Table 1) at the Oncology department of the Onze Lieve Vrouwe Gasthuis (The Netherlands). Of these, 20 patients received adjuvant endocrine therapy (mean age: 56 +/- 13 years) whereas the other 20 patients had been diagnosed with metastasized breast cancer and received palliative endocrine therapy (mean age: 59 +/- 17 years). Exclusion criteria were: previous thrombosis, the use of anticoagulant therapy, any form of oncologic breast surgery in the previous six months and/or treatment with chemotherapy or radiation therapy in the previous 6 months. All 40 breast cancer patients were using endocrine therapy. 15 patients (37.5%) were using anti-estrogens (tamoxifen, fulvestrant) and 25 patients (62,5%) were using aromatase inhibitors (anastrozole, lezetrole, exemestane). At the time of blood collection both patient groups had been

using endocrine therapy for a median of 20 months (IQR 6,3-34,3). The period since the last chemotherapeutic treatment in the adjuvant group was a median 26 months (IQR 10,8-32,5).

Table 1 Patient characteristics

	Controls	Breast cancer patients		
		Adjuvant	Metastatic	All
Number	20	20	20	40
Age (mean ± SD)	52±8	56±13	59±17	57±15
Hematological parameters (mean ± SD)				
Haemoglobin (mmol/L)	8.2±0.6	8.4±0.7	8.2±1.0	8.3±0.8
Leukocytes(10^9/L)	5.9±1.7	5.1±1.3	7.0±3.2	6.1±2.6
Thrombocytes (10^9/L)	235±49	220±41	234±80	227±63
D-dimer (µg/mL)	0.25±0.1*	0.39±0.2	0.71±0.7	0.56±0.55
Antithrombin (%)	97±10	94±10	102±23	98±18
Fibrinogen (g/L)	3.1±0.6	3.1±0.8	3.3±0.7	3.2±0.7
Thrombin-antithrombin complex (µg/L)	7.5±17.3	12.4±25.7	9.5±13.5	10.9±20.3
Prothrombin fragment 1 + 2 (pmol/L)	173±137.5*	229±190	272±264	250±228
Tumour size cm (Mean ± SD)**		2.7±1.2		
Metastases				
Skeletal			12 (60%)	
Liver			5 (25%)	
Pulmonary			8 (40%)	
Brain			0 (0%)	
Months to surgery (Median (IQR))		30.5(16-38)		
Months to chemotherapy (Median (IQR))		26.0(10.7-32.5)***		
Treatments (number (%))				
Anti-estrogens		7 (35)	8 (40)	15 (37.5)
Aromatase Inhibitors		13 (65)	12 (60)	25 (62.5)
Cardiovascular risk factors (number (%))				
Hypertension	4 (20)	1 (5)	6 (30)	7 (17.5)
Hyperlipidemia	2 (10)	2 (10)	5 (25)	7 (17.5)
Smoking	1 (5)	5 (25)	4 (20)	9 (22.5)

* $p<0.05$ between all breast cancer patients and controls
** In some patients, multiple tumours were excised.
*** n=14

Patient characteristics are shown in Table 1. The adjuvant group comprised of 20 patients in whom the primary tumour had been surgically removed. At diagnosis tumour stage had varied from T1 to T3. Of these patients, 65% had undergone lumpectomy with additional radiation therapy while 35% had undergone a modified radical mastectomy. All surgical and invasive procedures

as well as chemotherapy and radiation therapy were completed at least six months before enrolment. We consider the majority of the adjuvant group to be without active malignant disease. In the ATAC study [23] in breast cancer patients treated with endocrine therapy in the form of tamoxifen or anastrozole or a combination of these two therapies, the cancer recurred in either a localized or metastasized form in 7.2%, 8.5% and 9.1% of the patients respectively after three years. In the current study 1 patient (5%) in the adjuvant group was diagnosed with recurrence of the primary tumour, two years after admission and blood sampling.

In the metastasized group, which consisted of 20 breast cancer patients, at admission and blood sampling liver metastases had been found in 25%, skeletal metastases in 60% and pulmonary metastases in 40% of patients. Therefore, we consider this group as bearing a large tumour load. None of the breast cancer patients, neither in the adjuvant group nor in the group with metastasized disease, developed actual venous thrombosis during the course of disease or during the course of endocrine treatment.

In addition, we investigated 20 healthy female controls matched for age (mean age: 52 +/- 8 years), recruited from the hospital staff, who were not diagnosed with breast cancer and who were not treated with endocrine therapy in any form, including oral anticontraceptives. None of the healthy controls were on anti-platelet medication at the time of blood collection. All patients and controls agreed to participate in the study following informed consent. This study was approved by the local medical ethical committee (Medisch Ethische Commissie, OLVG, Amsterdam).

Blood collection and MP isolation

Blood samples were drawn with a 21-gauge needle without tourniquet. After discarding the first 4 mL, blood was collected into a 4 mL tube containing 3.2% citrate (BD, Plymouth, UK). MP rich plasma was prepared within 30 minutes after blood collection by centrifugation for 20 min at 1,550 x g at room temperature, without brake. Aliquots of plasma were snap frozen in liquid nitrogen, and then stored at -75°C until use. For MP isolation, 250 µL of plasma was thawed on ice for 60 min and then centrifuged for 30 min at 17,570 x g at 20°C. Subsequently, 225 µL of supernatant (i.e. MP-free plasma) was removed. The remaining 25 µL containing the MP pellet was resuspended in 225 µL of phosphate-buffered saline (154 mM NaCl, 1.4 mM phosphate, pH 7.4, PBS), containing 10.9 mM

trisodium citrate to prevent coagulation activation. Samples were centrifuged for 30 minutes at 17,570 x g at 20°C, thereafter, 225 µL of supernatant was removed and the MP pellet was resuspended in 125 µL of PBS.

Phenotypic analysis of plasma derived MP

Flowcytometric analysis was used to quantify and characterize plasma derived MP, as previously described [24]. Briefly, 5 µL of MP sample was diluted in 35 µl PBS containing 2.5 mM $CaCl_2$ (pH 7.4). Then, the samples were incubated for 30 minutes at room temperature in the dark with 5 µL Annexin V-APC (Caltag Laboratories, Burlingame, CA, USA) and/or 5 µL fluoresceinisotyocianate (FITC)-, and phycoerythrin (PE)- labelled anti-human monoclonal antibodies (mAb), or 5 µL isotype-matched control mAb.

For the phenotypic characterization of MP the following cell specific mAb were used: anti-CD14-PE (MΦP9, IgG_{2b}), anti-CD20-PE (L27, IgG_1), anti-CD31-PE (WM-59, IgG_1), anti-CD34-PE (8G12, IgG_1), anti-CD45-FITC (HI30, IgG_1,κ), anti-CD61-PE (VI-PL2, IgG_1), anti-CD142-PE (HTF-1, IgG_1, κ), anti-CD227-FITC (HMPV, IgG_1, κ) from BD (San Jose, USA); anti-CD54-PE (84H10, IgG_1), anti-CD62P-PE (CLB-Thromb/6, IgG_1) and anti-CD66b-FITC (80H3, IgG_1κ) from Immunotech (Marseille, France); anti-CD62E-PE(HAE-1f, IgG_1) from Kordia (Leiden, the Netherlands); anti-CD66acde-PE (CLB-gran/10, IH4Fc, IgG_1) from Sanquin (Amsterdam, the Netherlands); anti-CD106-FITC (1.G11B1, IgG_1) from Calbiochem (Darmstadt, Germany); anti-CD142-FITC (VD8, IgG_1) by American Diagnostics (Stanford, CT, USA); anti-CD144-FITC (BMS158FI, IgG_1) from Bender MedSystems (Vienna, Austria); anti-CD41-FITC (5B12, IgG_1, κ), anti-Glycophorin A -FITC (JC159, $IgG_{1\ \kappa}$) from DAKO (Glostrup, Denmark); anti-CD227-FITC (B24.1, IgG_1) from Genetex (Irvine, USA); anti-VEGF R2-PE (89106, IgG1) from R&Dsystems (Minneapolis, USA). Labelled isotype controls IgG_1 (X40) and IgG_{2a} (X39) were used from BD (San Jose, USA), and IgG_{2b}-PE (MCGb) from IQProducts (Groningen, the Netherlands).

After incubation, 360 µL PBS/calcium buffer was added and the samples were analysed on a FACS Calibur using Worklist Manager (BD) for 30 seconds during which the flowcytometer analysed approximately 30 µL of the suspension. Forward scatter and sideward scatter were set at logarithmic gain. To distinguish MP from events due to noise, MP were identified on the basis of their specific

forward scatter and sideward scatter characteristics, with gates set using in vitro platelet activation and MP generation data (results not shown), and by Annexin V positivity [25]. To identify Annexin V-positive events, a threshold was placed in a MP sample prepared without calcium. The number of MP/L plasma was calculated as previously described # $MP_{per\ minute}$ *(Volume(V) $_{tube}$/V_{minute}) *(V_{end}/V_{start}) * (1000/ $V_{labeled}$)= # events/mL [26]. Data were analysed with CellQuest-pro software (BD). The numbers of MP measured may vary up to 4 fold depending on FACS laser settings, which may alter due to the half yearly maintenance performed by the manufacturer. Therefore, MP measurements were always performed in series and patient and control plasma's were measured on the same days. In addition, standard pool plasma was measured daily to secure similarity in FACS flow sensitivity throughout the study. During measurements for this study, the numbers per mL were higher than previously observed [7] indicating the FACS was more sensitive for MP. However, the distribution of MP markers in the pool plasma was comparable.

Thrombin generation measurements

Thrombin generation (TG) was measured by means of the Calibrated Automated Thrombogram (CAT) method (Thrombinoscope BV, Maastricht, the Netherlands) [27,28]. TG was determined in the MP rich plasma samples or in the supernatant or pellet after centrifugation for MP isolation (as described above), TG (in 80 μL plasma or after addition of 10 μL MP pellet to 70 μL of normal pooled platelet-poor plasma, obtained from 80-90 healthy volunteers) was triggered by 1 pM TF and 4 μM phospholipids, 4 μM phospholipids alone, or buffer (no exogenous TF and phospholipids added). All reagents used were obtained from Thrombinoscope BV. TG was measured as fluorescence, read in a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland) equipped with a 390/460 filter set and thrombin generation curves were calculated with the Thrombinoscope software (Thrombinoscope BV).

Six parameters were derived from the thrombin generation curves: lag time (initiation phase of coagulation, defined as the time to reach 1/6 of the peak height), endogenous thrombin potential (ETP), time to peak, time to tail, peak height and velocity. Validation of the CAT method showed normalization of non-time-dependent parameters to be mandatory to obtain acceptable inter-assay variations [28]. Intra-assay variations for normalized parameters are typically

below 6%, inter assay variations below 8% [28]. Therefore, each thrombin generation measurement includes normal pooled plasma and both the ETP and peak height values are expressed as the ratio of value patient/value normal pooled plasma, expressed in percentages.

Markers of endothelial activation and plasmatic coagulation activation.

Mature von Willebrand factor (vWF) and vWF propeptide plasma levels were measured by ELISA as described previously [29]. Coagulation activation was assayed by measuring the concentration of prothrombin fragments 1+2 (F1+2), thrombin antithrombin (TAT) complexes and D-dimer. F1+2 levels were determined using the Enzygnost F1+2 (monoclonal) ELISA kit (Siemens Healthcare Diagnostics, Deerfield, USA). Normal F1+2 values are reported to range from 69-229 pmol/l (kit insert information as provided by the manufacturer). TAT was determined using the Enzygnost TAT micro testkit. Normal values are reported to range from 1-4.1 µg/l (kit insert information). D-dimer concentrations were determined on a Roche/Hitachi Modular P800 system using the Tina-quant assay (Roche Diagnostics, Indianapolis, USA). Decision limit in VTE exclusion protocols is 0.5 µg/ml (kit insert information). Antithrombin activity levels and fibrinogen concentrations (Claus method) were measured on a Sysmex CA-1500 System using the Berichrom Antithrombin III assay and Dade Thrombin Reagent with Dade Owrens Veronal Buffer, respectively (all Siemens Healthcare Diagnostics, Deerfield, USA).

Statistical analysis

We compared the difference of MP numbers, mature vWF and propeptide levels and results of thrombin generation between groups with non-parametric Mann Whitney *U* test or Kruskal Wallis test where appropriate. Bivariate correlations were estimated by Spearman's rank method (R). All tests for statistical significance were two-tailed and P values of <0.05 were considered significant. Analyses were performed using SPSS 16.0 for windows (SPSS Inc., Chicago, USA).

Results

Biological parameters

Most hematological parameters were similar between controls and breast cancer patients including haemoglobin, number of thrombocytes, antithrombin and fibrinogen values (Table 1). Breast cancer patients had significantly higher D-dimer and prothrombin fragments 1+2 levels than controls, indicating coagulation activation. Although not statistically significant, both parameters were highest in the group with metastatic disease. With regard to endothelial activation markers, breast cancer patients had higher levels of mature vWF and vWF propeptide compared with controls. Median (range) levels of mature vWF were 48 (26-100) nM for controls and 81 (35-245) nM for patients ($p<0.001$). For propeptide the median levels were 5.7 (4.1-10.3) nM for controls and 7.9 (3.6-29.2) nM for patients ($p=0.002$). This pattern of mature vWF levels being relatively more elevated than propeptide levels (Fig. 1) is compatible with chronic (as opposed to acute) endothelial activation [30].

Number and phenotypic characterization of MP

As is shown in Table 2 and Fig.2 the total numbers of Annexin V-positive MP were higher in breast cancer patients compared to controls ($p=0.03$) (median: adjuvant $10600x10^6$/L, metastasis $8875x10^6$/L, all patients $10000x10^6$/L, controls $6500x10^6$/L). MP of controls, as well as patients, were mainly platelet-derived (>98%). Accordingly, platelet-derived MP were also higher in breast cancer patients (CD41, $p=0.03$). With regard to MP derived from activated platelets, patients had higher numbers of MP expressing P-selectin (CD62P, $p=0.01$, median: adjuvant $325x10^6$/L, metastasis $400x10^6$/L, all patients $330x10^6$/L, controls $200x10^6$/L) (4.1 vs. 3.1% of total MP). The number of TF-positive MP ($p=0.001$ median: adjuvant $31x10^6$/L, metastasis $34x10^6$/L, all patients $33x10^6$/L, controls $15x10^6$/L) was higher in patients compared with controls, although the percentage of MP expressing TF is very low for both groups, namely <1%. The number of leukocyte-derived MP (CD45 positive) was higher in patients compared with controls ($p=0.005$, median: adjuvant $61x10^6$/L, metastasis $65x10^6$/L, all patients $61x10^6$/L, controls $42x10^6$/L). There was a correlation of leukocyte-derived MP with P-selectin positive MP ($R=0.35$, $p=0.005$) and with TF-positive MP ($R=0.53$, $p<0.001$). Endothelium-derived MP expressing CD144 were slightly increased in patients. Notably, MP expressing other endothelial markers (CD 62E, CD106 and CD54) did not differ significantly.

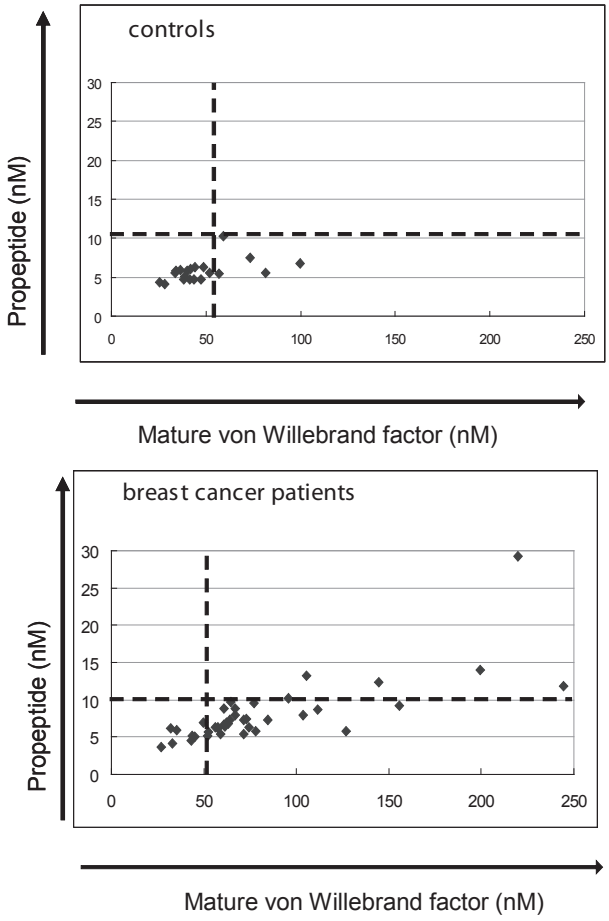

Figure 1: Relation between mature vWF and propeptide levels in patients with breast cancer and controls. Dotted lines represent the upper limit of the 95% confidence interval of, respectively, mature vWF and propeptide levels of the control group. Breast cancer patients have higher mature vWF ($p<0.001$) and propeptide levels ($p=0.002$) compared with controls.

For granulocyte-derived MP (CD66b, CD66e), B-cell-derived MP (CD20), monocyte-derived MP (CD14) and erythrocyte-derived MP (glycophorinA) no significant differences were found and all accounted for <1% of total MP count. To detect tumor cell-derived MP, CD227 (MUC1) expression was assessed. Only in breast cancer patients (four patients) very small numbers of CD227 positive MP were detected, accounting for <1% of total MP.

We found a positive correlation between CD227-positive MP and TF-positive MP, ($R= 0.68$, $p=0.001$), indicating that cancer-derived MP may carry TF.

Table 2. Numbers of MP with different phenotypic characteristics as determined by FACS analysis. MP were defined based on FSC/SSC pattern as well as Annexin V-positivity. Numbers are 10^6/L.

	Controls (n=20)	Breast cancer patients on endocrine therapy (n=40)	P-value (Mann Whitney U)
Total MP	6500	10000	0.03
Range	1100-13600	3450-37150	
%	100	100	
CD41+ MP	6400	9850	0.03
Range	1100-13200	2900-37000	
%	98.7	98.1	
CD62P+ MP	200	330	0.01
Range	18-450	10-900	
%	3.1	4.1	
CD45+ MP	42	61	0.005
Range	12-80	20-185	
%	<1	<1	
CD144+ MP	4	8	0.002
Range	0-180	0-125	
%	<1	<1	
Tissue Factor+MP	15	33	0.001
Range	0-25	8-200	
%	<1	<1	

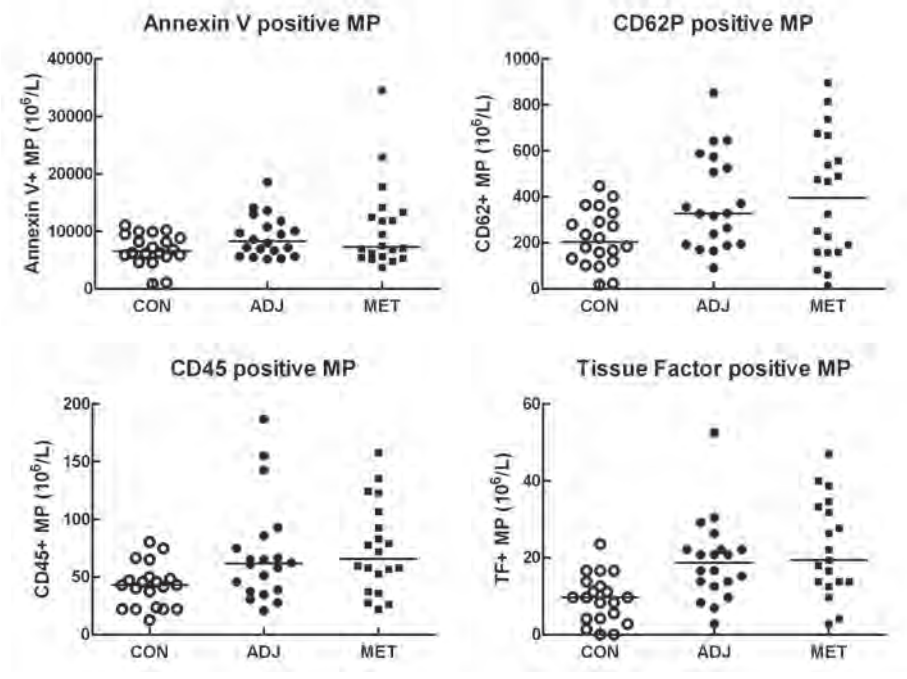

Figure 2: Numbers and subsets of MP in patients and controls.
Numbers of MP from specific cellular origin as defined by marker positivity in plasma from controls (CTR) and breast cancer patients using endocrine therapy divided in those without (ADJ) and with metastasizing (MET) disease. Lines represent median. Dotplots of total numbers of MP (Annexin V positive), activated platelet derived (CD62P+), leukocyte derived (CD45+) and TF positive MP are shown.

Relation of MP numbers and subsets with endocrine treatment and tumour load

No correlations were found between the numbers of MP from any cellular subset and the different types of endocrine therapy that patients used. In addition, no correlation was found between these parameters and the absence or presence of metastases.

Thrombin generation

The thrombin generation (TG) assay as performed with the CAT method allows variation in experimental conditions by variation of externally added TF and/or phospholipids.

As a first experiment, the TG was performed using the MP rich plasma as used for the FACS analysis. Using standard assay conditions i.e. initiation of the reaction with 1 pM TF and 4 μM phospholipids, a trend for higher endogenous thrombin potential (ETP) was observed in the patient groups compared with controls (p=0.09, Table 3). Since TF is a strong initiator of TG, it may render the test insensitive for small differences in endogenous TF. Therefore, the TG of MP rich plasma was tested after initiation with 4 μM phospholipids in the absence of exogenous TF, and again a higher endogenous thrombin potential (ETP) was observed for breast cancer patients compared with controls (p=0.05). Finally, in the absence of both exogenous TF and phospholipids, still slightly higher ETP was observed in plasmas from breast cancer patients compared with controls (p=0.13). Also, a higher peak height (p= 0.03) was observed in breast cancer patients compared with controls, indicating a role for the phospholipids content of the patient's plasma in hypercoagulability, probably consisting of MP, as is shown by a correlation (R=0.45, p=0.001) between peak height and the total number of MP (Figure 3).

As a control experiment in order to determine the role of the MP in the observed differences in TG, TG was performed in the supernatant plasma after removal of MP by centrifugation. In this supernatant, in the absence of both exogenous TF and phospholipids, TG was very low. ETP was 8%, peak height 6% and lagtime 162% of what was observed in MP rich plasma, indicating that MP contributed most to TG in MP rich plasma and may account for the differences found between patients compared with controls.

Table 3: Results of thrombin generation experiments
Results are shown as median (range) for MP-rich plasma (row 1-3, 5) and standard plasma to which isolated MP were added (row 4). Different conditions were used, adding 1 pM TF and 4 μM PL (row 1), 4 μM PL only (row 2) or without exogenous PL and TF added (buffer, row 3-5). P-values representing differences between all patients and controls.

	Controls	All patients	Adjuvant group	Metastasis group	P-value
ETP plasma (nM.min) (1 pM TF and 4 μM PL)	954 (669-1664)	1018 (536-1295)	1057 (799-1295)	980 (536-1250)	0.09
ETP plasma (nM.min) (4 μM PL)	888 (219-1538)	947 (325-1231)	967 (670-1231)	915 (325-1188)	0.046
ETP plasma (nM.min) buffer	899 (604-1663)	1014 (297-1347	1076 (743-1347)	958 (297-1174)	0.13
ETP microparticles (nM.min) (buffer)	1029 (822-1234)	1105 (893-1336)	1117 (893-1336)	1100 (990-1212)	0.03
Peak Height plasma (nM) (buffer)	72 (32-171)	115 (13-257)	119 (37-181)	106 (13-257)	0.03

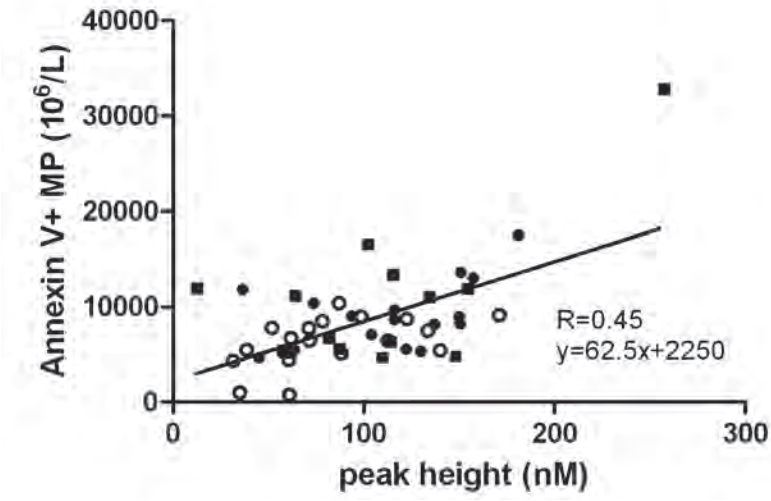

Figure 3: Correlation between peak height and the total number of MP in plasma in the absence of exogenous tissue factor and phospholipids**.**
Data are shown from plasma of breast cancer patients using endocrine therapy, with (■ n=15) and without (● n=17) metastases and of controls (o n=19). R=0.45, p=0.001.

Lastly, to verify the role of MP in TG and to exclude the role of the plasma, the isolated MP were added to normal pooled plasma and tested for TG. In these conditions a higher ETP was observed for breast cancer patients compared to controls (p=0.03) (Figure 4), suggesting that the differences observed were MP and not plasma related. Both disease progression and type of endocrine therapy used did not cause significant differences in thrombin generation.

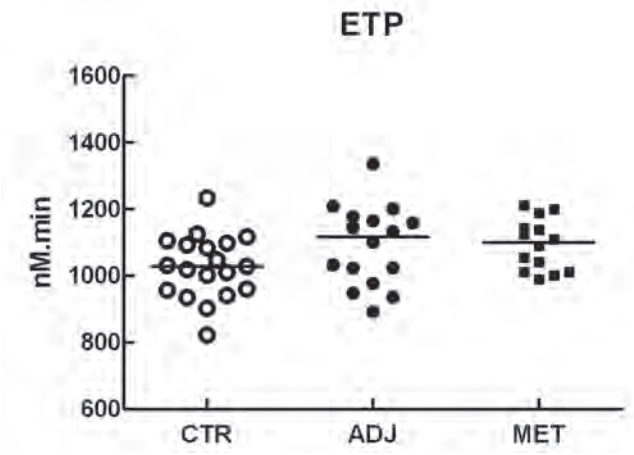

Figure 4: Thrombin generation of isolated MP added to standard plasma in the absence of exogenous TF and phospholipids. The result of the endogenous thrombin potential (ETP) is shown for controls (CTR o) and for breast cancer patients without (ADJ •) and with metastatic disease (MET ■). Lines represent median. Breast cancer patients (ADJ and MET together) have a significantly higher ETP (p=0.03) compared with controls.

Discussion

Our study assessed whether MP of patients with breast cancer treated with endocrine therapy would have a more prothrombotic profile as compared to those of healthy controls. In addition, we established whether the presence of metastasized disease would augment such a prothrombotic effect. Indeed, increased F1+2 fragment, TAT complex and D-dimer values in the patient group together with increased mature vWF levels accompanied by only a modest increase in vWF propeptide levels provided evidence for ongoing coagulation- and endothelial activation.

As parameters of MP procoagulant properties we used numbers and origin of MP by flowcytometry and MP-related procoagulant potential by thrombin generation analysis. Our data show that compared to non diseased individuals breast cancer patients (both the adjuvant- and the metastasized group) on endocrine therapy have higher absolute numbers of MP that are predominantly platelet derived (>98%), indicating normal proportion of platelet derived MP.

In addition, patients have significantly more CD62P-positive MP than controls indicating platelet activation as has been described for breast cancer patients

before [8,11,31]. CD62P or P-selectin is stored in secretory granules of resting platelet and endothelium and is rapidly translocated to the cell surface upon activation. The CD62P-positive MP may interact with leukocytes, inducing the formation of leukocyte-derived MP, which can express TF and so enhance clot formation [12,32,33]. Indeed a correlation between leukocyte-derived MP and P-selectin- as well as TF-positive MP was observed.

The patient MP were procoagulant since the ETP was increased in breast cancer patients on endocrine therapy, and this was related to the MP rather than to a plasma component since the increase of the ETP was retained when thrombin generation was performed with isolated MP. The increased MP-induced thrombin generation in patients may have several causes. Firstly, the larger quantity of MP in breast cancer patients provides more negatively charged phospholipid procoagulant surface. Secondly, MP of patients may be qualitatively different, specifically the increased numbers of leukocyte derived MP may have procoagulant properties, i.e. an increased amount of TF expression. In both scenarios, the increase in MP procoagulant surface or properties, the end result is a procoagulant state as reflected by the elevated thrombin generation parameters.

As most adjuvant patients remain disease free after 3 years (93% in ATAC study) [23], the lack of difference between patients with and without metastases was unexpected. The surprising element was the relative lack of abnormalities in the metastatic group. We observed markedly lower numbers of MUC1-positive MP than previously described by Tesselaar and colleagues [8]. One explanation for the low numbers could be that patients with metastasized breast cancer on endocrine treatment generally have a suppressed form of disease and therefore differ significantly from the patients included in the Tesselaar study at the time of diagnosis of metastasized cancer and not (yet) treated with endocrine - or other therapy. The same patient characteristic may also account for our observation that breast cancer patients with disseminated disease do not express higher numbers of MP compared to those without active malignant disease (the adjuvant group), which is in discordance with previous observations [8,11,31] all of which included patients before any therapy, including endocrine therapy, was started. Preanalytical and analytical methods, being similar, or a putative lack of statistical power are both unlikely to underlie this intriguing difference.

Numbers of patients with disseminated disease were higher in our study, which was designed to hold sufficient statistical power to detect differences between patients and controls of similar magnitude as described in these previous studies [8,11,31]. Apparently, by including stable metastatic breast cancer patients on endocrine therapy, we have selected a group more similar to patients with local disease, illustrated by the overall modest effects on MP profile that have also been demonstrated by others. The similarity between the two patients groups is also reflected in the finding that concurrent with total MP numbers, the relative size of MP subsets from various sources and thrombin generation did not differ between the metastasized and adjuvant group. It may be speculated that differences in MP and thrombin generation parameters between breast cancer patients on endocrine therapy and controls are linked to the use of endocrine therapy rather than to the malignant disease. However, the type of endocrine therapy used (anti-estrogens or aromatase inhibitors) was not related to any of the parameters studied.

Several limitations should be taken into consideration. Since we did not include a control group of healthy women treated with endocrine therapy or an untreated group of breast cancer patients, the exact contribution of endocrine therapy to the presence of (subsets of) MP remains to be elucidated. Also, we cannot rule out an effect of prior tumour presence, chemotherapy, lumpectomy or radiation therapy in the adjuvant group on MP numbers and subsets. Clearly, the small differences and the large scale of distribution of values imply that larger patient groups need to be included in future studies on MP profiles in breast cancer patients.

In conclusion, breast cancer patients receiving endocrine therapy have increased numbers of mainly platelet derived MP and a higher MP-dependent thrombin generation, irrespective of the presence of metastatic disease or the kind of endocrine therapy used. Altered MP subset characteristics in these patients, especially the higher number of activated- platelet derived MP and leukocyte derived MP, may in part explain a heightened procoagulant state in breast cancer patients treated with endocrine therapy.

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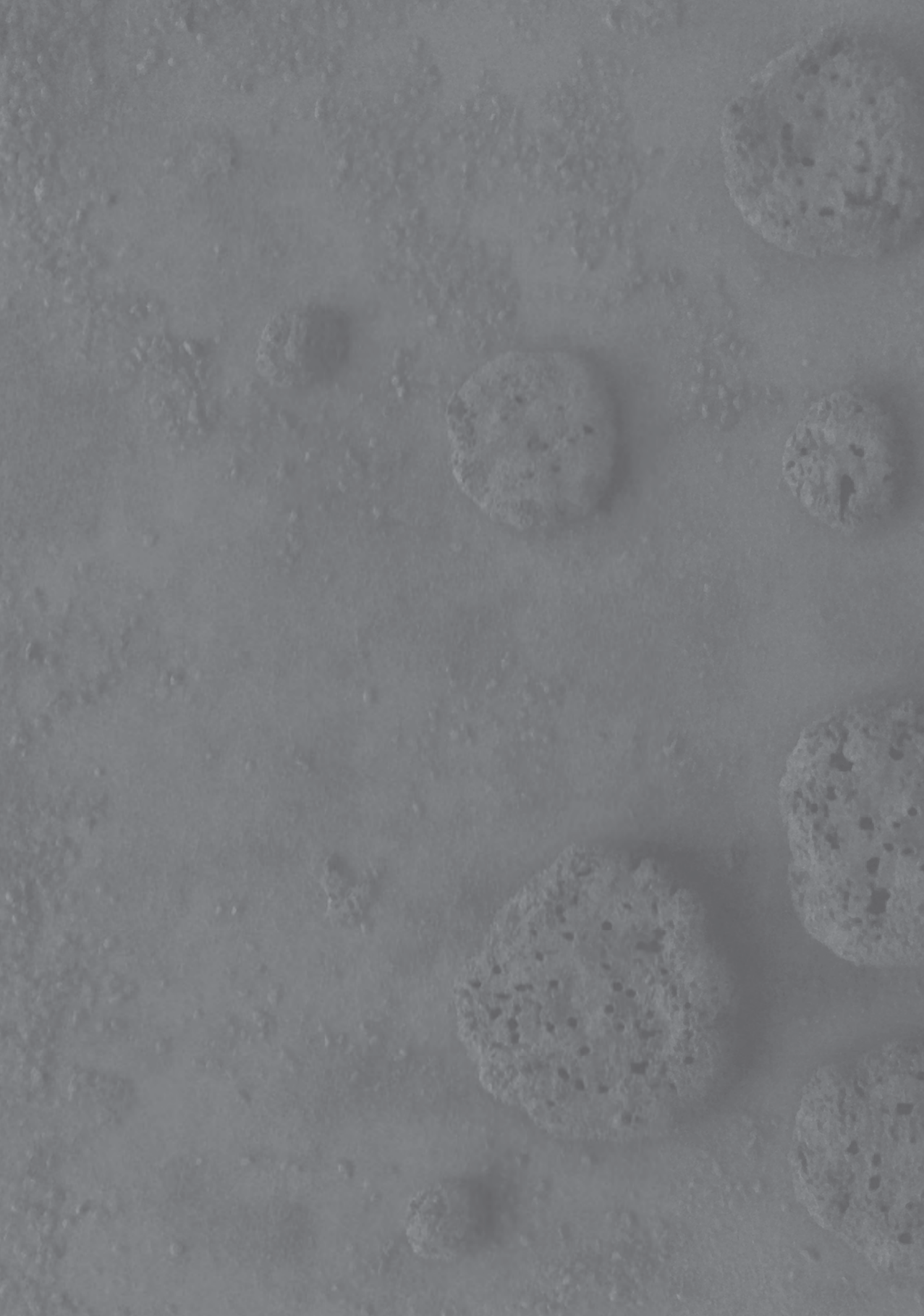

Chapter 6

Procoagulant myeloblast-derived microparticles in AML patients: changes in numbers and thrombin generation potential during chemotherapy

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Journal of Thrombosis and Haemostasis 2011; 9: 223-226

Several lines of evidence indicate that cell-derived microparticles (MP) are functional entities involved in thrombotic disease [1]. Negatively charged phosphatidylserine in the MP membrane may provide a surface for catalytic complexes such as the tissue factor (TF)/VIIa complex. In addition, MP may express TF and PSGL-1, the latter being able to bind platelet- and MP-bound P-selectin (CD62P), promoting thrombus development [2, 3].

Patients with cancer are at increased risk of thrombosis [4, 5]. This may involve MP [6,7], possibly via increased MP numbers and/or altered MP origin and functional properties in malignant disease. In a proof of concept study we addressed the changes in MP phenotypical and procoagulant (sub) population characteristics in three consecutive patients with acute myeloid leukaemia (AML), providing highly dynamic conditions of malignant cell proliferation and chemotherapy-induced cell death in the circulation. Blood samples were collected during routine blood sampling before, during and late after the first cycle of chemotherapy. All patients had been included in the HOVON 42 AML treatment trial (http://www.hovon.nl/trials) (approved by the local medical ethical committee [Medisch Ethische Commissie, OLVG, Amsterdam]), agreed to participate in the study following informed consent.

Preanalytical conditions and flowcytometric analysis of MP and the antibodies used, were as described [8]. Antibodies used specifically to define myeloblast origin were: anti-CD117-PE (104D2, IgG1) (Dako, Glostrup, Denmark); anti-HLA-DR-PE (L243, IgG2a), anti-CD13-PE (WM15, IgG1), and anti-CD34-PE (8G12, IgG_1) (BD, San Jose, California, USA). In addition, anti-CD162 (PSGL-1)-PE (KPL-1, IgG1) and Mouse Pure (X40, IgG1) (BD) were used. Because MP are small and labelling is often less efficient than with whole cells, expression of markers was determined as positive events (% of MP) and as mean fluorescent intensity (MFI) of MP. FACS data were analyzed using CellQuest software (BD). Statistical significance of MFI changes was analysed with Student's *t*-test.

To assess MP procoagulant properties as MP-specific thrombin generation, we adapted the Siemens Endogenous Thrombin Potential (ETP) assay on a BCS-XP analyser (Siemens Healthcare Diagnostics, Marburg, Germany). The MP-specific ETP reaction was initiated using 30 times diluted Actin FS (Siemens) instead of Innovin (Siemens), thus reducing the phospholipid content and not providing

reagent-derived TF in the ETP reaction mixture. Linear dose-response relations between MP numbers and the ETP-parameters ETP (area under the curve or total amount of thrombin) and lag-time (time from reaction start to start of thrombin generation), were observed when washed MP were added to MP-poor plasma (data not shown). ETP was determined in three different MP dilutions in MP-poor pooled plasma and MP-specific ETP/1.000 MP was calculated after correction for background ETP of MP-poor plasma during each analytical session.

Patient 1, a 28-year-old female, was diagnosed with AML FAB-subtype M2. On admission (day 0), her leukocyte count was 68.5 x 10^9/L (27% blasts), with a platelet count of 41 x 10^9/L and a hemoglobin concentration of 6.1 mmol/L. Treatment consisted of three cycles of chemotherapy with cytarabin and idarubicin, amsacrine and high dose cytarabin, and etoposide and mitoxantrone (HOVON 42 protocol; http://www.hovon.nl/trials).

Patient 2, a 56-year-old male was diagnosed with AML FAB-subtype M2. On admission his leukocyte count was 277.7 x 10^9/L (84% blasts), with a platelet count of 68 x 10^9/L and a hemoglobin concentration of 2.3 mmol/L. Induction therapy was as in patient 1.

Patient 3, a 21-year-old male presented with a relapse after treatment for AML FAB-subtype M4. His leukocyte count was 1.4 x 10^9/L (1% peripheral and 26% bone marrow blasts), with a platelet count of 46 x 10^9/L and a hemoglobin concentration of 6.9 mmol/L. Reinduction therapy was initiated with high dose cytarabin combined with intrathecal methothrexate.

Blast of patients expressed CD13, CD34, CD117 and HLA-DR. Late after chemotherapy, patient 1 and 2 were in complete remission (<5% bone marrow blasts, no peripheral blasts and a normal peripheral blood count). Patient 3 had a partial remission (8% bone marrow blasts).

The numbers of MP per µL plasma determined at day 0 of chemotherapy were 1206, 3980 and 2756 for patients 1,2 and 3, respectively. Number of MP in control pooled plasma was 3728 ±514 (SD). During chemotherapy (days 1-7), the numbers of MP declined in all three patients (nadir as sampled: 349, 2587 and 2312 in patients 1, 2 and 3, respectively). In their (partial) remission samples,

increased numbers of MP were observed (3108, 10111 and 8163 in patients 1,2 and 3, respectively).

In control plasma, 95-98% of all MP were platelet derived (CD41+) as described earlier [8]. Before treatment, this percentage was 60%, 43% and 99% in patients 1,2 and 3, respectively, indicating that significant subpopulations of MP of patients 1 and 2 but not patient 3 might derive from other, putatively AML blastoid, cellular sources.

Indeed, MP from patients 1 and 2 before treatment expressed myeloblast markers (Fig. 1) that were not found on MP of control pooled plasma or MP of patient 3. MP from patient 1 were positive for CD13 (60%), CD34 (16%) and CD117 (9%). MFIs of these markers were, respectively, 40.9, 12.3 and 8.5 on patient MP, vs. 3.2, 5.0 and a 3.2 on control MP (all $P < 0.001$). MP of patient 2 expressed CD117 (13%); MFI 10.2 ($P < 0.001$). In addition, MP of patients 1 and 2 expressed HLA-DR at a considerable intensity at the start of treatment (44% and 61% in patients 1 and 2, respectively, vs. 10% in control pooled plasma). MFIs were 47.6 and 109.8 on MP of patients 1 and 2, respectively, vs. 11.2 on control MP ($P < 0.001$). HLA-DR expression on MP appeared to be myeloblast derived and not related to an immune response, because in a control experiment it was not found on MP from three patients with sepsis and disseminated intravascular coagulation and three patients with metastasized breast cancer (data not shown). The various marker-positive MP population sizes indicated combined expression of platelet and leukocyte markers on single particles. Indeed, the myeloid markers were not only on CD41 negative MP, but also partly co-expressed with CD41 (Fig. 1).

Thus, in patients 1 and 2 before chemotherapy high numbers of circulating leukemic cells correlated with relatively low percentages of platelet-derived MP and large proportions of MP that appeared to be myeloblast-derived. MP of patient 3, who presented without detectable peripheral circulating myeloblasts, did not express immature myeloid markers, indicating that at least in this case, the bone marrow blasts did not release MP into the blood stream.

During chemotherapy the absolute and relative numbers of such MP declined (Fig. 1). Late after chemotherapy 4% of MP of patient 1 remained positive for

CD13 but no other myeloid markers were found on MP of any of the patients in their remission samples. Also, aberrant HLA-DR expression was not found in the remission samples of any of the patients. In addition, in all remission samples >95% of MP expressed CD41. These data illustrate the disappearance of myeloblast-derived MP following chemotherapy.

The presence of myeloblast-derived MP was related to an elevated ETP (Fig. 1). In pre-treatment samples of patients 1 and 2 but not patient 3, MP-specific ETP (ETP per MP, or slope of the dose-response curve) was higher than in control MP. When these MP disappeared during chemotherapy, the MP-specific ETP declined and in remission samples values approximated those of control MP.

Taken together, normal or low numbers of MP were present in AML patients before chemotherapy but presence of MP expressing immature myeloid markers was related to MP-specific ETP, indicating that these MP were highly procoagulant.

We propose that procoagulant myeloblast-derived MP may play a role in the thrombosis risk of AML patients. Combined expression of platelet and leukocyte markers on single particles that may result from fusion of MP, may change catalytic processes by colocalizing molecular actors [9]. Of the actors possibly involved [2,3], interestingly, PSGL-1 expression corresponded with the myeloid marker expression (data not shown). Although a direct role for PSGL-1 in the enzymatic processes leading to thrombin generation as measured here is unlikely, it is thought to play a role in thrombus formation *in vivo* and may thus contribute to a prothrombotic profile [2]. Prechemotherapy MP of patient 2 expressed TF (7%) and this was accompanied by reduced lag times (not shown) in addition to increased ETP, suggesting that TF on these MP may provide them with a procoagulant function, as has been described for other cancer-related MP [7,9]. However, TF was not found on MP of patient 1 and therefore the high procoagulant properties of these MP remain to be explained.

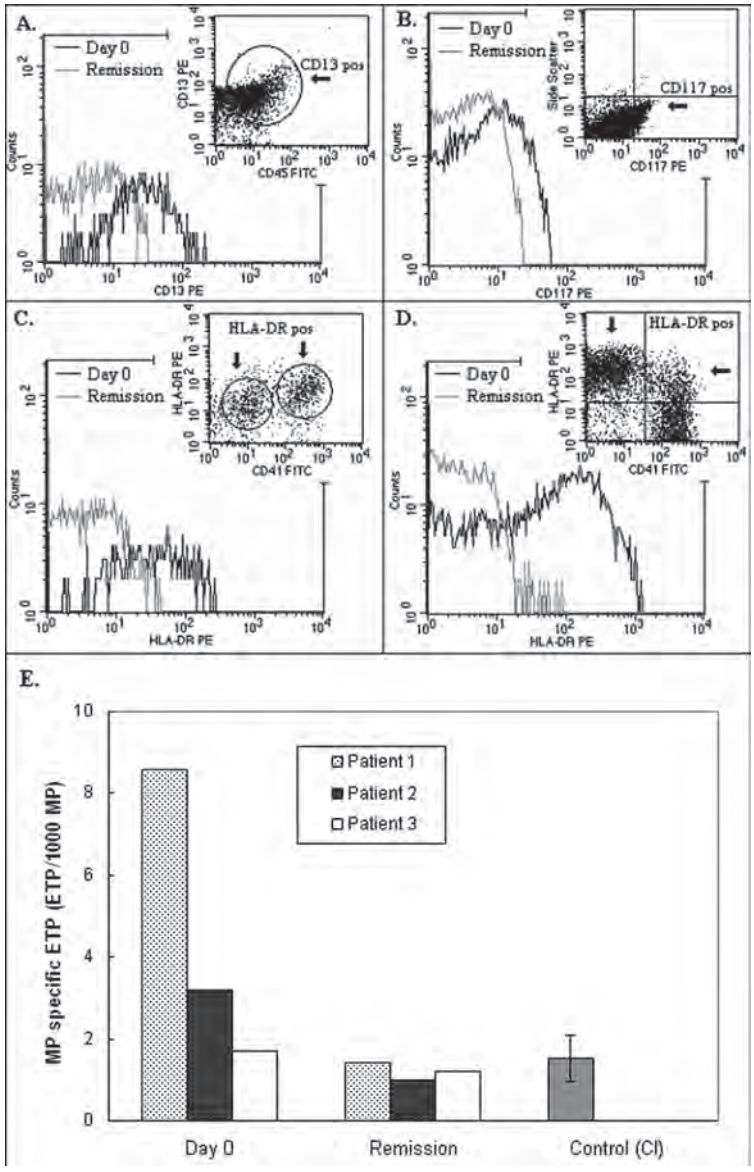

Fig 1.Presence of MP with aberrant expression of immature myeloid markers is related to an increased MP-specific ETP. (A/B) Histograms showing increased expression of myeloblast markers on MP of patients 1 (A; CD13) and 2 (B; CD117) before treatment compared with remission samples. Inserts show scatter plots to illustrate CD13/CD45 expression on MP of patient 1 (A) and CD117 expression on MP of patient 2 (B). (C/D) Histograms showing increased expression of HLA-DR before treatment compared with remission samples on MP of patients 1 (C) and 2 (D). Inserts show scatter plots illustrative of CD41 distribution on MP on day 0. HLA-DR was observed on CD41+ and CD41- MP of patient 1 (C) but mainly on CD41- MP of patient 2 (D). (E) Endogenous thrombin potential (ETP) of patient MP in normal MP-poor plasma. ETP per MP was much higher for MP of patients 1 and 2 before treatment, as compared with MP of control pooled plasma. ETP per MP declined during treatment and MP-specific ETP of remission samples was similar to that of MP of control pooled plasma.

Despite cell death inducing chemotherapy, an early -treatment-associated decrease in MP was observed. Therefore, either chemotherapy-associated cell death does not lead to an overt MP production, or such MP have a very high clearance rate. The increased numbers of MP in all the remission samples may be explained as a postchemotherapy effect, possibly reflecting sustained kinetic bone marrow proliferation abnormalities in early remission. Although the MP found in early remission did not have altered procoagulant properties compared with control MP, the high numbers in which they may circulate may contribute to a prothrombotic profile as well.

This is the first study describing increased MP-specific procoagulant activity in relation to a malignant myeloblast-derived population of MP. Further studies are necessary to identify the key players and any possible relation to the increased thrombotic risk of AML patients.

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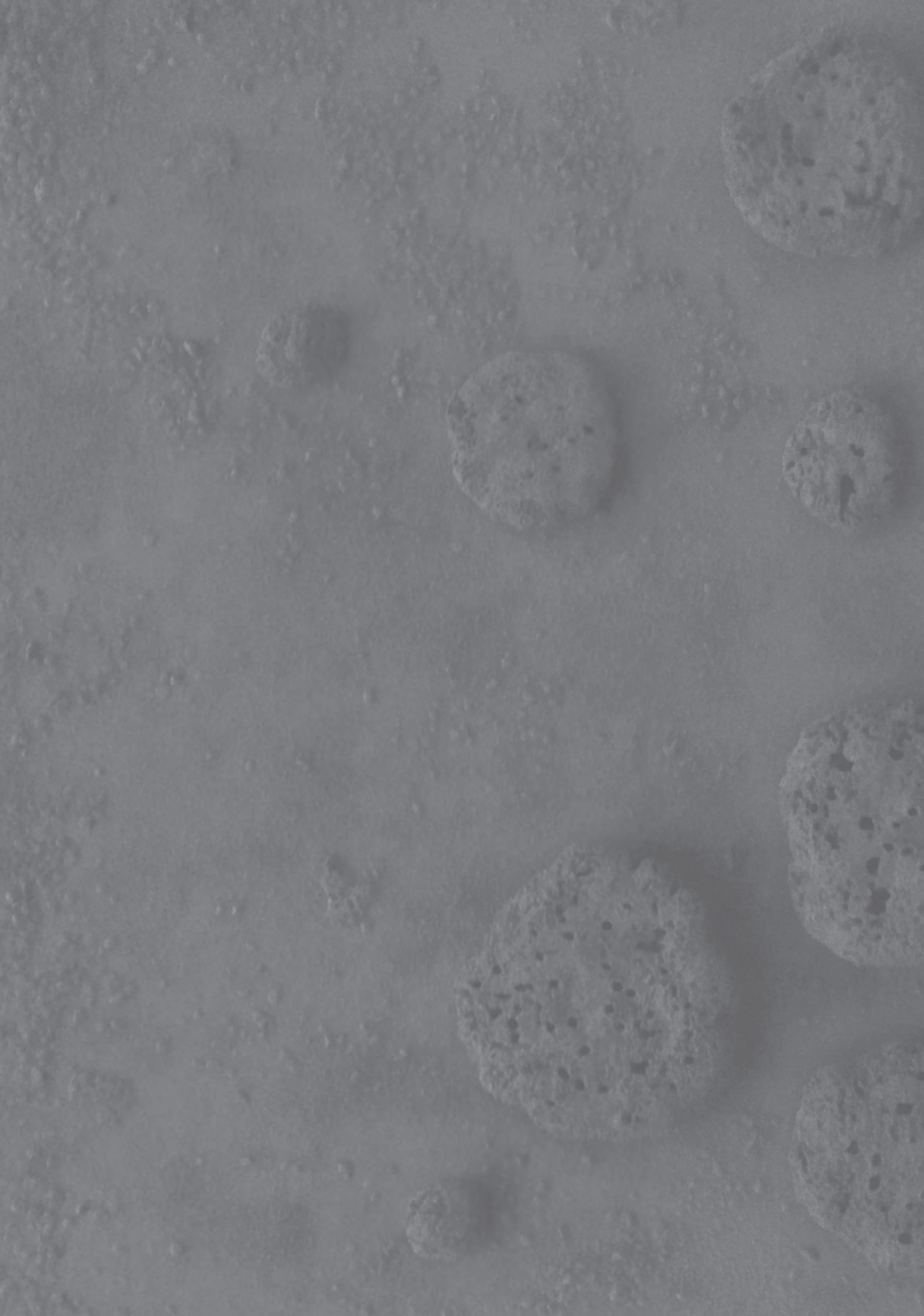

Chapter 7

Dynamics of microparticle populations during a bungee jump with or without beta-receptor blockade; a stress model

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Submitted

In response to stress all circulating blood cells as well as endothelial cells release cellular microparticles (MP) [1]. MP are membranous fragments less than 1 micrometer in diameter that express at least some of the antigenic markers distinctive of the parent cells. Cell activation by numerous agents stimulates their release, for example complement factors and cytokines, or cellular processes such as apoptosis, oxidative injury and shear stress [2]. MP are both cause and consequence of vascular changes, and play a role in thrombosis and haemostasis by providing a phosphatidylserine surface for procoagulant molecules including tissue factor [2]. However, the appearance of MP in response to stress is not well studied and an *in vivo* model is lacking. *Ex vivo* whole blood studies in which endotoxins are applied to establish MP release, showed an increase in MP count after 6-24 hours [3,4]. Aras *et al.* showed a 2-fold increase in MP levels three hours after intravenous endotoxin infusion in healthy volunteers and these MP levels returned to baseline after 8 hours [5].

We hypothesized that a bungee jump would be an adequate *in vivo* model to study the effects of acute stress on the generation of MP. Recently, this model was indeed used in a Dutch collaborative project focussing on immune- and haemostatic responses to stress. Twenty healthy male volunteers aged 18-35 years made a bungee jump from a sixty meter high crane. In order to study the effect of beta adrenergic receptor blockade, an effective agent for attenuating most physiological responses to stress, propranolol was administered to half of the volunteers for three days prior to the jump (forty milligram three times a day). We investigated the influence of the bungee jump on levels of circulating MP and their cellular origin. Results of the main study have been described previously [6,7]. In short, bungee jumps took place under supervision of an experienced commercial crew. The study was reviewed and approved by medical ethics committee of the Academic Medical Center in Amsterdam, The Netherlands and written informed consent was obtained from all subjects. On the morning of the study day an intravenous access catheter was placed in the cubital vein. Exactly two hours before the jump the first blood sample was drawn (20 ml), referred to as baseline sample. Subsequent samples were drawn directly before the jump (while elevated at jump level), immediately after the jump and two hours later.

For MP measurements, blood was collected into a 4 mL tube containing 3.2 % citrate (Becton-Dickinson, Breda, the Netherlands). MP isolation and phenotypic analysis of MP were performed as previously described [8]. To identify annexin V-positive events, a threshold was placed in a MP sample prepared without

calcium. In some samples (n=24) high numbers of erythrocyte-derived (glycophorin A-positive) MP were observed, and those samples appeared to have a high haemolysis index. As the numbers of erythrocyte-derived MP in these samples could not be corrected for haemolysis they are excluded from the present analysis. These samples were equally distributed between time points and between subjects.

To assess MP procoagulant properties as MP-dependent thrombin generation, we adapted the Siemens endogenous thrombin potential (ETP) assay on a BCS-XP analyser (Siemens Healthcare Diagnostics, Marburg, Germany) as previously described [9]. The reaction was initiated using 30 times diluted Actin FS (Siemens) instead of Innovin (Siemens), thus reducing the added amount of phospholipids and not providing reagent-derived TF in the ETP reaction mixture. The thrombin generation curves were analysed with Curves (v 1.0 spec. 3.2, Dade Behring), and three parameters were calculated: ETP (endogenous thrombin potential, or area under the curve that represents the total amount of thrombin generation), lag time (time from onset of the reaction to start of thrombin generation) and peak height (represents the peak level of thrombin generation). Linear dose-response relations between MP numbers derived from normal pooled plasma and the ETP parameters were observed within certain limits of numbers of MP added (data not shown). ETP was determined in two different MP dilutions (2.5x and 4x) in MP-poor pooled plasma for each subject, ascertaining that at least one data point would fit within the measuring range. Results are described as medians. We used the non-parametric Mann Whitney *U* test to compare groups and changes related to time to jump were analysed by one way analysis of variance (repeated measures).

Hemodynamic effects (heart rate and blood pressure) were published previously [6,7]. In short, mean arterial blood pressure (MAP) increased directly prior to the jump compared with baseline in all subjects. Directly after the jump, MAP had decreased again, which was followed by a further decrease 2 hours after the jump. Heart rate increased in a similar fashion, but this change was absent in subjects pre-treated with propranolol.

Stress as provided by a bungee jump resulted in large changes in MP numbers. In all subjects MP levels increased right after the jump (Figure 1) and after two

hours these numbers had returned to baseline levels. Right after the jump an immediate and major increase in MP numbers was observed for platelet-derived MP (CD41-positive, $3230x10^6$/L vs. $1850x10^6$/L, $p<0.001$), leukocyte-derived MP (CD45-positive $43x10^6$/L vs. $28x10^6$/L, $p<0.05$) and monocyte-derived MP (CD14-positive $41x10^6$/L vs. $9x10^6$/L, $p<0.001$). No differences were observed for endothelial-derived MP (CD62E and CD144), MP expressing markers of platelet activation (CD62P and CD63) and tissue factor-positive MP.

The increase of MP numbers was influenced by the pre-treatment with propranolol. Baseline MP levels did not differ between the propranolol and control group. However, right after the jump the propranolol group had even higher levels of platelet-derived MP (propranolol $4025x10^6$/L vs. controls $1920x10^6$/L; $p<0.05$). In contrast, monocyte-derived (CD14-positive) MP were lower in the propranolol group as compared to the controls ($37x10^6$/L vs. $93x10^6$/L; $p<0.05$).

Right after the jump increased numbers of MP resulted in an increased thrombin generation peak height (41 vs. 35 mA/min, Figure 1F). Peak height correlated with the total numbers of MP ($R=0.57$, $p<0.001$). No differences in ETP and lag time were observed, neither were differences observed between the propranolol group and the control group.

We studied whether an acute severe stress response as induced by a bungee jump influences MP numbers and subsets, and whether this was affected by beta-adrenergic blockade. Bungee jumping temporarily increased the numbers of circulating platelet-, leukocyte- and monocyte-derived MP. This increase was related to stress during the jump rather than to stress prior to the jump since the highest number of platelet-derived MP was observed right after the jump. Within 10 minutes an almost two-fold increase in leukocyte-derived MP was observed. This impressive increase of MP right after the jump could be due to cytokine release, oxidative injury or apoptosis [2]. Two hours after the jump MP levels had returned to baseline in all volunteers. Visual inspection of Figure 1A produced a crude estimate of the *in vivo* half-life of MP of 1 hour or less [5]. This is in line with previous studies that described a half-life of MP varying from 5 minutes to 3 hours [5,10].

The difference in MP levels in the propranolol and control group was observed right after the jump, and not at baseline, indicating that the effect of propranolol on MP levels is dependent on stress as induced by a bungee jump. Moreover, in this stress model propranolol has differential effects on MP-formation as platelet- and leukocyte-derived MP release was facilitated in the propranolol group, and monocyte- and endothelial-derived MP release was inhibited. As the beta receptor to which propranolol is targeted is present on the membrane of leukocytes [11], monocytes [12], endothelial cells [13] and platelets [14], they could all respond to propranolol.
The increase of platelet-derived MP in the propranolol group was unexpected since propranolol is known to suppress platelet aggregation [15]. Sims *et al.* [16] described that shedding of MP induced by platelet activators may be uncoupled from other platelet responses necessary for platelet aggregation, as different platelet agonists have differential effects on platelet MP composition and MP membrane components. The mechanisms by which platelets shed MP are largely unknown, but probably require an increase in intracellular calcium [17]. Cyclic adenosine monophosphate (cAMP) might also be involved as cAMP inhibited MP formation [18], probably by lowering intracellular calcium. There is a ß receptor-mediated cAMP increase in stress [18], which is inhibited by propranolol [14]. Thus, theoretically propranolol can lower cAMP in the cell by blocking the β receptor, thereby increasing MP shedding. Thus platelet aggregation and the shedding of platelet derived MP follow different pathways.

In conclusion, our results indicate that bungee jumping temporarily increased numbers of circulating platelet-, leukocyte- en monocyte- derived MP. Interestingly, pre treatment with propranolol resulted in an unexpected and marked additional increase in leukocyte- and platelet-derived MP as induced by acute stress. The beta receptor may therefore be involved in suppressing MP formation in leukocytes and platelets. The mechanisms by which propranolol influences the formation of MP remains to be elucidated.

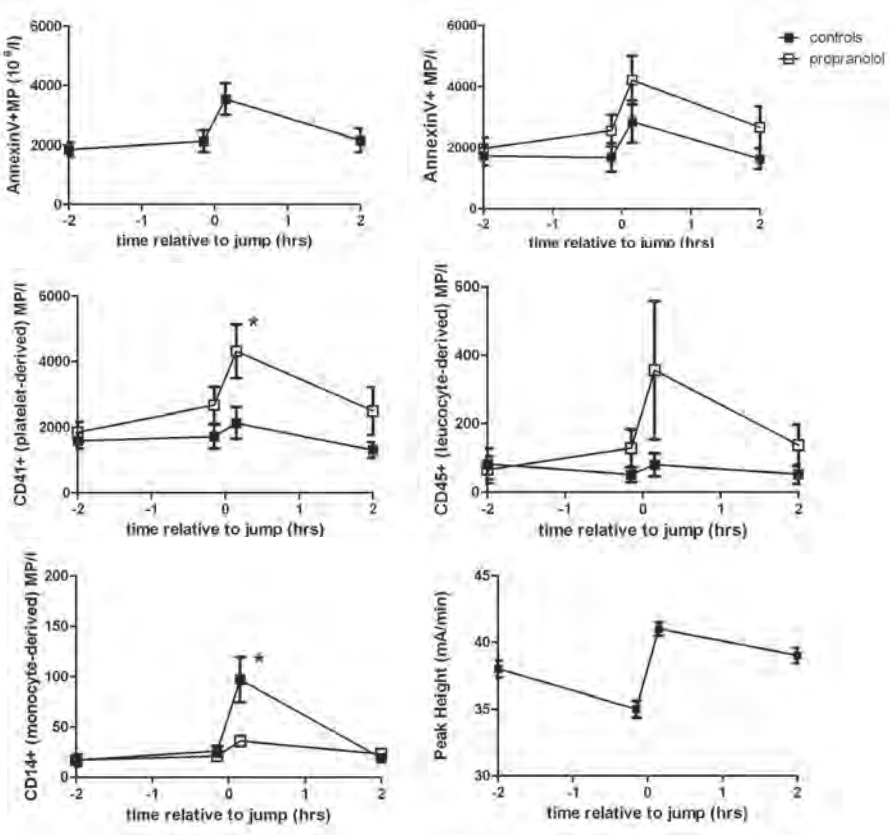

Figure 1A-E: Numbers of Annexin V-positive, platelet-, leukocyte- and monocyte-derived microparticles relative to the jump are shown. All values changed significantly during the jump ($p<0.05$). In platelet- and monocyte- derived MP a difference was observed between the propranolol and control group right after the jump. Figure 1F: Peak Height results relative to the jump are shown. Results are shown as medians and SEM .* $p<0.05$ propranolol vs. controls.

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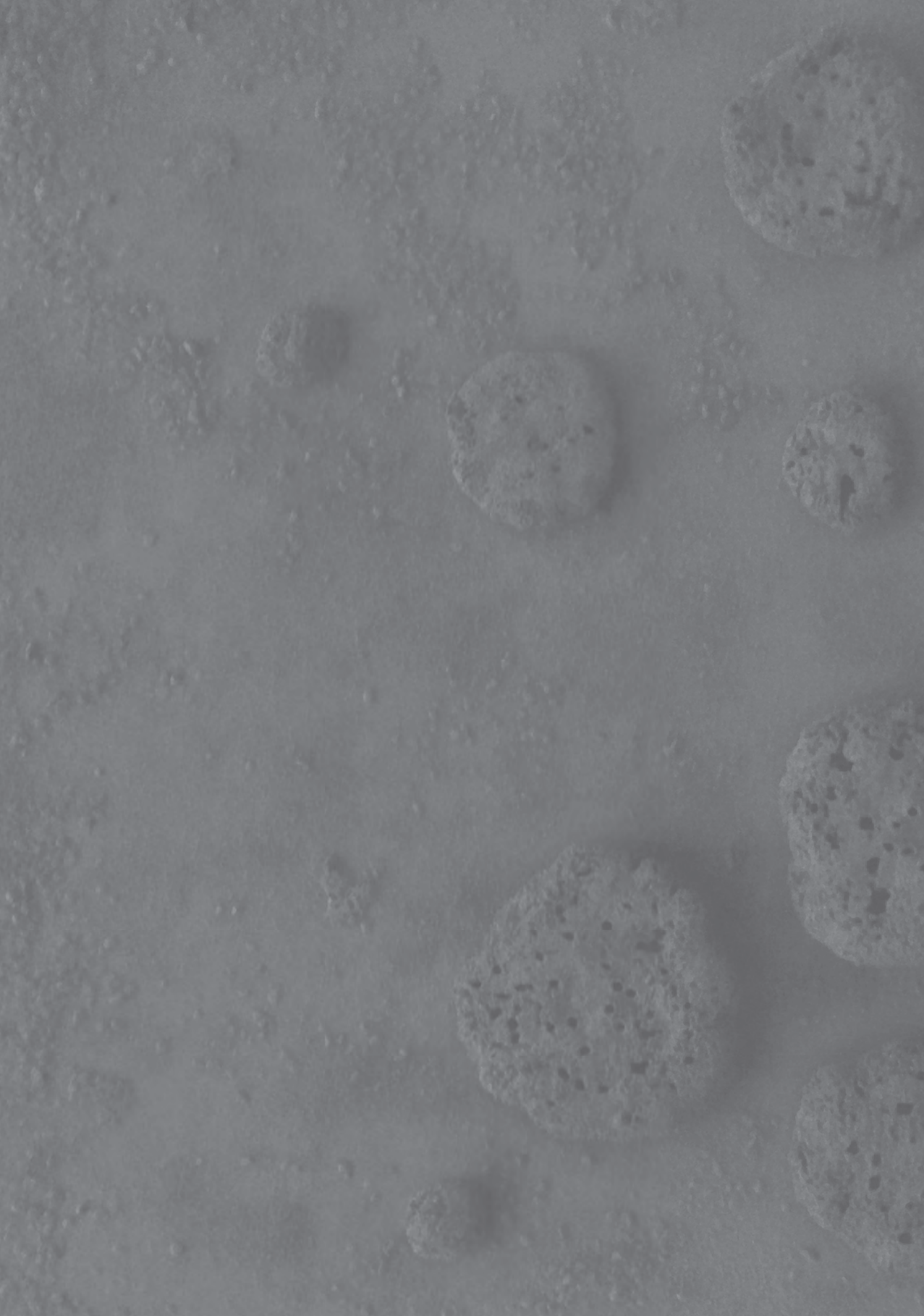

Chapter 8

Summary, general discussion and future perspectives

Summary

The potential influence of MP in many different thrombotic and bleeding diseases has been investigated. MP are procoagulant by nature as they express phosphatidylserine on their membrane, providing a surface for coagulation. Besides, expression of tissue factor, the initiator of coagulation, by MP contributes to clot formation as well. Moreover, expression of P-selectin and leukocyte activation markers like P-selectin glycoprotein ligand 1 (PSGL-1) by MP attributes to thrombosis [1,2].
In this thesis we investigated the contribution of MP to pre-thrombotic states in which patients have an increased risk of developing arterial and/or venous thrombosis. Both the number and phenotypic profile of MP was assessed as well as the procoagulant potential of MP.

In **Chapter 1** we describe the definition of MP, their formation and the technical difficulties involved in their analysis. In addition, we provide an overview of the literature on MP in thromboembolic disease.
In **Chapter 2** MP in patients with Essential Thrombocythemia (ET) are studied. ET is a clonal disorder with a large increase in platelet numbers and patients with ET have a high risk of thrombosis. ET patients had an increased number of MP compared with controls and these MP expressed markers of platelets and endothelial cells: expressing both CD62E, an endothelial marker and CD41, a platelet marker. These MP of bilineal origin might result from physical interaction between activated platelets and endothelial cells, inducing MP formation and transfer of proteins from one to the other membrane. These CD62E+/CD41+ MP were only increased in patients who had one or more clinical risk factors for thrombosis. Leukocyte-derived MP and MP expressing TF were increased as well. In a thrombin generation system, MP of patients with ET induced a shorter lag time and a higher peak height in thrombin formation, contributing to the clinical hypercoagulability of these patients.
In **Chapter 3** we described another study assessing MP in ET and Polycythemia Vera (PV). These are both myeloproliferative diseases in which patients have an increased risk of thrombosis, and patients with a JAK2V167 mutation and/or high leukocyte numbers are at an even higher thrombotic risk. ET is primarily associated with an increased platelet count, PV with an increased erythrocyte count. The only difference we observed between PV and ET was that patients

with PV had more leukocyte-derived MP, and this correlated with an increased leukocyte count and with JAK2 positivity. ET patients showed an impressive, and PV patients a moderate, increase in numbers of CD62E+/CD41+ MP, which was entirely absent in controls. When thrombin generation of MP was determined, patients with ET again had higher peak height and endogenous thrombin potential compared with controls, in line with the results described in Chapter 2. Although patients with PV had higher numbers of MP carrying leukocyte activation markers, such as PSGL-1 and CD11b, their thrombin generation results did not differ from controls. In this study we also assessed MP-specific thrombin generation, which we arbitrarily defined as the endogenous thrombin potential that could be generated by 1000 MP. This MP-specific thrombin generation did not differ between patients and controls, indicating that the increased thrombogenicity in patients is a result of increased numbers of MP, rather than a difference in functional properties.

In **Chapter 4** we determined MP levels and their procoagulant potential in patients with chronic renal failure. These patients were divided in three groups, one group with patients with chronic kidney disease stage IV, one with patients on peritoneal dialyses and one with patients on haemodialysis. All patients showed endothelial activation, which was of a different nature in peritoneal dialysis and chronic kidney disease stage IV compared with haemodialysis. All patient groups had a large increase in MP of platelet, leukocyte and endothelial ancestry. MP levels could not be used to differentiate patient groups from one another. Moreover, a haemodialysis session did not result in an increase of MP, showing that renal failure is a more important determinant of MP-profile in these patients than physical blood cell activation by the dialysis membrane or flow through the artificial kidney. In peritoneal dialysis and chronic kidney stage IV a pattern compatible with chronic endothelial activation was observed. However, in haemodialysis acute endothelial activation was observed after a haemodialysis session. MP in patients with chronic renal failure had reduced thrombogenicity in the thrombin generation assay. We speculate that thrombopathic platelets in these patients, a well-known phenomenon in renal failure due to uremic toxins, result in platelet-derived MP with similarly reduced procoagulant potential.

MP in cancer and the effect of therapy

MP are increased in solid malignancies, especially when metastases are present. These studies include patients before therapy is started [3-5], but it is unknown

what happens to MP levels during therapy. In **Chapter 5** we describe a cross-sectional study in 40 breast cancer patients using endocrine therapy. These breast cancer patients were divided into two groups, 20 patients had had curative therapy and were considered disease free, and 20 patients had metastatic disease. All patients used either an aromatose inhibitor or an anti-estrogen. It is known that anti-estrogens, such as tamoxifen, increase the risk for thrombosis. We investigated whether patients who used anti-estrogens had another MP profile than those who used aromatase inhibitors, however no differences were observed. Moreover, no differences in MP numbers or thrombin generation results could be observed between the adjuvant group and the group with metastatic disease. The observation that patients with metastatic disease did not have higher MP levels was unexpected, and could reflect the more or less 'stable disease' of these patients while on endocrine therapy. On the other hand, patients with or without metastasis on any endocrine therapy had slightly higher numbers of MP and higher thrombin generation results compared with controls, indicating a hypercoagulable state, possibly resulting from endocrine therapy itself. To answer the question whether endocrine therapy itself influences MP subsets and their procoagulant potential more clearly, a prospective study in this patient group before, during and after therapy should be performed.
In **Chapter 6** three patients with acute myeloid leukemia (AML) are described in which MP levels and their procoagulant potential was measured in subsequent samples obtained before and during chemotherapy and in remission. At diagnosis these AML patients had a high percentage (>30%) of myeloblast-derived MP, and this percentage diminished during chemotherapy, showing that changes in MP subsets can be measured using flowcytometry. Upon remission myeloblast-derived MP could not be detected anymore. It has been suggested that apoptotic cell death during chemotherapy might result in a massive increase in MP, in this small study such an increase was not measured in the peripheral blood. The ETP/1000MP was higher at diagnosis than in remission, and diminished during therapy, indicating that the myeloblast-derived MP which were present at diagnosis are more procoagulant than control MP.

MP formation during stress

A bungee jump study that was designed to investigate the effect of stress on the immune system enabled us to investigate the effect of stress on the formation of MP as described in **Chapter 7**. Twenty healthy volunteers

performed a bungee jump from a 60 meter high crane, half of them were pre-treated with propranolol three days prior to the jump. We determined the half-life of MP, which was approximately one hour. We observed a large increase in MP numbers immediately after the jump that had returned to baseline two hours after the jump. Moreover, volunteers who had used propranolol had a larger increase in leukocyte- and platelet-derived MP. In contrast, numbers of monocyte-derived MP at the other hand were higher in the group who had not used propranolol. We hypothesized that the effect of propranolol is due to a decrease of intracellular cyclic AMP by blocking the beta-receptor. The beta-receptor therefore seems to be involved in the formation of MP. The use of propranolol did not alter MP-induced thrombin generation.

General discussion

The hypothesis of this thesis was that MP contribute to the pre-thrombotic state as observed in patients with thromboembolic disease. The number and phenotypic profile of MP was studied and their procoagulant potential assessed. The procoagulant properties of MP rely mainly on the expression of anionic phospholipids, especially phosphatidylserine, and of TF, the major cellular activator of the clotting system [6]. The presence of phosphatidylserine on MP is shown by their ability to bind Annexin V. In line with literature [7], we observed increased numbers of Annexin V-positive MP in our patient groups. Higher numbers of TF positive MP were observed in patients with chronic renal failure and ET. TF-positive MP are known to be significantly higher in patients with cancer [3,8] and with cardiovascular disease [6]. However, the overall numbers of TF-positive MP in our studies were low, less than 1% of all MP. Tesselaar *et al*. found a percentage of TF+ MP in breast cancer patients of 6%, which is much higher than the percentages we observed; this difference might be explained by the fact that in this study metastasized patients were included before therapy was started [3]. The cellular origin of TF-positive MP might include platelets, cancer cells and monocytes [9]. We observed co-expression of TF and platelet markers, suggesting the platelet as source for TF-positive MP.

In concordance with previous observations [3,4,10], in almost all subjects studied the majority of MP was platelet –derived. These numbers varied from 43% in an AML patient with a large proportion of MP being myeloblast-derived to 93-98% in patients with renal failure, breast cancer patients and ET/PV patients. MP from leukocytes, endothelial cells and erythrocytes were present at much lower levels. Changes in phenotypic profiles of patients were observed, which could either be small or large. In ET and PV there was a large (27%) percentage of CD41+/CD62E+ MP, which were absent in controls. This might reflect a novel mechanism involved in the increased risk on thrombosis in MPN patients, which needs further research. In AML patients before therapy was started a large percentage (30%) of myeloblast-derived MP were observed. Large percentages of tumor-derived MP have also been observed by Ghosh *et al.,* who showed that patients with the earliest stage of CLL (Rai 0) had 84% platelet-derived MP and only small levels of MP derived from leukemic B-cells [11]. In patients with advanced stages of CLL (Rai III/IV) the percentage of platelet-derived MP had

dropped to 50% and the proportion of MP of leukemic B-cells had increased to around 40%. In breast cancer patients and in chronic renal failure only small diffences in phenotypic profile were observed. These patients had more CD62P-positive MP compared with controls; similar findings were obtained by Toth *et al.* and the significance of this finding is unknown [3]. P-selectin positive MP might attribute to the procoagulant potential of MP as they can bind PSGL-1, which promotes clot formation [1].

The effect of medical treatment on MP numbers and phenotypic profile was studied as well. We showed that therapy in cancer patients influenced MP levels. Despite cell death inducing chemotherapy, an early -treatment-associated decrease in MP was observed, especially in the number of myeloblast-derived MP. Therefore, either chemotherapy-associated cell death does not lead to an overt MP production, or such MP have a very high clearance rate. In breast cancer patients we did not observe the expected increase in MP in patients with metastatic disease, which was in contrast with previous studies [3-5]. We hypothesized this was due to the more or less 'stable disease' these patients had while using endocrine therapy, but prospective studies in which larger breast cancer patient groups need to be included are needed to study MP profiles and the effects of therapy.

This thesis adds to other research on MP, improving our understanding of the pathogenesis of thrombosis. Experimental work provided a basis for understanding the significance of MP in the onset and progression of thrombosis. During the initial phase of thrombus development in vivo TF derived from hematopoietic cells is delivered by MP [12]. Activated platelets at the site of injury express P-selectin. In P-selectin null mice MP failed to accumulate in the thrombus [1]. Therefore the accumulation of monocyte MP bearing TF in the thrombus is dependent on the interaction of platelet P-selectin and its counterreceptor P-selectin glycoprotein ligand 1 (PSGL-1) on the surface of MP [1]. Mice that overexpressed soluble P-selectin had increased numbers of leukocyte-derived MP bearing TF and had shortened clotting times [13]. When inhibitory PSGL-1 antibodies were infused into these mice the clotting time prolonged and thrombus size decreased [13,14]. In patients with PV we measured increased numbers of PSGL-1-positive MP. This did not, however, result in increased thrombin generation. Possibly, because PSGL-1-postive MP

were present at very low numbers minor effects escaped detection or that their main effects are mediated through interaction with the vessel wall.

The procoagulant potential of MP was investigated using a thrombin generation assay, in which three parameters were assessed; lag time is the time from onset of the reaction to start of thrombin generation, peak height represents the maximum speed of thrombin generation and endogenous thrombin potential (ETP) represents the total amount of thrombin generation. We have performed thrombin generation assays in three different conditions; in MP rich plasma, in the MP pellet resuspended in human pooled plasma and in the supernatant. Using these different conditions we were able to investigate the different thrombogenic properties of MP in this test. Patients with renal failure, breast cancer and ET had increased MP-dependent thrombin generation compared with controls. Previous studies had shown that patients with recurrent venous thromboembolism and with myeloproliferative disease have increased MP-associated thrombin generation [15,16]. Patients with thromboembolic disease have higher MP-dependent thrombin generation, this indicates that MP contribute to the prothrombotic profile in these patients.
We further investigated whether MP in some diseases were more procoagulant than in controls and whether MP had a qualitative or a quantitative effect in thrombin generation. Qualitative effects f.e. could be achieved by increased expression of TF, the main determinant of lag time, or by increased phospholipid content on MP. Quantitative effects could be achieved by increased numbers of MP. Based on our findings, we tend to conclude that both qualitative and quantitative aspects are involved. In AML we observed a qualitative effect, consisting of increased thrombin formation per MP at diagnosis as compared to remission. In ET and breast cancer a quantitative effect was found. Thrombin generation in these patients was higher, however ETP/1000MP was the same. In chronic renal failure both effects appeared to be operational. Due to a large increase in MP numbers in patients their MP-dependent thrombin generation was slightly higher, on the other hand ETP/1000MP was lower, leading to the conclusion that circulating MP possessed reduced ability to induce thrombin generation in patients. This suggests that MP contribute to thrombosis by different functional mechanisms.
Research in the functional properties of MP in thrombosis is compromised as there are only few functional tests. Thrombin generation assays, specified

to MP, could provide more information about the functional capacities of MP. Although of great interest, we were not able to investigate whether the ancestry of MP results in differences in procoagulant ability. For example, are monocyte-derived MP more procoagulant than platelet-derived-MP? Monocyte-derived MP express TF, and shorten lag time and increase peak height in a TF-dependent manner [17,18]. Van der Meijden *et al.* observed that platelet-derived MP, as well as erythrocyte-derived MP not only propagate coagulation by exposing phosphatidylserine but also initiated thrombin generation indepedently of TF in a Factor XII-dependent manner [18]. Platelet-derived MP and monocyte-derived MP differentially modulate thrombosis, the physiological relevance of this finding, especially in various disease states, requires further investigation.

Future perspectives

Although the initial studies on cellular MP considered these vesicles as 'platelet dust', studies from the past decades have dramatically altered this view. MP are nowadays known to contribute to thrombosis, infection, angiogenesis, cell-cell interactions and can fuse with other cells delivering both proteins and mRNA. With all applications of MP detection, it remains of great importance that standardization of MP measurement and pre-analytical conditions is being accomplished. The characterization of MP remains difficult because of their small size and heterogeneity in terms of phospholipid content and antigenic composition. In addition, there is no consensus on the best markers for defining MP subpopulations such as endothelium-, leukocyte-, or cancer-derived MP. Important efforts in standardizing the pre-analytical and analytical variables have been developed on behalf of the International Society on Thrombosis and Haemostasis standardization subcommittee in vascular biology [19,20]. Hopefully, in the future, standardization of available methodologies can be achieved, this will be needed to advance the field.

Although not all mechanisms by which MP contribute in the pathogenesis of thrombosis are revealed yet, and are still under investigation, they play an important role [21]. Recent studies report that MP not only are procoagulant, but also inhibit anticoagulant functions. Subpopulations of MP might express tissue factor pathway inhibitor and might convey fibrinolytic properties. Therefore MP might act as a regulator in hemostasis. The possible clinical relevance of this finding remains to be demonstrated [21]. Clinically, the relevance of MP for diagnostic stratification of patients in high and low risk for thrombosis is under investigation. For arterial thrombosis a few recent prospective studies demonstrated that the level of endothelial-derived MP is an independent predictor of major adverse cardiovascular events [22] and mortality [23]. The level of endothelial-derived MP might be used to monitor response to therapy in the future.

For venous thromboembolism (VTE) retrospective studies found increased levels of TF-positive MP in patients with VTE compared with those without risk stratification in venous thrombosis [3,24]. Recently the first randomised-controlled phase II trial with MP was performed, in which TF-bearing

microparticles (TFMP) were successfully used to predict the risk of VTE. In this study by Zwicker *et al.* [25] patients with cancer were divided in a group with high and a group with low numbers of TF-positive MP. After two months the cumulative incidence of VTE in the high TF- positive MP group was substantially higher than in the low TF-positive MP group (27.3 vs. 7.2%). The use of enoxoparin in the high TF-positve MP group reduced the incidence of VTE to a rate comparable to the low TF-positive MP group (5.6%). Hopefully more clinical prospective trials in which MP are used for diagnosis or to predict the risk of thrombosis will be performed in the future.
It remains to be shown whether the addition of MP to existing diagnostic strategies including clinical risk profiles (such as the Vienna risk score) and other laboratory markers like d-dimer, adds to better risk assessment.

It is not expected for MP to have a role in therapeutic regimens for thrombosis in the near future, but future therapeutic targets on MP might be P-selectin for the prevention of thrombosis in cancer [9], and phosphatidylserine for the prevention of metastases [26].

MP are now established contributors in the pathogenesis of thrombosis. Further research is needed before the full potential of MP can be realized. In the future, circulating MP may serve as a reservoir of prognostic and predictive biomarkers in thrombosis, therapeutic targets and may be used to monitor response to treatment.

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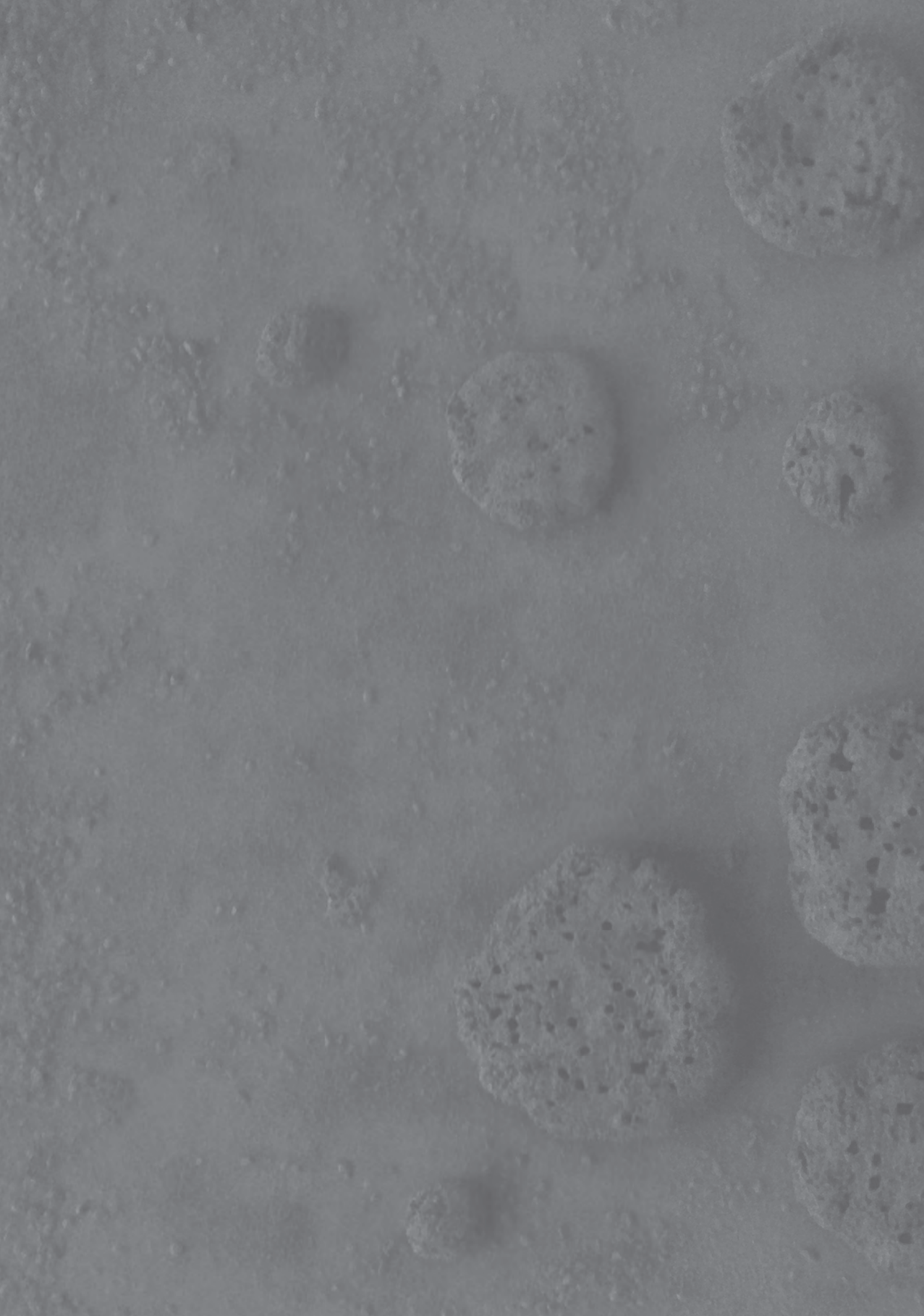

Samenvatting
Dankwoord
Curriculum Vitae

Samenvatting

Micropartikels zijn kleine onregelmatig gevormde blaasjes, die kleiner zijn dan 1 nanometer (ofwel $1x10^{-9}$ meter). Ze ontstaan wanneer cellen in het bloed in apoptose gaan, dit is geprogrammeerde celdood, of als cellen geactiveerd raken. Bloedplaatjes, witte bloedcellen, rode bloedcellen, endotheelcellen en ook kankercellen kunnen micropartikels afsnoeren als ze geactiveerd raken. Micropartikels hebben op hun membraan dezelfde eiwitten als de cel waarvan ze afkomstig zijn, en daarvan kan ook afgeleid worden van welke cel ze afkomstig zijn.
Er zijn verschillende manieren om micropartikels te meten. Voor het onderzoek in dit proefschrift hebben we de flowcytometer gebruikt, en dit is over het algemeen ook de meest gebruikte methode voor het bepalen van micropartikels. Flowcytometrie is een techniek voor het tellen en bestuderen van in een stromende vloeistof voorkomende microscopisch kleine deeltjes. Laserlicht wordt gericht op de stromende vloeistof. Het type, de grootte en andere eigenschappen van het deeltje dat zich in de lichtstraal bevindt bepalen de mate van verstrooiing van het licht, en deze lichtweerkaatsing en fluorescentie wordt door meerdere detectoren waargenomen. Micropartikels in de flowcytometer hebben via toegevoegde antistoffen verschillende fluorescente markers (merkstoffen) aan zich gebonden, en aan de hand van deze markers kan de cellulaire herkomst bepaald worden.

Toen micropartikels voor het eerst beschreven werden in 1967 dacht men dat deze partikels helemaal geen rol hadden, maar de laatste 20 jaar heeft er zeer veel onderzoek naar de functies van micropartikels plaatsgevonden en heeft men ontdekt dat ze belangrijke zijn in verschillende processen. Ze zijn betrokken bij stolling, infecties, nieuwvorming van bloedvaten (angiogenese) en interacties tussen cellen. Daarnaast kunnen ze ook mRNA en eiwitten van de ene naar de andere cel overbrengen door te fuseren met een cel.

Het beste bestudeerd is de rol van micropartikels in stolling. Micropartikels hebben het phospholipide phosphatidylserine in hun buitenmembraan, en daarmee vormen ze een oppervlak waarop stolling kan plaatsvinden. Daarnaast brengen sommige micropartikels ook tissue factor tot expressie op hun oppervlak. Tissue factor is de initiator van de stolling, en ook hierdoor

kunnen micropartikels bijdragen aan de vorming van een stolsel. Bovendien kunnen er ook andere eiwitten op micropartikels aanwezig zijn zoals P-selectine en P-selectin glycoprotein ligand 1 (PSGL-1) die van invloed zijn in de stolselvorming.
Het doel van dit onderzoek was om bij patiëntengroepen van wie bekend is dat ze een verhoogd risico hebben op het krijgen van een arteriële of veneuze trombose te kijken wat de rol van micropartikels was. Dit hebben we gedaan door de aantallen micropartikels te meten, en te kijken naar de cellulaire herkomst van deze micropartikels; oftewel of ze van bloedplaatjes afkomstig zijn, of juist van witte bloedcellen enzovoort. Daarnaast werd er gekeken of ze ook maakten dat het bloed makkelijker stolde. Dit werd onderzocht door middel van een trombine generatie test. In zo'n trombine generatie test wordt er gemeten hoeveel trombine er in een bepaalde tijd wordt gegenereerd. Trombine is een enzym dat van belang is in de bloedstolling. Het zorgt ervoor dat fibrinogeen wordt omgezet in fibrine. Fibrine is het basisbestanddeel van een stolsel. Er zijn verschillende parameters die men kan beoordelen als men een trombine generatie test gebruikt; de lag time, peak height en endogenous thrombin potential. Lag time is de tijd die het duurt van de start van de reactie tot het moment dat er trombine wordt gevormd. Peak height, of piekhoogte, staat voor de maximale snelheid waarmee trombine wordt gevormd, en endogenous trombine potential (endogene trombine potentiaal) staat voor de totale hoeveelheid trombine die gevormd wordt. Door onder verschillende condities te meten hebben we goed het effect van micropartikels in de trombine generatie kunnen testen. Samenvattend hebben we gekeken of er bij de onderzochte patiënten een toename van micropartikels was, of deze een andere herkomst hadden dan micropartikels bij gezonde proefpersonen en of micropartikels bijdroegen aan de verhoogde stollingsneiging van deze patiënten.

Hoofdstuk 1 is de introductie. Hierin wordt beschreven op welke manieren micropartikels gemeten kunnen worden en wat de functies van micropartikels zijn. Daarnaast wordt er een literatuuroverzicht gegeven over de rol van micropartikels in trombo-embolische aandoeningen waarbij patiënten een verhoogd risico hebben op arteriële of veneuze trombose.
In **Hoofdstuk 2** worden micropartikels beschreven bij patiënten met essentiële trombocytose. Dit is een beenmergziekte, zich voornamelijk uitend in een

toename van het aantal bloedplaatjes, waarbij patiënten een verhoogd risico hebben op trombose (bloedstolsel). Bij patiënten met essentiële trombocytose werden verhoogde aantallen micropartikels gevonden. Deze micropartikels brachten eiwitten tot expressie die zowel van bloedplaatjes als endotheelcellen (vaatwand cellen) afkomstig waren, zo brachten ze CD62E tot expressie wat alleen gezien wordt op endotheelcellen, en CD41 wat van bloedplaatjes afkomstig is. Deze CD62E /CD41 positieve micopartikels hadden dus een bilineale herkomst, wat inhoudt dat ze van twee verschillende cellen afkomstig zijn. Dit kan zijn door interactie tussen bloedplaatjes en endotheelcellen waardoor micropartikels gevormd worden en transfer van oppervlakte-eiwitten plaatsvindt. Deze micropartikels waren alleen verhoogd bij patiënten die één of meer klinische risicofactoren hadden voor het ontstaan van trombose. Ook micropartikels afkomstig van witte bloedcellen en micropartikels die tissue factor tot expressie brachten waren verhoogd bij patiënten met essentiële trombocytose. Deze micropartikels zorgden er in de trombine generatietest voor dat de lag time korter was en de piekhoogte hoger bij patiënten, en lijken hierdoor bij te dragen aan de verhoogde hypercoagulabiliteit van deze patiënten.

In **hoofdstuk 3** worden micropartikels vergeleken tussen patiënten met essentiële trombocytose en polycythemia vera. Dit zijn allebei myeloproliferatieve aandoeningen; chronische ziekten waarbij het beenmerg te veel bloedplaatjes, witte bloedcellen of rode bloedcellen aanmaakt. Zoals eerder beschreven wordt in essentiële trombocytose een verhoogd aantal bloedplaatjes gevonden, en in polycythemia vera met name een verhoogd aantal rode bloedcellen. Beide aandoeningen worden gekenmerkt door een verhoogd risico op trombose. Dit risico kan verder worden verhoogd door de aanwezigheid van de JAK2V167 mutatie (een puntmutatie die de cel gevoeliger maakt voor groeifactoren) en de aanwezigheid van verhoogde aantallen witte bloedcellen in het bloed. Opvallend was dat bij onderzoek van de micropartikels in beide patiëntgroepen geen verschil werd gevonden in aantallen micropartikels afkomstig van bloedplaatjes en rode bloedcellen, terwijl de aantallen bloedplaatjes en rode bloedcellen wel verschillend waren tussen beide groepen. Het grootste verschil tussen beide patiëntgroepen was dat patiënten met polycythemia vera meer micropartikels hadden die afkomstig waren van witte bloedcellen, en dit correleerde met verhoogde aantallen witte bloedcellen en de aanwezigheid van de JAK2V167 mutatie. In de trombine generatietesten werden de resultaten uit hoofdstuk 2 bevestigd;

patiënten met essentiële trombocytose hadden een hogere piekhoogte, en een hogere totale hoeveelheid gevormd trombine (ETP). De trombine generatie resultaten van polycythemia vera patiënten waren niet verschillend van die van essentiële trombocytose patiënten en controles. Als we keken naar de specifieke trombine generatie door micropartikels, die was gedefinieerd als de hoeveelheid trombine die gegenereerd kan worden door 1000 micropartikels, was er geen verschil tussen patiënten en controles. Dit betekent dat de gevonden verschillen in trombine generatie verklaart worden door de toename in micropartikels, en daarbij de toename in phosphatidylserine op het oppervlak van micropartikels, en dat er geen verschil lijkt te zijn in functionele eigenschappen van de micropartikels bij deze patiënten.

In **Hoofdstuk 4** worden micropartikels en hun mate van procoagulantie (stollingsbevorderende eigenschappen) beschreven in patiënten met chronische nierinsufficiëntie (verminderde werking van de nieren). Deze patiënten hadden verschillende stadia van nierinsufficiëntie, er was een pre-dialyse groep, een groep die peritoneaal dialyse kreeg en een groep die hemodialyseerde. Alle patiënten met chronische nierinsufficiëntie lieten verhoogde endotheelactivatie (activatie van de vaatwand) zien in vergelijking met controles, maar in de peritoneaal dialyse groep en de pre-dialyse groep had dit een ander patroon dan in de hemodialyse groep. Deze laatste groep had een patroon van acute endotheelactivatie na een dialyse, terwijl de andere twee groepen het beeld van chronische endotheelactivatie lieten zien. Alle patiënten lieten fors verhoogde aantallen micropartikels zien, die zowel van bloedplaatjes, witte bloedcellen en endotheelcellen afkomstig waren. Er was geen verschil tussen de patiëntengroepen onderling, en er was ook geen verschil voor en na dialyse. Hieruit kan geconcludeerd worden dat het verhoogde aantal micropartikels in hemodialyse patiënten komt door het nierfalen zelf, en niet door activatie door contact met de dialysemembraan of bloedstroom door het dialyse apparaat. De micropartikels waren per 1000 micropartikels minder trombogeen dan de micropartikels van controles. Micropartikels in patiënten met chronisch nierinsufficiëntie hadden ook een lagere mean fluorescence intensity, wat inhoudt dat ze minder phosphatidylserine tot expressie brengen in verhouding tot controles. Over de oorzaak kan alleen gespeculeerd worden, maar het zou kunnen dat de bloedplaatjes, die in chronische nierinsufficiëntie minder goed plakken doordat er door de verminderde werking van de nier meer uremische toxines zijn, ook tot minder trombogene micropartikels leiden.

Micropartikels in patiënten met kanker en het effect van medicamenteuze behandeling

Het is bekend uit de literatuur dat micropartikels verhoogd zijn bij maligniteiten (kwaadaardige tumoren), met name wanneer er ook sprake is van metastasen (uitzaaiingen). Deze studies hebben micropartikels gemeten voordat er met behandeling werd gestart, derhalve is het niet bekend wat het effect van behandeling is op de aantallen micropartikels.

In **Hoofdstuk 5** wordt een cross-sectionele studie beschreven, ofwel dwarsdoorsnedeonderzoek; dit is een vorm van observationeel onderzoek waarbij op een bepaald tijdstip gegevens over risicofactoren en/of uitkomsten in een populatie worden verzameld. In deze studie betrof het 40 patiënten met borstkanker die behandeld worden met hormoontherapie. Deze patiënten zijn onderverdeeld in twee groepen, 20 patiënten zijn curatief behandeld en krijgen de hormoontherapie als adjuvante behandeling, en 20 patiënten hebben gemetastaseerde ziekte. Anti-oestrogenen en aromataseremmers zijn beiden hormoontherapeutica en worden bij patiënten die borstkanker hebben gehad gebruikt om de kans op een recidief te verlagen, en als er sprake is van uitzaaiingen bij borstkanker worden ze gebruikt om de groei van de tumor te remmen. Het is bekend dat anti-oestrogenen, zoals tamoxifen, een verhoogd risico op trombose geven, en er werd onderzocht of de patiënten die anti-oestrogenen gebruikte een ander micropartikel profiel hadden dan degenen die aromataseremmers gebruikten. Dit kon niet worden geobserveerd. Daarnaast werden er ook geen verschillen gevonden in aantallen micropartikels of resultaten van trombine generatie testen tussen de adjuvante groep en de groep met gemetastaseerde ziekte. Dit was een onverwachte bevinding, en kan een uiting zijn van de min of meer 'stabiele ziekte' die patiënten met gemetastaseerde ziekte hebben door het gebruik van hormoontherapie. Aan de andere kant hadden patiënten wel meer micropartikels in vergelijking met controles, en ook een hogere ETP (totale hoeveelheid gevormd trombine) bij trombine generatie testen, passend bij hypercoagulabiliteit, mogelijk door de hormoontherapie geïnduceerd. Om dit goed te kunnen bekijken zou een prospectief onderzoek uitgevoerd moeten worden, dat wil zeggen een onderzoeksopzet waarbij een bij het begin van het onderzoek geïdentificeerde groep patiënten die gaat starten met hormoontherapie wordt opgevolgd met betrekking tot veranderingen in procoagulante micropartikels en effecten in trombine generatie.

In **hoofdstuk 6** beschrijven we drie patiënten met acute myeloide leukemie bij wie voor, tijdens en na chemotherapie de aantallen micropartikels en de mate van procoagulantie van deze micropartikels werden bepaald. Op het moment dat de diagnose werd gesteld hadden de twee patiënten die circulerende myeloblasten (voorlopercellen van witte bloedcellen) in het bloed hadden ook meer dan 30% micropartikels die van deze myeloblasten afkomstig waren. Dit percentage nam af tijdens therapie, en toen patiënten in remissie waren werden deze micropartikels in het geheel niet meer gevonden. De ETP per 1000 micropartikels was op het moment van diagnose hoger dan tijdens remissie, waaruit geconcludeerd kan worden dat micropartikels afkomstig van myeloblasten meer procoagulant zijn dan controle micropartikels.

De formatie van micropartikels tijdens stress

In een bungeejump studie die was opgezet om het effect van stress op het immuunsysteem te onderzoeken hebben we micropartikels bepaald om het effect van stress op de vorming van micropartikels te onderzoeken, zoals beschreven staat in **Hoofdstuk 7.** Twintig gezonde mannelijke proefpersonen deden een bungeejump vanaf 60 meter hoogte. De helft van hen was voorbehandeld met propranolol (een beta-blokker) de drie dagen voor de sprong. Er werd op 4 verschillende tijdstippen rondom de sprong micropartikel aantallen bepaald, en hieruit konden we de halfwaarde van micropartikels bepalen die een uur bedroeg. Er was een grote toename in aantal micropartikels vlak na de sprong. Proefpersonen die propranolol hadden gebruikt hadden significant hogere aantallen micropartikels afkomstig van bloedplaatjes en witte bloedcellen. Micropartikels afkomstig van monocyten daarentegen waren juist hoger bij de proefpersonen die geen propranolol hadden gebruikt. Hieruit kan geconcludeerd worden dat de beta-receptor is betrokken bij de vorming van micropartikels. Er is verder onderzoek nodig om duidelijk te krijgen op welke manieren propranolol precies de vorming van micropartikels beïnvloedt.

Toekomstperspectieven

Hoewel de eerste studies naar micropartikels nog weinig waarde aan hun aanwezigheid hechtten, is door de vele onderzoeken van de laatste twintig jaar het belang van micropartikels duidelijk geworden. Het feit dat micropartikels klein zijn, en de groep micropartikels een grote diversiteit kent aan eiwitten die ze op hun oppervlak tot expressie brengen, maakt dat het karakteriseren van micropartikels lastig is. Ook is er nog geen consensus wat de beste markers zijn om micropartikels afkomstig van endotheel, witte bloedcellen of kankercellen te definiëren. Daarnaast zijn er veel verschillende manieren om deze micropartikels te meten, en het zal de volgende jaren van groot belang zijn om tot een standaardisatie van deze metingen te komen, zodat resultaten beter met elkaar vergeleken kunnen worden. Een commissie van de 'International Society on Thrombosis and Haemostasis' spant zich in om tot deze standaardisatie van metingen te komen, om hiermee het veld van micropartikel onderzoek verder te ontwikkelen.

Het is nog niet volledig duidelijk op welke manieren micropartikels bijdragen aan de pathogenese van trombose, dit zal de komende jaren zeker duidelijker worden. Het is de vraag of micropartikels ook een rol kunnen spelen in het voorspellen van trombose of in de behandeling hiervan. Er is een aantal studies geweest van patiënten met arteriële trombose waarbij hoge aantallen van micropartikels afkomstig van endotheelcellen een voorspeller waren voor morbiditeit en sterfte. Het aantal micropartikels afkomstig van deze endotheelcellen zou dan in de toekomst mogelijk ook gebruikt kunnen worden om de therapie te monitoren. Voor veneuze trombose zijn er studies geweest waarin de aanwezigheid van micropartikels die tissue factor tot expressie brengen bijdraagt aan het voorspellen wie er een veneuze trombose krijgt. Wat de toegevoegde waarde van micropartikels is bovenop al bestaande risico-scores moet de toekomst uitwijzen. Op lange termijn zouden micropartikels wellicht zelfs een rol kunnen spelen in behandeling van trombose.

Dankwoord

In 2007 begon ik met onderzoek naar micropartikels in het Onze Lieve Vrouwe Gasthuis (OLVG). Het is zeker niet als promotie-onderzoek begonnen, maar ik ben blij dat het wel als zodanig geëindigd is. Het onderzoek heb ik al die jaren met heel veel plezier heb gedaan, en er zijn veel mensen die ik hierbij graag voor hun bijdrage zou willen bedanken.

Allereerst professor ten Cate, beste Hugo, jij raakte betrokken bij het onderzoek door de trombine generatie testen en ik ben blij dat jij mijn promotor bent geworden. Hoewel op afstand, voelde je dichtbij door je immer snelle antwoorden. Je hebt altijd heldere commentaren op mijn stukken, en gaf me het vertrouwen dat ik nodig had. Dank ook voor je geduld. Ik moet de laatste tijd vaak uitleggen waarom ik helemaal in Maastricht ga promoveren, maar jij bent een hele goede reden. Wim Terpstra en Anja Leyte, mijn co-promotoren. Wim, toen ik jouw praatje zag over micropartikels werd ik gegrepen door je enthousiasme, en besloot dat ik graag met jou en met Anja onderzoek wilde doen in het OLVG. Je bent een zeer toegewijde hematoloog, en vanwege je grote betrokkenheid bij je patiënten en je kennis ben je voor mij een groot voorbeeld als dokter. Je bent daarnaast een echte onderzoeker, altijd geïnteresseerd in nieuwe resultaten. Ik wil je bedanken voor je enthousiaste begeleiding en je heldere analyses. Anja, in onze samenwerking heb ik je toegewijde begeleiding altijd zeer gewaardeerd. Ik kan altijd bij je terecht, en je bent ongelooflijk zorgvuldig in de dingen die je doet. Ik zou graag willen dat ik ooit mijn bevindingen net zo duidelijk en to-the point zou kunnen verwoorden als jij. Daarnaast was het ook buiten het werk gezellig. Ongeveer gelijktijdig met mijn vertrek uit het OLVG werd jij hoofd van het HKCL en ik heb veel bewondering voor hoe je dat doet.

Ik wil de leden van de promotiecommissie hartelijk danken voor het zitting nemen in deze commissie; professor Reutelingsperger, professor Kees Brinkman, professor Biessen, doctor Anna Falanga en professor Kooman. Anna Falanga, I greatly appreciate you are in my assessment committee. I'm grateful for our nice cooperation on our work on microparticles in essential thrombocythemia. Kees (Brinkman), jij bent degene die mij op het spoor van het onderzoek heeft gezet omdat je tijdens een beoordelingsgesprek van mij als AIOS aangaf dat ik toch ook echt onderzoekservaring moest opdoen. Dank daarvoor!

Veel dank voor mijn mede-onderzoekers. Muriel, jij bent degene geweest die mij de praktijk van het onderzoeken heeft geleerd in alle facetten; van hoe ik een pipet moest vasthouden, tot hoe ik een excel-sheet maakte, of resultaten op moest schrijven. Ik heb heel, heel veel van je geleerd en een supergezellige tijd met je gehad. We hebben zoveel uren naast de FACS gestaan dat we zelfs tegen haar praatten als ze het zwaar had met al onze metingen;-) Ik ben er heel blij mee dat je straks als paranimf naast me zal staan. Henri, jij bent voor mij de man van de trombine generatie, die met veel geduld mijn vele vragen beantwoordde. Ook heb ik genoten van je grappige mailtjes en verhalen, en we moeten eigenlijk nog een keer een echte Limburgse vlaai gaan eten. Michiel, jij was een goede versterking op het onderzoek. Jij hebt geweldig werk gedaan voor de studie met AML en PV/ET. Het was goed om te zien hoe jij direct enorm heel veel kennis van een onderwerp had. Daarnaast heb je een heel aanstekelijk gevoel voor humor en ik ben blij voor je dat je nu promotie-onderzoek doet in het AMC. Pascal, jij was absoluut een vrolijke noot op het lab, en ook altijd heel relaxt. De man van de BCS, en ik was niet jaloers op je, want wat een kuren had dat apparaat. Ik hoop dat je het goed naar je zin hebt in je nieuwe baan. Lea, jij bent mijn steun en toeverlaat in de statistiek. Als ik met mijn handen in het haar zat omdat ik een power berekening nodig had, of moest corrigeren voor multiple testing, dan wist jij gelukkig altijd de oplossing. Ook was het gezellig om samen te lunchen, of met de kinderen af te spreken. Jij nog heel veel succes met je promotie-onderzoek. Bas de Laat en Carel Eckmann wil ik danken voor de von Willebrand factor en propeptide metingen die ze voor ons hebben gedaan bij Sanquin. Kylie, Ankie, Melvin, Jacomien, Bob en andere stagiaires op het lab van het HKCL fijn dat jullie konden meehelpen met metingen en andere dingen, Jacomien fijn dat je zoveel hebt onderzocht over het effect van propranolol op de vorming micropartikels, en Bob wat zou ik graag zulke mooie figuren kunnen maken als jij.

Het teaching hospital van het OLVG wil ik bedanken voor al hun ondersteuning in de vorm van hele nuttige cursussen voor onderzoekers en ondersteuning door de bibliotheek. Joep Maeijer, sorry voor alle keren dat we (net te laat) onze postertekst bij je aanleverden, en jij daar wel altijd een mooie poster van wist te maken. Dank daarvoor.

Alle medewerkers van het HKCL wil ik bedanken voor hun gastvrijheid en medewerking. Ik voelde me heel erg thuis bij jullie op het lab, en heb een hele leuke tijd gehad. Cieleke, Dick, Frans, Marjan, Martijn, Mina en Wendy, het was fijn werken bij jullie op de speciële hematologie, Soenita, er is niemand die zo aanstekelijk over eten kan praten als jij, laten we snel weer eens een Bollywood avond afspreken, en Marja, ik mis je verhalen.

De internisten groep in het OLVG, ik waardeer het zeer dat ik bij jullie in het OLVG onderzoek heb gedaan, en jullie hebben allemaal op de één of andere manier er ook aan bij gedragen. Bart en Onno, fijn dat jullie zoveel patiënten voor me konden includeren, Carola, we hebben prettig samengewerkt aan artikel over micropartikels bij nierfalen, en Jos, ik vind het heel bijzonder dat ik in 'jouw Maastricht' ga promoveren. Veel dank ook voor de verpleegkundigen in het OLVG van de oncologie/hematologie en dialyse die hebben geholpen met gegevensverzameling en bloedafnames. Ik bedank alle patiënten voor hun medewerking aan de studies.

Alle oud-collega's van het OLVG, ik heb een hele goede tijd met jullie gehad. Jessica, Josephine, Edwin, Femke en Christine, we zijn samen begonnen in het OLVG, dat schept een band voor het leven, ook alle anderen van de NTBR groep; altijd gezellig om bij te kletsen, laten we snel weer eens gaan eten. Edwin en Fenneke, jullie zijn me voorgegaan op het onderzoekspad in het OLVG, en ik heb veel te danken aan jullie inspanningen. David, thanx dat we mee konden doen met je bungee jump studie. Collega's van het AMC, Mentrum en Slotervaart ziekenhuis, dank voor de fijne samenwerking.

Lieve vrienden, jullie zorgen voor veel gezelligheid en afleiding. Kjell en Judith, fijn dat we zo dicht bij elkaar wonen dat we makkelijk bij elkaar langs kunnen, Nieke en Rogier, onze stranduitjes waren een welkome afleiding, en ik heb nu al zin in de skivakantie, Matthijs en Margo, we verheugen ons op een weekendje samen in Duitsland! Hannah, Anouk en Haike, Lisette en Rogier, Andrieke en Egle-Jan, Astrid en Andy, wat fijn dat jullie er zijn om leuke dingen mee te doen. Lieve vriendinnen, Lily, Liedewij, Miriam, Mirjam en Sanne, jullie zijn heel belangrijk voor mij, jullie zorgen voor veel lol, maar ook voor steun, en ik ben trots op alle stoere dingen die jullie doen. Lily, als jij iets bedenkt gebeurt het ook, en ik maak graag gebruik van je nuchtere adviezen, Liedewij, we gaan al

zo ver terug, het is fijn te weten dat je er altijd bent als ik je nodig heb, en je maakt de beste grappen. Ik geloof in Nieuwerzijds! Miriam, ik geniet ervan je verhalen te horen, en ik vind het zo stoer dat je altijd bezig bent je dromen waar te maken, heel veel succes nu met Inktfisk. Sanne mede-dokter, heel fijn om met jou het leven en werk te bespreken, wie weet komen we ooit ook wel in Noord wonen, en Mirjam, ik ken niemand die zo sterk en dapper is als jij. Hoe jij door moeilijke tijden heen gaat bewonder ik enorm, ik vind het fijn dat jij naast me staat als mijn paranimf.

Lieve schoonfamilie wat ben ik blij met jullie, Ludo, jij bent alweer vier jaar één dag per week vast onderdeel van ons gezin, onwijs bedankt daarvoor en voor je wijze lessen en de lekkere maaltijden. Barbara en Jan, geweldig dat jullie altijd voor ons klaar staan om ons te helpen, en voor alle keren dat jullie door op te passen ons enorm hebben geholpen. Zo kunnen we hard werken, maar ook leuke dingen doen. Rob en Nancy dank voor jullie gezelligheid, en Rob, dank voor de prachtige kaft. Lieve Oma's Onderwater en van Tergouw, wat fijn dat jullie er zijn, en dat ik van al jullie levenservaring kan leren. Lieve broer en zus; Nelleke en Sergi, wat fijn om mijn zusje Amsterdam te hebben, ik ben heel blij met onze goede band, broer Kees, schoonzus Else met nichtje Sara, altijd gezellig om jullie te zien, fijn dat jullie voor leuke afleiding zorgen tijdens mijn drukke onderzoekstijd. Lieve papa en mama, dank dat jullie me altijd gesteund hebben in alles wat ik heb gedaan, en voor een hele fijne thuisbasis hebben gezorgd. Fantastisch ook dat jullie de afgelopen jaren altijd klaar staan om voor de meisjes te zorgen.

Allerliefste Janna en Lena, jullie maken mijn leven zo veel mooier en iedere dag tot een feestje. Het is heerlijk om jullie te zien opgroeien, en ik zie er naar uit dat ik straks weer extra veel tijd heb om met jullie te spelen. Tom, je bent mijn liefste, en je bent een fantastische steun geweest tijdens het schrijven van dit boekje. Dank voor je vertrouwen en liefde, je bent de allerbeste die ik me kan wensen!

Curriculum Vitae

Marijke Trappenburg was born on march 13, 1977 in Rotterdam. She graduated from the Erasmiaans Gymnasium in Rotterdam in 1995. Subsequently she started her medical education at the University of Amsterdam. Her first scientific research was on the normal values of calcium and PTH at the laboratory in the Academical Medical Centre in Amsterdam. In 2000 she travelled in South America for half a year. At the end of 2003 she finished medical school and from 2004 she worked as a resident in internal medicine at the Onze Lieve Vrouwe Gasthuis (Dr. P.H.J. Frissen) where she started her internal medicine training in 2006, after building a community centre in Managua, Nicaragua, in the winter of 2006. In 2007 she started research on microparticles at the Onze Lieve Vrouwe Gasthuis, which resulted in the described PhD project on the contribution of microparticles in pre-thrombotic states, which was supervised by Dr. A. Leyte and dr. W.E. Terpstra. In 2011 she started her training in the division of internal medicine section of geriatrics in the Academic Medical Centre (prof. dr. S.E.J.A. de Rooij). In November 2013 she was registered as an internist specialized in geriatrics. She lives in Amsterdam with Tom and their two daughters, Janna and Lena.