From the Department of Clinical Sciences,
Danderyd Hospital, Division of Cardiovascular Medicine

Karolinska Institutet, Stockholm, Sweden

CELL DERIVED MICROPARTICLES: METHOD DEVELOPMENT, AND CLINICAL AND EXPERIMENTAL STUDIES

Fariborz Mobarrez

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“Discovery is to see what everybody has seen
and think what nobody has thought”

Albert Szent-Györgyi, Nobel Laureate 1937

To my beloved family

ABSTRACT

Background
Cell derived microparticles (MP) are released from the cell membrane upon activation or apoptosis. They resemble their parent cell by exposing similar proteins or surface receptors. This enables identification of their cellular origin. MP are considered to facilitate cross-talk between cells, and to be involved in coagulation, inflammation and vascular function. Elevated circulating MP have been shown in previous studies. Assessment of MP is, however, difficult due to methodological issues.

Aims
To evaluate pre-analytical procedures and a flow cytometric method for detection of microparticles. To study the effects of statin treatment and inflammation on phenotype and functional properties of microparticles.

Methods and Results
In **Paper I** we describe a flow cytometric method for measurements of platelet derived microparticles (PMP) exposing CD62P or CD142. Mean fluorescence intensity measurements were more reproducible than concentration measurements. The presence of platelet fragments could be detected with the peptide phalloidin. This approach can be used as a quality control of samples. Samples frozen and stored as platelet-free plasma generated lowest number of platelet fragments upon flow cytometric analysis. Using our flow cytometric protocol we found two times higher exposure of CD62P and CD142 on PMP in plasma from type 1-diabetes patients compared to healthy controls.

In **Paper II** and **III** we investigated the effects of atorvastatin on MP. Nineteen patients with atherothrombotic disease were randomized to treatment with atorvastatin or placebo in a cross-over fashion. Thrombin generation and exposure of CD61, CD62P, CD142 and phosphatidylserine (PS) were assessed on PMP (**Paper II**). Endothelial derived MP (EMP) were assessed by CD144 or $CD144^+$ $CD142^+$ exposure (**Paper III**). During atorvastatin treatment both thrombin generation and exposure of CD61, CD62P, and CD142 on PMP decreased. No effect was seen on PS exposure. Furthermore, we demonstrated that MP enhanced thrombin generation through PS and CD142 exposure. Unexpectedly, circulating EMP measured as CD144 or $CD144^+$ $CD142^+$ increased significantly during atorvastatin treatment.

In **Paper IV** we investigated and characterized *in vivo* release of MP from 15 healthy volunteers administered lipopolysaccharide (LPS) in the presence of hydrocortisone with or without inhaled nitric oxide. MP from platelets (CD42a or CD41), endothelial cells (CD144 or CD62E) and monocytes (CD14) were studied. Nuclear content in MP was assessed (SYTO 13 binding) as well as HMGB1 exposure. Irrespective of

treatment, LPS led to an increase in numbers of all MP, as well as the number of PMP and monocyte MP positive for anti-HMGB1 and SYTO 13.

Conclusions

We describe a flow cytometric method to measure MP in plasma, and we demonstrate that MP from platelets and endothelial cells respond differently to statin treatment, reflecting the complexity of MP formation. Furthermore, we show that experimental inflammation leads to elevated circulating MP, and that MP may be a source of extracellular HMGB1. MP may be used as biomarkers, an idea that deserves to be investigated more extensively in future studies.

LIST OF PUBLICATIONS

I. **Mobarrez F,** Antovic JP, Egberg N, Hansson M, Jörneskog G, Hultenby K, Wallén H. A multicolor flow cytometric assay for measurement of platelet-derived microparticles.
Thromb Res. 2010 Mar;125:e110–6.

II. **Mobarrez F**, He S, Bröijersen A, Wiklund B, Antovic A, Antovic JP, Jörneskog G, Wallén H. Atorvastatin reduces thrombin generation and expression of tissue factor, P-selectin and GPIIIa on platelet-derived microparticles in patients with peripheral arterial occlusive disease.
Thromb Haemost. 2011 Aug;106:344–52.

III. **Mobarrez F**, Egberg N, Antovic JP, Bröijersen A, Jörneskog G, Wallén HN. Release of endothelial microparticles in vivo during atorvastatin treatment; a randomized double-blind placebo-controlled study.
Thromb Res. 2012 Jan;129:95-7.

IV. Anne Soop, Lars Hållström, Claes Frostell, Håkan Wallén, **Fariborz Mobarrez**, David S. Pisetsky. Effect of endotoxin administration on the number, phenotype and content of nuclear molecules in blood microparticles of normal human subjects.
Manuscript

The articles will be referred to in the text as **Papers I–IV** and are reproduced in full as appendices.

ADDITIONAL PUBLICATIONS

The following articles are not discussed in this thesis

I. **Fariborz Mobarrez**, Danijela Mikovic, Aleksandra Antovic, Jovan P. Antovic. Is a decrease of microparticles related to improvement of hemostasis after FVIII injection in hemophilia A patients treated on demand?
J Thromb Haemost. 2012 Dec 11. [Epub ahead of print]

II. **Fariborz Mobarrez**, Rolf Nybom, Viktoria Johansson, Christina M. Hultman, Håkan Wallén, Mikael Landén, Lennart Wetterberg. Microparticles and microscopic structures in three fractions of fresh cerebrospinal fluid in schizophrenia: case report of twins.
Schizophr Res. 2013 Jan;143:192-7

III. Anne C. Zachau, Mikael Landén, **Fariborz Mobarrez**, Rolf Nybom, Håkan Wallén, Lennart Wetterberg. Leukocyte-derived microparticles and scanning electron microscopic structures in two fractions of fresh cerebrospinal fluid in amyotrophic lateral sclerosis; a case report.
J Med Case Rep. 2012 Sep 3;6(1):274

IV. Skeppholm M, **Mobarrez F**, Malmqvist-Knudsen K, Wallén NH. Platelet derived microparticles during and after acute coronary syndrome.
Thromb Haemost. 2012 May 31;107:1122-9.

V. Vikerfors A, **Mobarrez F**, Bremme K, Holmström M, Ågren A, Eelde A, Bruzelius M, Antovic A, Wallén H, Svenungsson E. Studies of microparticles in patients with the antiphospholipid syndrome (APS).
Lupus. 2012;21:802-5.

VI. Tehrani S, **Mobarrez F**, Antovic A, Santesson P, Lins PE, Adamson U, Henriksson P, Wallén NH, Jörneskog G. Atorvastatin has antithrombotic effects in patients with type 1 diabetes and dyslipidemia.
Thromb Res. 2010 Sep;126:e225-31

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AFM	atomic force microscopy
ATP	adenosine-5'-triphosphate
CAT	calibrated automated thrombogram
CD	cluster of differentiation
CSF	cerebrospinal fluid
CV	coefficient of variation
DAMP	damage-associated molecular patterns
EMP	endothelial (derived) microparticles
FS	forward-scatter
GP	glycoprotein
HCAEC	human coronary artery endothelial cells
HMGB1	high mobility group box 1
HUVEC	human umbilical endothelial cells
LMP	leukocyte (derived) microparticles
LPS	lipopolysaccharide
MESF	molecules of equivalent soluble flurochrome
MFI	mean fluorescence intensity
MMP	monocyte (derived) microparticles
MP	microparticles
NTA	nanoparticle tracking analysis
PAMP	pathogen-associated molecular patterns
PAOD	peripheral arterial occlusive disease
PAR	protease-activated receptor
PC	phosphatidylcholine
PDI	protein disulfide isomerase
PE	phosphatidylethanolamine
PFP	platelet-free plasma
PMP	platelet (derived) microparticles
PPP	platelet-poor plasma
PRP	platelet-rich plasma
PS	phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand 1
RBCMP	red blood cell (derived) microparticles
RT	room temperature
SM	sphingomyelin
SS	side-scatter
TEM	transmission electron microscopy
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TLR4	Toll-like receptor 4
TNFα	tumor necrosis factor-alpha

1 INTRODUCTION

1.1 HISTORY

In 1947, West and Chargaff reported that following high-speed centrifugation of plasma the clotting time was significantly prolonged [1]. Interestingly, when the pellet was added back to the supernatant of the centrifuged plasma the clotting time was reduced, showing the same reduction as when adding thromboplastic protein, later known as tissue factor (TF).

Twenty years later Peter Wolf further characterized the pro-coagulant content of plasma that had been centrifuged at high speed. In these samples he identified particles from activated platelets by electron microscopy [2]. He described these particles as "platelet dust" and showed that they consisted of phospholipids and had pro-coagulant properties.

In the past decades, our knowledge of "platelet-dust" has increased significantly, mainly due to development of more advanced methods. The particles described by Wolf have been named microparticles (MP) or microvesicles. It has become apparent that they are not only released by platelets, but may also be derived from other cells [3].

MP can be found in the blood as well as in other body fluids such as urine [4], synovial fluid [5] and cerebrospinal fluid [6,7]. Indeed, MP are not only side effects of cellular activation processes, but may also act as biological vectors and be of importance in physiology and pathophysiology.

1.2 TYPE OF VESICLES

The most common circulating particles or vesicles described in the literature are of three types: exosomes, MP and apoptotic bodies (Fig. 1). There are several morphological differences between these vesicles (see Table 1).

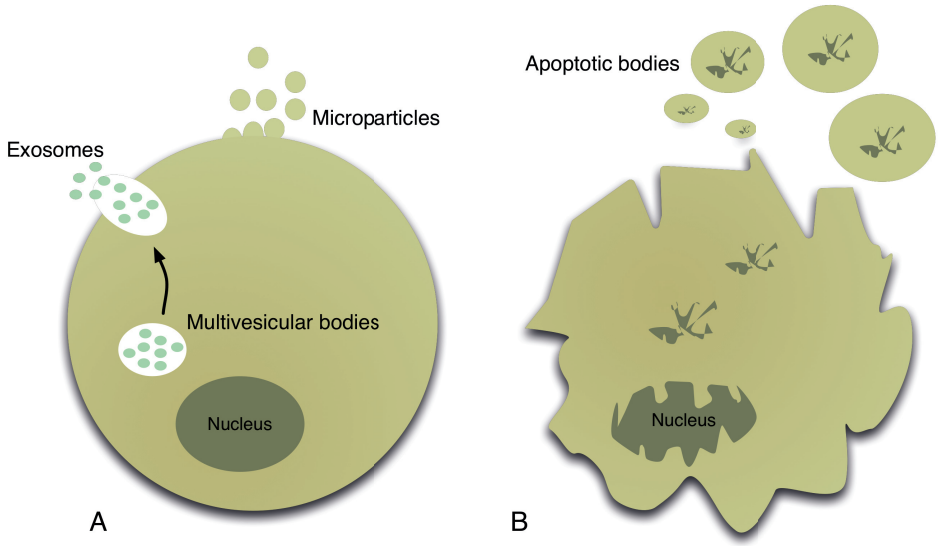

Figure 1. Schematic presentation of different types of vesicles. Exosomes are derived from the interior of the cell, whereas microparticles are formed directly from the cell membrane (A). Apoptotic bodies containing fragmented DNA are generated in the late phase of apoptosis (B).

Table 1. Common characteristics of intercellular vesicles

Characteristic	Exosomes	Microparticles	Apoptotic bodies
Size (diameter)	0.03−0.1 μm	0.1−1.0 μm	1.0−5.0 μm
Origin	Multivesicular bodies	Plasma membrane	Unknown
Mechanism of release	Cell activation	Cell activation and early phase of apoptosis	Late phase of apoptosis
Composition	Cholesterol, sphingomyelin, ceramide, low phosphatidylserine exposure	Expose phosphatidylserine	Expose phosphatidylserine
Centrifugation force	100 000 – 200 000 x *g*	~ 20 000 x *g*	800 − 110 000 x *g*
Markers used to detect the particles	Tetraspanins (CD63, CD9)	Integrins, selectins, other antigens of parental cell	DNA fragments, histones
Methods for detection	Electron microscopy, Western blotting, mass spectrometry, flow cytometry	Flow cytometry, capture-based functional assays	Flow cytometry
Reference	8, 9	10, 11	12, 13

1.2.1 Exosomes

Exosomes are phospholipid bilayer vesicles around 30–100 nm in diameter, and are derived from endosomal compartments within the cell. These compartments are known as multivesicular bodies which may fuse with either lysosomes, where they are subjected to proteolytic degradation, or with the cell membrane, where the exosomes within are released (Fig. 1A) [8,14]. Exosomes contain mainly cytosolic components, including tetraspanins, heat shock proteins, microRNA and phospholipids such as phosphatidylserine (PS). It seems though that exposure of PS is lower in exosomes than in MP [15].

Many cell types release exosomes both constitutively, and in a more regulated form through multivesicular bodies, and are best characterized in immune cells such as dendritic cells, T cells, B cells and macrophages [16]. Exosomes are found in a variety of body fluids, including blood, urine, saliva, bronchoalveolar lavage fluid, synovial fluid and breast milk. Key mechanisms by which they exert their biological functions on cells have been ascribed to be 1) direct contact with molecules on the surface of cells, 2) endocytosis of the vesicles and 3) membrane fusion between vesicles and the cell membrane [9,10]. Importantly, exosomes are known to expose major histocompatibility complex molecules, resulting in stimulation of T cells in an antigen-specific manner [8].

Detection of exosomes is performed by means of flow cytometry, Western blotting or electron microscopy, but also by more advanced techniques such as nanoparticle tracking analysis (NTA) or mass spectrometry. However, there are some major methodological concerns regarding isolation and detection of exosomes. Centrifugation is a common pre-analytical procedure to isolate exosomes, but there is increasing evidence that centrifugation is not optimal regarding MP and exosome isolation and separation [14,17,18]. Furthermore, CD63 (tetraspanin), a common exosome marker, is also exposed on platelet derived MP (PMP) upon platelet activation, thus making it not exosome-specific [19].

1.2.2 Microparticles

MP are released from the cell membrane upon activation of cell surface receptors, or during the early phase of apoptosis by a blebbing or shedding process, resulting in the generation of particles of 0.1–1.0 μm in diameter [10,11,20]. The lipid and protein composition of MP resembles that of the parent cell. This enables identification of the cellular origin. The outer layer of MP contains exposed PS which, however, normally resides on the cytoplasmic surface of the resting parent cell [21]. Recent studies also suggest the presence of MP that do not expose PS [22,23]. Internally, MP could carry a broad variety of cytoplasmic and nuclear components, including DNA and RNA[24,25].

Circulating MP are predominantly products of platelets, leukocytes and endothelial cells, and are considered to have various biological effects on coagulation, inflammation and vascular function [26-28]. Commonly used isolation and analytical protocols for MP include differential centrifugation followed by flow cytometry or capture-based assays [29,30].

1.2.3 Apoptotic bodies

Apoptotic bodies are approximately 1–5 μm in diameter and are released as blebs from cells undergoing apoptosis. They are characterized by size and PS exposure, and may contain fragmented DNA [12,20].

The apoptotic process is mediated by proteolytic enzymes called caspases, which trigger cell death by cleaving specific proteins in the cytoplasm and the nucleus. The activation process is initiated by either the "extrinsic-pathway" (receptor-mediated, for example involving tumor necrosis factor receptor 1 or CD95), or the "intrinsic-pathway" (mitochondrial-mediated), which causes intracellular molecules to aggregate and activate pro-caspases like caspase-8 and caspase-3 [31]. Morphologically, apoptosis is characterized by cell shrinkage, collapse of the cytoskeleton and nuclear fragmentation of DNA. Subsequently the apoptotic cell breaks up into fragments, termed apoptotic bodies, which are rapidly recognized and engulfed by neighboring cells or macrophages [32].

Standardized pre-analytical and analytical protocols are essentially lacking and most studies are performed in cell cultures. Measurements involve either supernatant or pellet after ultracentrifugation. Commonly used methods include flow cytometry together with annexin V labeling (binds to PS), or electron microscopy [12,33,34].

1.3 COMPOSITION OF MICROPARTICLES

1.3.1 Lipids

Phospholipids are important components of the cell membrane and of the membrane of MP. Phospholipids are asymmetrically distributed in the inner and outer leaflets of the membrane, with phosphatidylcholine (PC, uncharged molecule) and sphingomyelin (SM, uncharged molecule) predominantly in the outer leaflet and PS (negatively charged molecule) and phosphatidylethanolamine (PE, uncharged molecule) in the inner leaflet of plasma membrane [35]. The regulation of phospholipid asymmetry is maintained by three different enzyme types: flippase, floppase and scramblase [36]. Flippase uses adenosine-5'-triphosphate (ATP) to move PS and PE from the outer leaflet to the inner leaflet of the plasma membrane, against a concentration gradient. Floppase, uses ATP to transport PC and SM against concentration gradients in the opposite direction. Scramblase is ATP-independent and

triggers bi-directional movement of phospholipids between the two membrane leaflets. During cell activation or apoptosis, intracellular increase in calcium leads to inactivation of flippase and activation of floppase and scramblase, which shuttles phospholipids between the bilayers and subsequently PS molecules are exposed at the outer leaflet of the cell membrane [35,36] (Fig. 2). When platelets expose PS upon activation, it facilitates blood coagulation through the binding of positively charged coagulation factors to the platelet surface [37]. PS exposure on nucleated cells (e.g. leukocytes and endothelial cells) is essential for recognition and removal of apoptotic cells by macrophages [32].

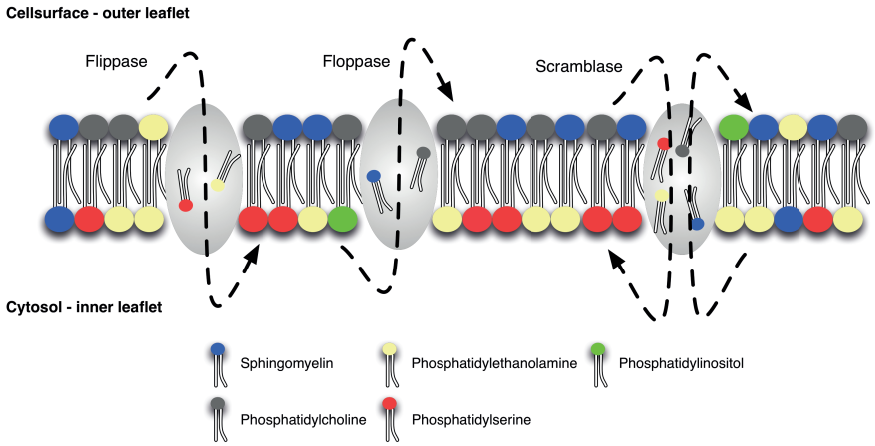

Figure 2. Phospholipid distribution and related translocating enzymes in the plasma membrane.

As described above, scramblase activity followed by either cell activation or apoptosis results in exposure of the negatively charged phospholipid, PS on the outer cell surface. PS exposure on cells and MP can be detected using fluorescence-labeled annexin V or lactadherin. Annexin V is a protein of ~35 kDa that binds to PS in the presence of calcium ions. Lactadherin is a glycoprotein of ~50 kDa, which binds to PS in a calcium-independent manner and has recently been reported to detect PS exposure in a more sensitive manner than annexin V [38]. As pointed out previously, not all MP expose PS. For example, MP from healthy donors failed or were less prone to bind annexin V [22] and the same tendency was observed in MP isolated from synovial fluid from patients with rheumatoid arthritis [39]. Another approach in the detection of MP is by labeling PE on the outer leaflets of MP. Duramycin, a 19-amino acid belonging to the lantibiotics (antimicrobial peptides produced by gram-positive bacteria), binds to PE with high affinity [40]. When MP are labeled with duramycin, MP from red blood cells, endothelial cells and monocytes, can be detected in higher numbers than if annexin V is used [41].

1.3.2 Content

MP display a variable spectrum of bioactive substances, membrane-anchored receptors and adhesion molecules on their surface, allowing specific interaction and crosstalk with various target cells. Some surface antigens may be specific to the parent cell. This enables further characterization of different MP subtypes through the use of labeled antibodies. In Table 2 some frequently used antigens, used to determine the cellular origin of MP are shown.

Table 2. Some common CDs/CD combinations used to identify the cellular origin of circulating microparticles.

Antigen	Parent cell	Alternative Name
CD45	Leukocyte	Protein tyrosine phosphatase, receptor type C. "Leucocyte common antigen"
CD14	Monocyte	-
CD66b	Granulocyte	-
CD4, CD8, CD20	Lymphocytes	-
CD235	Red blood cell	Glycophorin A
$CD31^{+}CD42^{-}$	Endothelial cell	Platelet endothelial cell adhesion molecule 1 (PECAM-1)
CD62E	Endothelial cell	E-selectin
CD144	Endothelial cell	Vascular endothelial (Ve) cadherin
CD34	Endothelial cell	-
CD54	Endothelial cell	Intercellular cell adhesion molecule 1 (ICAM-1)
CD51	Endothelial cell	Integrin alpha V
CD106	Endothelial cell	Vascular cell adhesion molecule 1 (VCAM-1)
CD146	Endothelial cell	Melanoma cell adhesion molecule (MCAM)
CD41	Platelet	Glycoprotein (GP) IIb
CD42a	Platelet	GPIX
CD42b	Platelet	GPIbα
CD61*	Platelet	GPIIIa
CD62P*	Platelet	P-selectin
$CD31^{+}CD42^{+}$	Platelet	PECAM-1

** Not entirely cell-specific; CD61 can also be exposed on megakaryocytes [42] and endothelial cells [43]. CD62P may also be exposed on endothelial cells [44,45].*

Depending on whether or not the parent cell is resting or in "an activated state", the released MP can differ in composition. For example, resting and activated endothelial cells both release MP which expose CD31 (PECAM-1), whereas MP from endothelial cells that have been activated also expose CD62E (E-selectin) in much higher levels [46].

Moreover, PMP generated from activated platelets expose more CD62P (P-selectin) compared with PMP from non-stimulated platelets [47].

Circulating MP also seem to "pick up" molecules from the external milieu. This has been discussed as being one mechanism behind the exposure of TF on PMP. Thus, TF from monocytes or monocyte derived MP (MMP) could be transferred to platelets and then, following platelet activation and PMP release, circulate as TF^+ PMP [48].

Proteomics provides comprehensive information on MP protein composition. Following ADP-induced platelet activation and PMP generation, 578 proteins were identified in PMP [49]. Interestingly, 380 of these proteins had not been previously detected in platelets. Some of the unidentified proteins were nuclear proteins most likely derived from megakaryocytes. Other PMP proteins described seemed to be transmembrane and cell surface proteins [49].

Recent studies have revealed that MP, in addition to their content of membrane proteins, also contain nuclear matter. MP generated *in vitro* from apoptotic Jurkat and HL-60 cells (human promyelocytic leukemia cells) have been shown to contain both DNA and RNA [50]. In these experiments MP were labeled with propidium iodide (used to stain DNA and RNA) after permeabilization and fixation, and analyzed by flow cytometry. Further experiments with gel electrophoresis and PCR reveled higher concentrations of ribosomal RNA and messenger RNA in MP compared to DNA [50]. Another approach is to label MP with SYTO 13 dye, which, in contrast to propidium iodide, is cell-permeable. SYTO 13 binds to both DNA and RNA but with a preference for double stranded DNA [25].

More recently, microRNAs have been found in MP as well [51]. MicroRNAs are short, nucleotide RNA sequences that bind to messenger RNA, and act as endogenous post-translational gene regulators [52]. MicroRNAs found in blood have been described to be very stable, even under tough conditions such as boiling, low pH and long-term storage. Later it was discovered that microRNAs are "protected" by vesicles (exosomes, MP and apoptotic bodies) [52]. The presence of microRNAs in vesicles suggests some interesting concepts. One is cell-to-cell communication. MP derived from activated THP-1 cells (a human acute monocytic leukemia cell line) containing microRNA-150 was actively delivered to HMEC-1 cells (cultured human microvascular endothelial cells) upon incubation [53]. However, the exact mechanisms behind the concept of intercellullar communication are not fully known. Secondly, since microRNAs in vesicles are in the circulation, they could potentially be used as biomarkers. Distinctive circulating microRNAs have been found in patients with myocardial infarction, heart failure, atherosclerotic disease, type 2 diabetes mellitus and hypertension, but much more research is needed in this field [51,52].

1.4 FORMATION OF MICROPARTICLES

Dogma dictates that MP are released in response to activation or apoptosis. Although there might be differences in the mechanisms resulting in MP formation, there are no consistent data on whether or not there are differences in terms of size, lipid or protein composition depending on the mechanism(s) of generation or release.

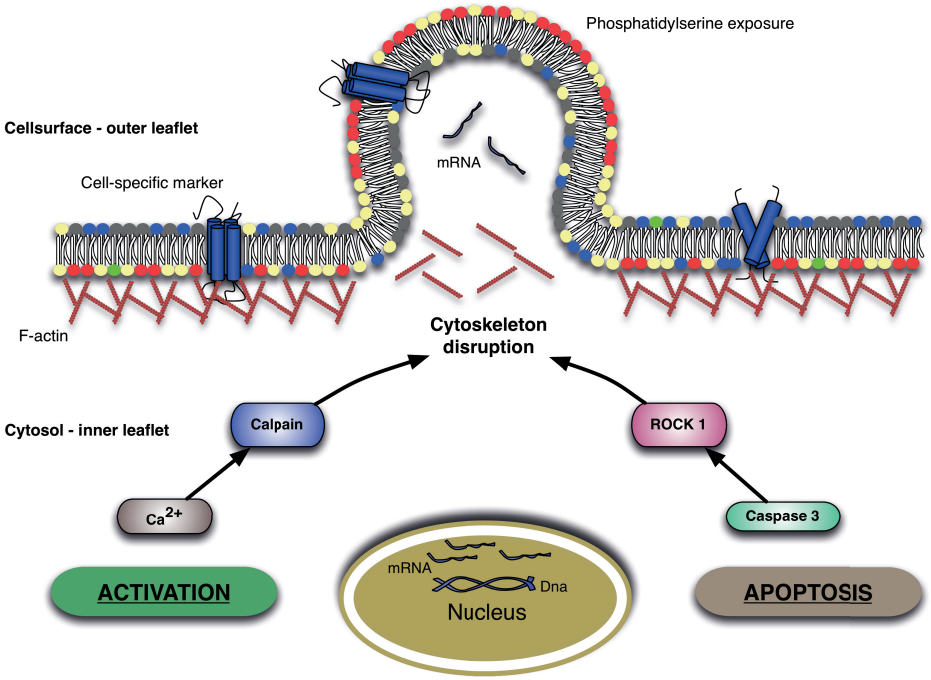

Figure 3. Schematic representation of microparticle formation.

1.4.1 Activation

Increasing intracellular calcium levels brought about through cell activation inactivates flippase and activates floppase and scramblase, leading to exposure of PS. In addition, the increase in cytosolic calcium activates protein kinases and phosphatases such as calpain, a calcium-dependent cysteine protease [11,54,55]. Calpain will trigger local degradation of cytoskeletal proteins, such as spectrin and actin and as a result, blebbing occurs and blebs (vesicles) detach from the plasma membrane (Fig. 3) [21]. Inhibition of calpain in platelets with calpeptin decreases PMP formation [56].

MP release can be a receptor-mediated event. For example, thrombin activates platelets by binding to a G protein-coupled class of receptors named protease-activated receptors (PAR) on the cell membrane of the platelet (PAR1 and PAR4). Collagen, on the other hand, can bind directly to GPIa/IIa or GPVI, all of which leads to PMP release [57]. Similarly, stimulation of leukocytes with lipopolysaccharide (LPS) leads to release of leukocyte derived MP (LMP) [58]. LPS is a component of the outer membrane of gram-negative bacteria which initially binds to a lipopolysaccharide-binding protein, which transfers it to CD14 and later forms a complex with MD2 and finally binds to toll-like receptor 4 (TLR4) [59].

1.4.2 Apoptosis

Apoptosis is commonly known as programmed cell death and this phenomenon is also associated with MP formation. The mechanism behind MP formation during apoptosis seems to depend mostly on activation of caspase 3 [60]. Caspase 3 cleaves Rho-associated kinase I (ROCK I), removing its inhibitory domain and thereby producing a constitutively active truncated form. ROCK I is involved in cytoskeletal rearrangement, including cell contraction by phosphorylation of myosin regulatory light chain (MLC). Phosphorylation of MLC has been shown to be required for membrane blebbing (Fig. 3) [61].

Discussing apoptosis and its mechanisms in platelets may be viewed as controversial. The platelet has no nucleus, and thus it is not a cell, rather a vesicle (or a large particle) formed from a megakaryocyte. There are reports showing that platelets contain caspases 3 and 8 [59,62], but whether activation of caspases leads to platelet "activation" or "apoptosis" is not known and if so, the "correct wording" could be discussed.

1.5 FUNCTIONS OF MICROPARTICLES

1.5.1 Hemostasis

The cell-based model of coagulation consists of three phases, which occur on different cell surfaces: 1) initiation on TF-bearing cells and 2) amplification and 3) propagation on activated platelets [63]. PS exposed on activated platelets together with calcium facilitates two important reactions of the coagulation cascade, the "tenase" and "pro-thrombinase" reactions [64]. The small amount of thrombin formed in the initiation phase will lead to further platelet activation, and complex formation and activation of FV, FVIII and FIX (the latter two forming the tenase complex), which activates FX, which subsequently, coupled to activated FV, generates more thrombin [63]. The importance of platelet PS exposure in normal hemostasis is reflected by the

Scott syndrome, which is a rare bleeding disorder. This syndrome is characterized by reduced PS exposure on activated platelets. In addition to reduced thrombin formation on the platelet surface, the Scott syndrome is also associated with reduced MP formation which may be due to attenuated calpain activation [64,65]

It is well established that MP possess the same pro-coagulant properties as platelets and TF-bearing cells, through the exposure of PS and TF [28,66]. The surface density of PS on MP is equal to or higher than on activated platelets and therefore MP may have up to 50- to 100-fold greater pro-coagulant properties, according to the results of some studies[67]. PS exposed on MP facilitates interaction between the positively charged γ-carboxyglutamic domains of coagulation factors FVII, FIX, FX and prothrombin, and the negatively charged PS [68]. Moreover, PS exposure together with TF considerably enhance the procoagulant activity of MP [68,69]. Other procoagulant mechanisms involve exposure of CD62P (P-selectin) and its ligand, P-selectin glycoprotein ligand 1 (PSGL-1). In animal studies it has been shown that activated monocytes release MMP which expose TF and PSGL-1, and these MMP can bind to CD62P on activated platelets, thus concentrating TF at the thrombus site [70].

MP may also possess anti-coagulating properties. *In vitro* studies of cultured endothelial cells stimulated with TNFα revealed generation of MP that exposed both TF and tissue factor pathway inhibitor (TFPI). Furthermore, TFPI-exposing MP have also been demonstrated in patients with acute myocardial infarction [71]. Other anti-coagulating molecules have also been reported on MP, such as endothelial protein-C receptor [72].

The contribution of pro- and anti-coagulant properties of MP to hemostasis needs further investigation carried out *in vivo*, as these properties could be limited to the experimental setups used.

1.5.2 Inflammation

Inflammation is triggered by innate immunity through the recognition of pathogen-associated molecular patterns (PAMP) and by reaction to tissue damage molecules called damage-associated molecular patterns (DAMP) [73]. DAMP or alarmins consists mainly of intracellular proteins which can be released or exposed during activation or cell death [74]. One example of a DAMP is high-mobility group box 1 (HMGB1). HMGB1 is found in all nucleated cells and platelets [75,76] and is a nuclear protein that binds to nucleosomes and promotes DNA bending. Extracellular HMGB1, depending on the redox state, can be active by itself or in combination with cytokines such as IL-1, and stimulate a wide array of immune responses, resembling LPS or TNFα stimulation [75,77].

MP released from various cells are believed to be involved in communication

between cells during inflammation. Indeed, several *in vitro* experiments have shown that MP can carry a broad variety of both pro- and anti-inflammatory bio-active molecules [78-81]. Additionally, incubating MP with different cell lines has shown that they are capable of binding and activating cells to release more cytokines. However, as previously stated, many of the results found in the literature are limited by the experimental model.

1.6 MICROPARTICLES IN DISEASE

There are numerous studies showing elevated levels of MP during different pathophysiologic processes. As described above, MP have "biological effects" *in vitro*. However, it is still not clear whether elevated numbers of MP found *in vivo* are the cause or a consequence of the disease. Table 3 summarizes some diseases in which MP levels or patterns are altered.

Table 3. Diseases in which MP levels in blood or CSF have been described as being altered.

Disease	MP phenotype	Body fluid	MP Levels	Reference
Acute coronary syndrome	PMP, EMP	Blood	↑	82, 83
Congestive heart failure	EMP	Blood	↑	84
Ischemic stroke	PMP, EMP	Blood	↑	85, 86
Type 1 diabetes	PMP, EMP	Blood	↑	87
Type 2 diabetes	PMP, EMP	Blood	↑	87–89
Peripheral artery disease	PMP	Blood	↑	47
Venous thromboembolism	EMP	Blood	↑	90
Pre-eclampsia	PMP, EMP, LMP, RBCMP	Blood	↑	91
Systemic lupus erythematosus	PMP, EMP, LMP	Blood	↑ and ↓	92-94
Anti-phospholipid syndrome	PMP, EMP	Blood	↑	95, 96
Sepsis	PMP, EMP, LMP	Blood	↑	97
Schizophrenia	EMP, LMP	CSF	↑	7
Amyotrophic lateral sclerosis	EMP, LMP	CSF	↑	8

1.7 MEASUREMENT OF MICROPARTICLES

MP can be studied by various methods, but flow cytometry is the most commonly used one. Table 4 shows most of the available methods and also highlights some of their advantages and disadvantages.

Table 4. Methods available for microparticle measurement

Methods	Advantages	Disadvantages
Flow cytometry	Availability. Small sample size. Multiple antibodies used simultaneously.	Low size sensitivity.
Atomic force microscopy	Accurate sizing with three-dimensional view of MP structures	Availability. Requires specialized antibody-coated surfaces.
Nanoparticle tracking analysis	Accurate sizing. Enables quantification.	Availability. No phenotyping
Transmission electron microscopy	High resolution. Phenotyping by immunogold labeling.	Availability. Cannot determine concentrations
Functional assays	Enable assessment of biological activity. Availability.	No size determination. Indirect quantification.

1.7.1 Flow cytometry

In flow cytometry, physical properties (size and internal complexity) and fluorescence characteristics of single cells are measured. A schematic figure of the flow cytometry technique is shown in Figure 4. Briefly, the detection of cells or particles is performed by passing them through a flow chamber and through one or several laser beams depending on the flow cytometry model. One detector measures size and is placed in line with the laser beam as it measures the forward-scattered light (FS). To prevent the laser beam directly illuminating the FS detector an obscuration bar, approximately 1 mm wide, is placed in front of the FS detector. Thus, only light that has been refracted or scattered enough from its original direction will be recorded. The FS detector detects scattered light at an angle of approximately 1–70°, depending on the setup of the instrument [99,100]. A higher collection angle reflects higher FS sensitivity [101]. Side-scattered light (SS) is scattered light at 90 degrees to the laser beam and the SS collection angle is approximately 47−133°. In addition to FS and SS measurements, flow cytometers are also able to detect fluorescence signal emissions from fluorescent conjugated antibodies. The fluorescent molecules are excited to a higher energy state, and upon returning to their resting states the fluorochromes emit light energy at higher wavelengths. The light signals are detected by photomultiplier tubes and digitized for computer analysis. The resulting information is usually displayed in dot-plots or histograms. Labeling cells or vesicles with several antibodies, each with a different fluorochrome or “color”, allows several cell properties to be measured simultaneously [99].

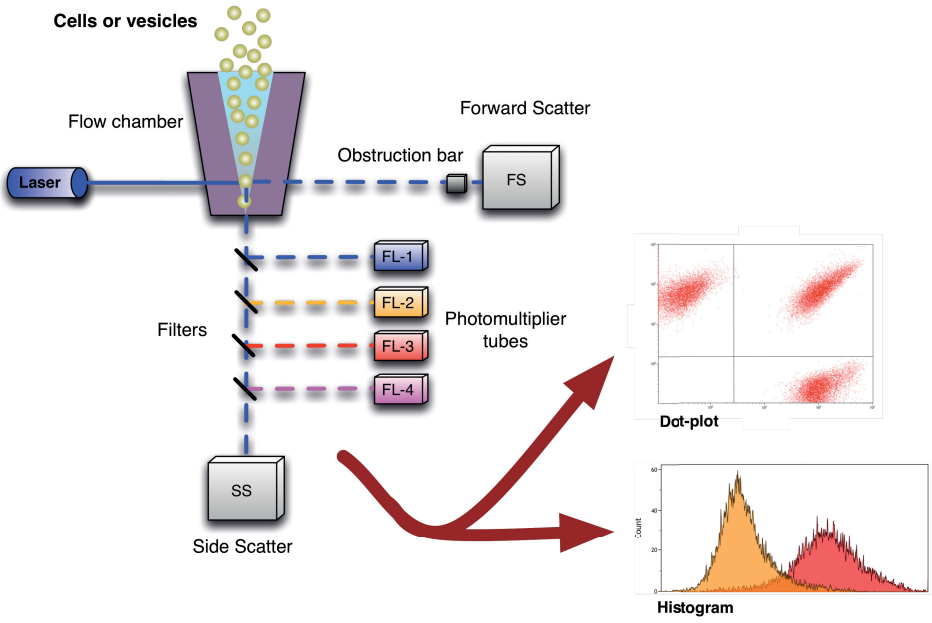

Figure 4. The principle of flow cytometry

There are several commercially available flow cytometers on the market today, all of which have different characteristics and sensitivities as regards measuring MP. Flow cytometers have a FS size limit (around 0.4 µm), which is due to the laser light wavelength (488 nm). This limits accurate MP measurement as MP are heterogeneous in size (from 0.1 to 1 µm) and assessment of MP populations in the lower size range thus may be inaccurate. New flow cytometers such as the Beckman Coulter Gallios™ and Apogee A50 have, however, have been designed to address this issue by increasing the FS collection angle. Table 5 lists some commonly used flow cytometers. Improvements, in addition to increased FS collection angle, include additional lasers, enabling the use of more fluorochrome-labeled antibodies simultaneously, and increase in number of decades. An increased number of decades enables better differentiation of small populations of vesicles due to gain in signal intensity.

Table 5. Comparison of some commonly used flow cytometers.

Model	Company and production year	FS detector angle	Total no. of lasers	No. of Decades
FACScan™ *	Becton Dickinson (1988)	1 − 8°	1	4
FACSCalibur™	Becton Dickinson (1995)	0.7 − 10°	2	4
FACSCanto™ *	Becton Dickinson (2005)	0.7 − 10°	2	5
FACSCanto II ™	Becton Dickinson (2007)	0.7 − 10°	3	5
Epics XL	Beckman Coulter (1999)	1 – 19°	1	4
FC 500 Analyzer	Beckman Coulter (2010)	2 – 16°	2	5
Gallios™ *	Beckman Coulter (2009)	1 – 19°	3	5
A50 Micro	Apogee Flow Systems (2011)	1 − 70°	4	4.5

** Flow cytometers used in this thesis.*

1.7.2 Transmission electron microscopy

Transmission electron microscopy (TEM) is often used to visualize MP and to validate flow cytometric data. Briefly, in TEM a beam of electrons is transmitted through a thin specimen and then the electrons are focused in order to create an image on a screen or on a film with high resolution (< 1 nm) [102]. Additionally, immunogold staining can be applied to TEM for phenotyping. A primary antibody against a specific protein is attached to a secondary antibody which in turn is bound to a gold particle. Samples for TEM studies have to undergo several pre-analytical steps, such as centrifugation (to obtain a pellet), dehydration and fixation. These procedures may, however, alter the size and morphology of MP, which is a potential limitation of the technique [102].

1.7.3 Atomic force microscopy

Atomic force microscopy (AFM) is high-resolution scanning microscopy (< 0.1 nm) and is mainly used for size detection of sub-micron particles. AFM involves a cantilever which has a very sharp tip attached at the end. The cantilever scans the surface of the sample by progressively moving backward and forward. The force exerted on the tip varies with the difference in the surface height and thus leads to bending of the cantilever. A laser beam is constantly reflected from the top of the cantilever towards a position-sensitive photodetector. This laser beam detects the bend occurring in the cantilever and the actual position of the cantilever is calculated, providing a 3D profile of the surface. The surface may also be coated with antibodies prior to adding the samples, thus providing phenotyping possibilities [103]. Some drawbacks of AFM are that it is time- and labor-intensive. Also, since the availability if the method is low it is not suitable for measuring MP in larger clinical trials [103].

1.7.4 Nanoparticle tracking analysis

Measurement of MP by means of nanoparticle tracking analysis (NTA) provides analysis of vesicles in the range 0.02−1.0 μm, and the technique can thus be an alternative to flow cytometry, especially when it comes to determination of MP number [104]. Briefly, in NTA particle size is measured in liquid placed under a laser beam, with simultaneous video tracking of the vesicles. All vesicles in the sample move under Brownian motion. Smaller vesicles move faster than larger particles and via the Stokes–Einstein equation, particle size can be calculated, after system calibration with beads of known size and concentration. NTA does not however, detect cellular origin, which is a disadvantage [104].

1.8 FUNCTIONAL ASSAYS

The methods presented previously all provide information about size and/or origin of MP. However, no information is obtained about their biological function, but there are indeed alternative methods to measure MP function. A number of commercial assays are available. They measure the pro-coagulant activity of MP by means of PS or TF exposure. In general, functional assays have two major limitations: 1) no size determination can be performed; samples could contain not only MP but also other vesicles such as exosomes or apoptotic bodies, or even cells or cell fragments, 2) the assays do not assess the concentration of MP in the sample but only quantify activity of the PS or TF content. Advantages are: 1) all MP, no matter what size or origin, will be measured, and 2) many of these assays are easy to perform and measurements of a large number of samples can easily be carried out in most laboratories without expensive equipments.

Pro-coagulant activity based on PS exposure
One example is the Zymphen MP activity ELISA assay (HYPHEN BioMed), which is intended to assess PS exposure. The method involves use of conventional 96-well microplates which are coated with annexin V. After addition of the sample and a washing procedure, a mixture containing the clotting factors FXa, FVa and FII together with calcium is added. PS exposed on MP enables the prothrombinase complex to be formed and thrombin is generated. A chromogenic substrate for thrombin is added to assess thrombin activity. Results are presented as nanomolar (nM) PS equivalents. The standard curve is generated by using known amounts of phospholipids.

Another method is the ELISA assay from Stago (Procoag-PPL), in which MP activity is measured by adding the patient or test sample to phospholipid-free porcine plasma together with FXa. Clotting time is measured and results are calculated from a standard curve with known amount of phospholipids.

Pro-coagulant activity based on TF exposure

A TF-activity assay from HYPHEN BioMed (Zymuphen MP TF assay), works in the same way as their PS-activity assay, except that wells are coated with monoclonal anti-TF antibodies instead of annexin V. Another method involves the use of wells coated with cell-specific (capturing) antibodies. TF activity is later measured by adding FVIIa and FX into the wells filled with plasma [105]. To ensure "true" TF activity the assay is performed in the presence and absence of a polyclonal anti-TF antibody. The results are later calculated by subtracting the pro-coagulant activity obtained in the sample containing TF antibody from the sample without TF antibody. A standard curve is constructed using known concentrations of FXa incubated with a chromogenic substrate.

Pro-inflammatory and other activities of MP

Other possibilities to measure MP activity is to assess its effects on various cells in culture with respect to cytokine release or biological activities reflecting cell growth or angiogenesis. For example, MP obtained by *in vitro* stimulation or isolated directly from patient samples can be added to cultured cells such as jurkat cells or HUVEC [78,106]. The generation of various pro-inflammatory or anti-inflammatory cytokines can then be measured [107].

2 AIMS

The overall aims of this study were:

- To describe and evaluate a flow cytometric method for measurement of microparticles in plasma, including the importance of pre-analytical sample handling.
- To study the effects of treatment with a drug with documented beneficial effects on atherothrombotic complications, on circulating microparticles and their function.
- To study the effects of inflammation on microparticle formation *in vivo*.

3 MATERIAL AND METHODS

3.1 PATIENTS AND HEALTHY CONTROLS

3.1.1 Paper I

To validate the flow cytometric method described in Paper I we analyzed plasma samples collected from 24 patients with diabetes mellitus type 1 and 18 healthy controls. The patients had no medical history of cardiovascular events. The healthy controls had no history of cardiovascular disease or diabetes and were matched for age and gender (Table 6).

Table 6. Characteristics of patients with type 1 diabetes and healthy controls presented in Paper I; ns, no statistically different significance between groups.

	Patients	**Controls**	***p*-value**
n	24	18	-
Age (years)	53 (41–75)	56 (25–71)	ns
Gender (F/M)	12/12	9/9	ns
Smoker/non-smoker (*n*)	2/1	0/0	-
Platelet count (x 10^9/L)	228 ± 39	213 ± 42	ns
Plasma glucose (mmol/L)	9.8 ± 4.0	5.4 ± 0.4	< 0.05
HbA1c (%)	6.4 ± 1.0	-	-
Total cholesterol (mmol/L)	4.6 ± 0.6	5.1 ± 1.0	ns
Triglycerides (mmol/L)	0.6 ± 0.3	0.9 ± 0.3	< 0.05
LDL-Cholesterol (mmol/L)	2.5 ± 0.6	3.4 ± 0.8	< 0.05
HDL-Cholesterol (mmol/L)	1.7 ± 0.5	1.4 ± 0.3	< 0.05

3.1.2 Paper II–III

In Papers II and III, 19 patients (14 females and 5 males) with peripheral arterial occlusive disease (PAOD) and hypercholesterolemia were recruited from the Department of Surgery, Karolinska University Hospital, Huddinge. Clinical diagnosis of intermittent claudication was verified by either duplex ultrasonography or angiography. Exclusion criteria were angina pectoris, previous acute myocardial infarction, ischemic stroke or previous coronary revascularization (i.e. coronary artery bypass grafting [CABG] or percutaneous coronary intervention [PCI]). Patients already on lipid-lowering and/or aspirin therapy were also excluded.

Following a run-in phase, patients were randomized to placebo or active treatment as shown below in Figure 5. All patients were given 320 mg aspirin (ASA; Alka Seltzer®) once daily after inclusion, and they continued with this treatment for the

entire study period. The patients were randomized to either 80 mg atorvastatin (Lipitor®) or matching placebo in a cross-over fashion, as shown below. The treatments were given for eight weeks each and separated by a four-week wash-out period. Blood sampling was performed at baseline (prior to start of aspirin treatment) and after the first and second treatment period (Fig. 5).

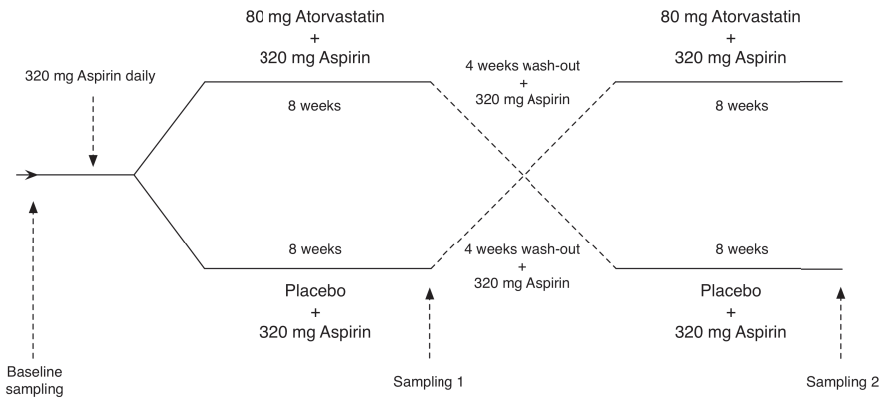

Figure 5. Study design, Papers II and III.

3.1.3 Paper IV

In Paper IV, 15 healthy volunteers (5 females and 10 males) were included, and they had no continuous medication for two months preceding the study. All volunteers were given LPS and glucocorticoids (Gc) and randomized in a double-blind fashion to inhalation of nitric oxide (iNO) or N_2 (placebo). The study had a cross-over design and the two treatments (iNO and placebo) were separated by at least six weeks. Blood samples were taken at baseline (-2.5 h), before the start of LPS infusion (-0.5 h) and at 2 h, 5 h and 24 h (Fig. 6).

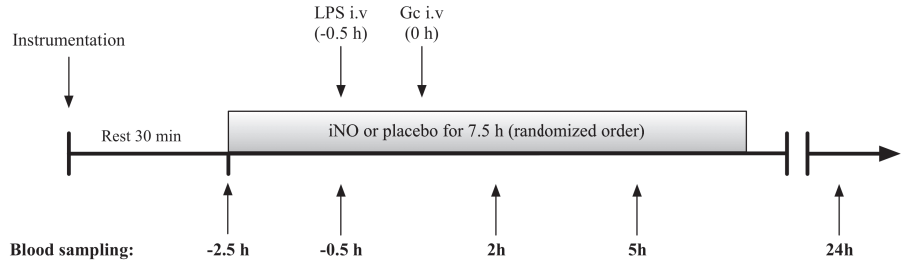

Figure 6. Study design, Paper IV. LPS: lipopolysaccharide, Gc: glucocorticoids, iNO: inhaled nitric oxide.

3.2 BLOOD COLLECTION

In Papers I–III all samples were obtained through clean puncture of an antecubital vein with no or minimal stasis, and after at least 15–20 minutes of rest in a supine position. Venous blood was collected into 5 mL vacutainer tubes containing 0.5 mL 0.129 M sodium citrate (Becton Dickinson, BD, Plymouth, UK) using 21-gauge needles (BD Vacutainer needles). The samples were then immediately centrifuged at 2000 × *g* for 20 minutes (Paper I) or 10 minutes (Papers II & III) at 15 °C, to obtain platelet-poor plasma (PPP). Aliquots of 500 µL PPP were later dispensed into plastic tubes and frozen at -80 °C until analysis.

In paper IV all blood samples (except the 24 h sample) were collected through an intravenous line (BD Venflon). All samples were collected in test tubes containing 0.5 mL 0.129 M sodium citrate. The samples were centrifuged at 2000 × *g* for 10 minutes at 4 °C, to obtain PPP. Aliquots of 500–1000 µL of PPP were later dispensed into plastic tubes and frozen at -80 °C until analysis.

3.3 MICROPARTICLE PREPARATION

In Paper I & II a MP-enriched pellet was prepared through high-speed centrifugation. Briefly, frozen aliquots of PPP were thawed at 37 °C for roughly 5 min and then centrifuged for 45 min at 20 800 × *g* at 10 °C. The supernatant was then removed, leaving 50 µL of a MP-enriched suspension which was further diluted with 450 µL of Tris-saline (50 mM Tris, 154 mM NaCl, pH 7.6) and centrifuged again for 45 min at 20 800 × *g* at 10 °C. Again, 450 µL of the supernatant was removed and the MP-enriched pellet was resuspended in the remaining 50 µL. Twenty µL of sample were then incubated with antibodies and prepared for flow cytometric analyses.

In paper III & IV we changed our flow cytometric protocol somewhat, and used the supernatant instead of a pellet. This necessitated a modification in MP preparation. Thus, the previously frozen PPP sample was thawed and centrifuged at 2000 × *g* for 20 minutes at room temperature (RT). The supernatant was then re-centrifuged, at 13 000 × *g* for 2 minutes, again at RT. Twenty µL supernatant was then incubated with antibodies and prepared for flow cytometric analyses.

3.4 METHODS

3.4.1 Flow cytometry

Paper I

In Paper I we present a two-color flow cytometric protocol for measurement of PMP. Twenty µL of plasma was incubated in the dark with 5 µL of CD42a-PE (GPIX; BD, Clone ALMA.16) and CD62P-FITC (P-selectin; AbD Serotec, Clone AK-6) or CD42a-PE and CD142-FITC (TF; AbD Serotec, Clone CLB/TF-5). After 20 minutes, the incubation was stopped by adding 500 µL of BD Cellfix™ (Becton Dickinson

Immunocytometry Systems, CA, USA). A BD FACScan with CELL Quest software (Becton Dickinson Immunocytometry Systems, CA, USA) was used and parameters were set at logarithmic gain. FS was set against FL1, and FL1 was chosen as threshold. Proper gating was established through Megamix beads (0.3 µm and 1.0 µm beads, American Diagnostica, Stamford, USA) and later optimized with fresh PRP, where platelets and PMP could clearly be distinguished. Conjugate isotype-matched immunoglobulin (IgG1-FITC, IgG1-PE) with no reactivity against human antigens was used as a negative control to define the background noise of the cytometric analysis.

The mean fluorescence intensity (MFI) after 30 seconds of measurement was translated into Molecules of Equivalent Soluble Fluorochrome (MESF) using calibration standard type IIIB (Schwartz 98), Quantum™ 26–FITC (Bang Laboratories, Fishers, IN, USA). This calibrator contains five equally sized bead populations. One population has no fluorochrome ("reference blank") and the other four populations of beads contain various amount of fluorochrome (Fig. 7). The software accompanying the beads translates then the fluorescence intensity of each population into corresponding MESF values and a calibration curve is obtained Samples of unknown fluorescence intensities were then measured and plotted against this calibration curve.

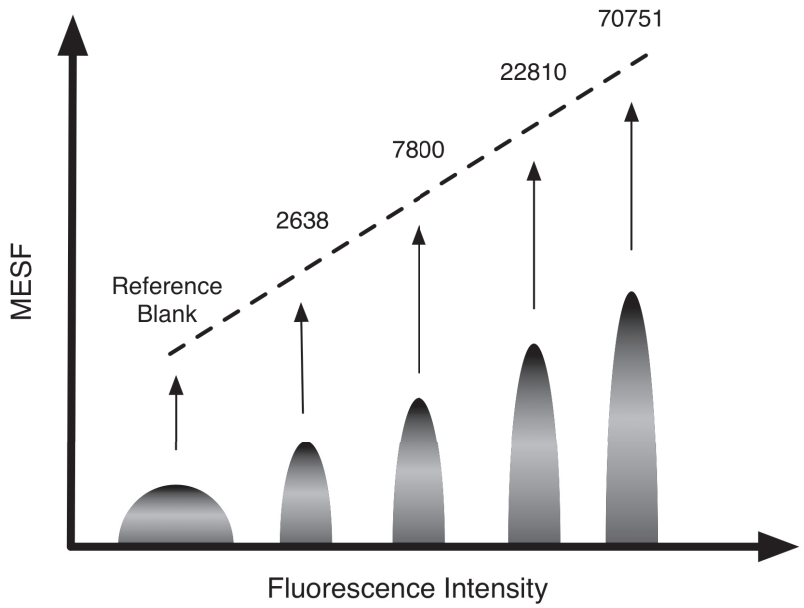

Figure 7. Molecules of Equivalent Soluble Fluorochrome (MESF) calibration plot. Five bead populations, i.e. blank and four populations with known amount of fluorochrome per bead. With the obtained calibration plot the mean fluorescence intensity is translated into MESF values.

Paper II

In Paper II we used two different flow cytometers to detect PMP. Measurements of $CD42a^+$ $CD142^+$ PMP were performed on the BD FACScan and measurements of PS or $CD42a^+$ $CD61^+$ or $CD42a^+$ $CD62P^+$ PMP were performed on the Beckman Coulter Gallios™ flow cytometer. Briefly, a MP-enriched pellet was obtained by high-speed centrifugation from plasma samples as described earlier, and twenty µL of sample was incubated for 20 min at RT with 5 µL of annexin V-FITC (AbdSerotec, Clone VAA-33, Oxford, UK), lactadherin-FITC (MFG-E8, Haematologic Technologies, Essex Junction, VT, USA), CD42a-PE (BD, Clone ALMA.16, Franklin Lakes, NJ, USA), CD142-FITC (AbDSerotec, Clone CLB/TF-5), CD61-FITC (BD, NJ, USA) or CD62P-FITC (BD, Franklin Lakes, NJ, USA). To measure the amount of platelet/cell fragments, samples were incubated with CD42a-PE and phalloidin-FITC (Sigma-Aldrich, St. Luis, MO, USA). FS was set against FL1, and FL1 was chosen as threshold. Proper gating was established through Megamix beads (0.3 µm and 1.0 µm beads, American Diagnostica, Stamford, USA) and later optimized with fresh PRP, where platelets and PMP could clearly be distinguished. Fresh PRP, which has a low phalloidin level, was used to determine the cut-off point for phalloidin-positive events in the MP gate. Conjugate isotype-matched immunoglobulin (IgG1-FITC, IgG1-PE) with no reactivity against human antigens was used as a negative control to define the background noise of the cytometric analysis. MFI after 30 seconds of measurement was translated into MESF values.

Paper III

In Paper III we carried out a comparison of MP results, presenting them as MESF values (i.e. amount of expression of a specific antigen) or concentration. Twenty µL of supernatant was incubated for 20 minutes in the dark with 5 µL of phalloidin-Alexa 660 (Invitrogen, Paisley, UK), lactadherin-FITC (Haematologic Technologies, VT, USA), CD144-APC (AH diagnostics, Stockholm, SWE) and CD142-PE (BD, NJ, USA). Samples were analyzed in a Beckman Coulter Gallios™ flow cytometer, and the EMP gate was determined using Megamix beads (0.5 µm, 0.9 µm and 3.0 µm, BioCytex, Marseille, FR). EMP were defined as particles < 1.0 µm in size, negative to phalloidin, positive to lactadherin and CD144 together with (and without) CD142. Conjugate isotype-matched immunoglobulins (IgG1-FITC, IgG1-PE and IgG1-APC) with no reactivity against human antigens were used as negative control. The results are presented as both concentrations of EMP ((EMP counted × standard beads added/L)/standard beads counted) (FlowCount, Beckman Coulter) and as MESF values (Bang Laboratories, Fishers, IN, USA).

Paper IV

In Paper IV we measured MP from platelets, monocytes and endothelial cells using a five-color protocol. Twenty µL of supernatant was incubated for 20 minutes in the dark with 5 µL of phalloidin-Alexa-660 (Invitrogen, Paisley, UK), lactadherin-FITC

(Haematologic Technologies, Vermont, USA), CD42a-PE (BD, Clone ALMA.16), CD14-PC7 (Beckman Coulter, Dublin, Ireland) and CD62E-APC (Becton Dickinson Immunocytometry Systems, CA, USA). In addition to CD62E, EMP were identified through labeling with CD144-APC (AH diagnostics, Stockholm, SWE). CD14-positive MP (i.e. MMP) were also stained with anti-HMGB1-PE (R&D Systems, Minneapolis, USA). MP were measured by flow cytometry on a Beckman Coulter Gallios^{TM} (CA, USA). The threshold was set on FS, and the MP gate was determined using Megamix beads (0.5 µm, 0.9 µm and 3 µm, BioCytex, Marseille, France. MP were defined as particles < 1.0 µm in size. Conjugate isotype-matched immunoglobulins (IgG1-FITC, IgG1-PE, IgG1-APC and IgG1- PC7) with no reactivity against human antigens were used as negative controls to define the background noise of the cytometric analysis. The results are presented as concentrations of MP ((MP counted × standard beads added/L)/standard beads counted) (FlowCount, Beckman Coulter).

We also measured nucleic acid content in platelet and monocyte MP by labeling them with SYTO 13. This is a dye that binds both DNA and RNA with high fluorescent yield, although it has a preference for double-stranded DNA. However, we had to modify our flow cytometric protocol somewhat. MP were identified through gating according to size (FS *vs.* SS) and SYTO 13 binding instead of PS exposure (lactadherin binding). The latter approach was used as a result of incompatibility between lactadherin-FITC and the SYTO 13 dye. Twenty µL of supernatant was incubated in the dark for 20 minutes with 5 µL SYTO 13 (250 nM, Molecular Probes, Eugene, OR) and anti-HMGB1-PE (R&D Systems, Minneapolis, USA) together with either CD14-PC7 (Beckman Coulter, Dublin, Ireland) or CD41-PC7 (Beckman Coulter, Dublin, Ireland); CD41 had to be used instead of CD42a in these experiments because of a lack of anti-CD42a antibodies with compatible labeling. MP were measured with a Beckman Gallios flow cytometer as described above. The results were presented as both concentrations of SYTO 13^+ ((MP counted × standard beads added/L)/standard beads counted) (FlowCount, Beckman Coulter) or as MFI.

3.4.2 Transmission Electron Microscopy

In Paper I we also examined our MP-enriched pellets by TEM. PRP was prepared by centrifugation of blood samples at 150 × *g* for 10 minutes at 15 °C (0.129 M sodium citrate tubes). One aliquot was centrifuged in order to obtain a PMP-enriched pellet, as described in Paper I, directly without any platelet activation. The second aliquot, collagen (5 µg/mL; CHRONO-PAR, Diagnostica Stago, Asnière, France) was added to the PRP and platelets were left to aggregate in a Platelet Aggregation Profiler (PAP-4, BIO/ DATA Corporation, Horsham, USA) for 15 minutes at 37 °C and with stirring at 1200 rpm. In order to obtain a PMP-enriched pellet, the "activated" PRP sample was centrifuged as described previously (i.e. two centrifugations at 2000 × *g*

for 20 min, and one centrifugation at 20 800 × *g* for 45 min). The MP-enriched pellet obtained from resting and activated platelets was immediately fixed in 4% paraformaldehyde, and PMP were visualized by TEM using negative staining. In brief, an aliquot of 4 µL was added to a grid with a formvar supporting film coated with carbon for 5 min. The excess solution was soaked off with filter paper, and the sample was stained with 0.5% uranyl acetate in water for 10 sec and air-dried. Samples were visualized in a Leo 906 electron microscope at 80 kV. Digital images were taken with a Morada camera (SiS Münster, Oberkochen, Germany).

3.4.3 Prothrombin fragment concentrations

In Paper II, prothrombin fragment (F1+2) concentrations in plasma were measured by using Enzygnost® F1+2 (monoclonal) kits (Dade Behring) according to the manufacturer's instructions.

3.4.4 Calibrated Automated Thrombogram

Thrombin generation in plasma was determined by using a calibrated automated thrombogram (CAT) as described by Hemker et al. [108] and according to the instructions of the manufacturer (Thrombinoscope BV, Maastricht, the Netherlands). Thrombin generation in this assay was initiated by the addition of 20 µL of both PPP reagent and FluCa reagent to 80 µL of plasma, giving final concentrations of 5 pM TF and 4 µM phospholipids, respectively. The calculated area under the curve represents the total amount of thrombin generated over time, and is called endogenous thrombin potential (ETP). Time to the start of thrombin generation (lag time), maximal concentration of thrombin generation (peak thrombin) and time to maximal thrombin generation (time to peak) are also assessed in CAT analysis (Fig. 8).

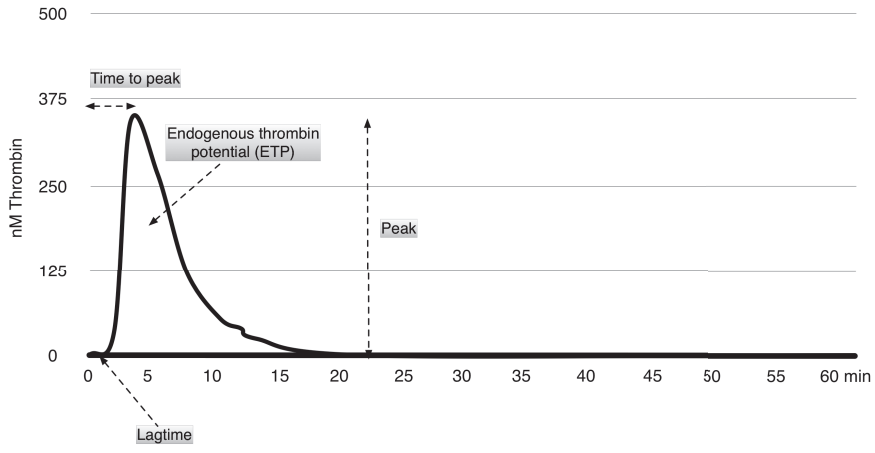

Figure 8. Calibrated Automated Thrombogram (CAT).

3.4.5 Effect of microparticles on thrombin formation

In order to study the influence of MP on thrombin generation, *in vitro* experiments were performed using CAT analysis. MP-enriched pellets were obtained from five patient samples collected during placebo treatment (Paper II), as described earlier. The pellet was then added to previously centrifuged commercial normal plasma (20 000 x *g* for 45 min at RT, Haemochrom, Diagnostica, Essen, Germany), but with no addition of TF or phospholipids, and thrombin generation was measured for 60 minutes. In order to further investigate the possible mechanisms of MP action in thrombin generation, experiments were also performed after addition of either a polyclonal antibody (American Diagnostica) or a monoclonal antibody against TF (AbDSerotec, Clone CLB/TF-5). A negative/isotype control mouse antibody (IgG1, AbDSerotec) was used to investigate possible unspecific effects of addition of an antibody in the CAT assay. The effects of the TF antibodies were also determined by adding them to recombinant TF in normal pool plasma. For comparison, blocking of the negatively charged PS was also investigated by testing the effects of addition of recombinant annexin V (final concentration 22 μM) or lactadherin (final concentration 40 μM) in commercial normal plasma.

3.4.6 Other laboratory methods

3.4.6.1 11-dehydro-thxromboxane B_2

Overnight excretion of 11-dehydro-thxromboxane B_2 (11-dehydro TxB_2) in urine, which reflects platelet function *in vivo* and is a sensitive monitoring technique of aspirin treatment, was determined by using a commercially available enzyme immunoassay (Cayman Chemical Co., Ann Arbor, MI, USA), and a sample procedure developed by Perneby et al. [109].

3.4.6.2 Routine analyses

Concentrations of plasma lipids, plasma glucose, glycosylated hemoglobin (HbA1C) and platelet counts in venous blood were all analyzed by routine laboratory techniques at the Clinical Chemistry Laboratory at Karolinska University Hospital, Solna.

4 STATISTICAL ANALYSES

Statistical calculations throughout Papers I–IV were performed using SPSS (version 16.0/19/21, SPSS Inc., Chicago, IL, USA) or Graphpad Prism software (version 5.0b/5.0c/6.0, La Jolla, CA). All figures were created by using Graphpad Prism software. We considered normality to be present if skewness was above -1 and below 1. Normally distributed data are presented as means ± SD, whereas skewed data are presented as medians and 25th and 75th percentiles.

Paper I:
Patient and control data are presented as median and interquartile range. Differences between patients and healthy controls were evaluated by analysis of variance (ANOVA). Coefficients of variation (CV) were calculated from the ratio of standard deviation and mean.

Paper II:
Power calculation was based on P-selectin expression on PMP in patients with claudication (baseline sample). It was estimated that 20 patients were needed to detect a 30% difference between treatments with a power of 80% at a significance level of $p < 0.05$ (two-sided test). Normally distributed data were analyzed by Student´s paired t-test. Skewed data (only F1+2 data) were logarithmically transformed and then analyzed by Student´s paired t-test.

Paper III:
Results are presented as median and interquartile range. Horizontal lines in the figures show the median. All data were analyzed by using Wilcoxon's matched-pairs signed rank test. For correlation tests, regression analysis was used (Spearman's Rank Correlation).

Paper IV:
All statistical analyses were performed between -0.5 hours (at LPS administration) and 24 hours. To evaluate the effect of treatment (iNO or placebo; factor 1) and changes over time (the effect of LPS; factor 2) a two-factor repeated-measures analysis of variance (ANOVA) was performed. Differences in MFI between two types of particles were assessed by using two within-factors repeated-measurements ANOVA. The two within-factors consisted of: 1) type of particle and 2) effect over time, and the interaction type of particle and time.

5 RESULTS AND DISCUSSION

5.1 PAPER I

Pre-analytical handling

In order to refine our flow cytometric assay, and especially the pre-analytical steps preceding the flow cytometric measurements, we were interested in distinguishing between PMP and disturbing platelet fragments. We thought the latter could be found in significant amounts in plasma samples not adequately processed. We hypothesized that platelet fragments could overlap in size and express the same antigens as PMP and thus "falsely" be detected as PMP in the PMP gate. A simple experiment was performed where fresh PRP was obtained by centrifugation of citrate blood samples at RT at 150 × *g* for 10 minutes. One aliquot was analyzed directly after sample preparation ("fresh PRP") and the other aliquot was frozen and stored for 24 h at -80 °C and then thawed in a water bath at 37 °C for 10 min. Prior to flow cytometric analysis, platelets in PRP were labeled with CD42a-PE and phalloidin-FITC. Phalloidin is a peptide isolated from the death cap mushroom and it binds with high affinity to filament actin (f-actin) [110].

The results showed that in fresh PRP 0.3 ± 0.2% of the platelets were $CD42a^+$ $phalloidin^+$, and in the frozen/thawed PRP 39 ± 3.9% of the platelets were $CD42a^+$ $phalloidin^+$ (n=10). This indicated that during the freezing/thawing procedure, a significant number of platelets had their cell membranes destroyed and f-actin molecules became exposed and bound to phalloidin (Fig. 9).

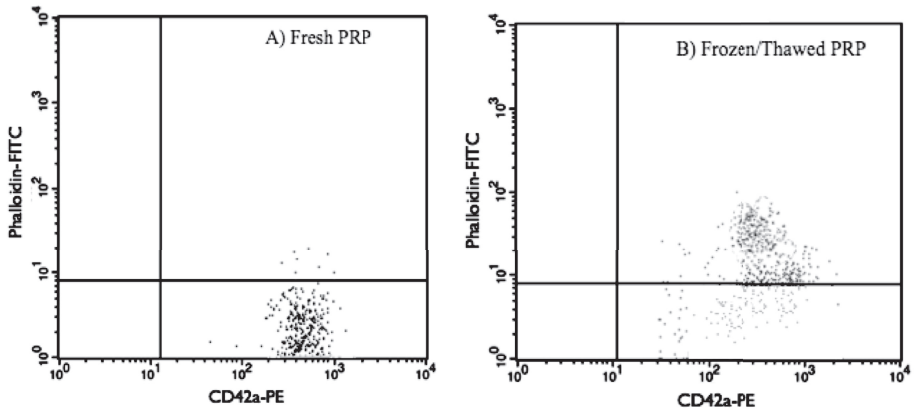

Figure 9. Effects of freezing and thawing platelet-rich plasma. The left plot shows analysis of a fresh PRP sample (A) and the right plot a frozen/thawed PRP sample (B).

Further on, we investigated the possibility of optimizing the centrifugation procedure prior to freezing a sample intended for MP analysis. Three samples with different levels of "platelet contamination" (i.e. PRP, PPP and PFP) were prepared and measurements were carried out before and after freezing. All samples were labeled with CD42a and phalloidin and the supernatant was used for measurements. All $CD42a^+$ $phalloidin^+$ events in the platelet gate were collected and the results showed that double centrifugation prior to freezing (i.e. centrifugation twice at 2000 × *g*) was associated with minimal CD42a- and phalloidin-positive events (Table 7).

Table 7. Effects of different centrifugation protocols and storage forms on the number of events positive for CD42a and phalloidin in the supernatant.

CD42a/Phalloidin	Fresh	RT, 24h	-20 °C, 24h	-80 °C, 24h
Platelet-rich plasma (PRP; 150 × *g, 10 min*)	2 ± 1.2	12 ± 4.9	467 ± 99.3	738 ± 113.8
Platelet-poor plasma (PPP; 2000 × *g, 20 min*)	0 ± 0.4	1 ± 0.9	11 ± 2.4	33 ± 3.2
Platelet-free plasma (PFP; 2 × 2000 × *g, 20 min*)	1 ± 0.6	1 ± 0.3	0 ± 0.2	0 ± 0.4

Calculation of intra- and interassay coefficients of variation (% CV) for the PMP measurements were carried out on samples from seven healthy individuals. The samples were collected on two different days, and after obtaining a MP-enriched pellet the samples were labeled with CD42a and CD62P, or CD42a and CD142. The intra- and interassay CVs for $CD42a^+$ $CD62P^+$ with regard to MESF values were 6.2% and 7.2%, respectively, and with regard to the number of positive events the corresponding CVs were 14.3% and 16.5%, respectively. The intra- and interassay CVs for $CD42a^+$ $CD142^+$ were 7.1% and 6.4% for MESF values, respectively, and 18.2% and 19.9% for number of positive events, respectively (Table 8).

Table 8. Intra- and inter-assay CVs for MESF and number of events positive for CD62P and CD142 (TF).

	Intraassay	Interassay
MESF CD62p	6.2%	7.2%
Events CD62p	14.3%	16.5%
MESF TF	7.1%	6.4%
Events TF	18.2%	19.9%

All particles were $CD42a^+$

We also investigated if results expressed as MESF values were comparable between laboratories and instruments. We measured one PMP plasma sample on two flow cytometers, a BD FACSCanto and an older BD FACScan at two different laboratories. Before measuring the sample we ensured that the settings of both cytometers were as similar as possible, and both cytometers were calibrated by using Quantum™ calibration beads. When the results were expressed as MESF we obtained a CV of 2.8%, as compared with 29.6% when the results were expressed as numbers of events (Fig.10).

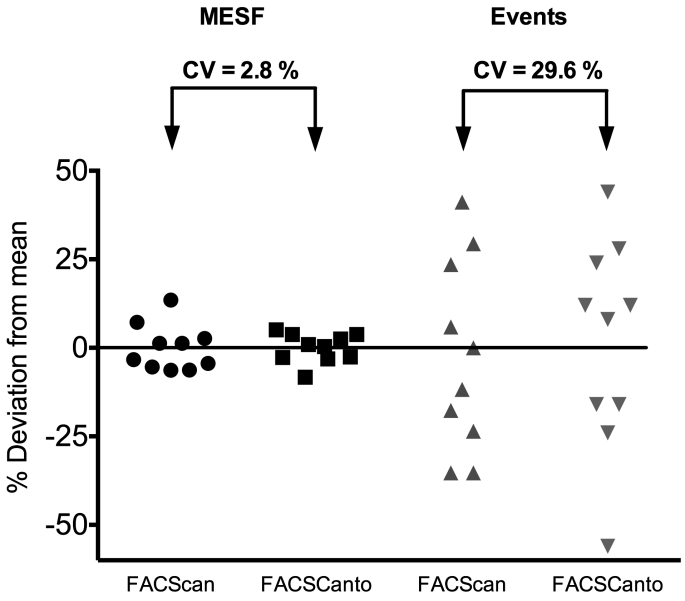

Figure 10. Repeated measurements of PMP in a plasma sample from a healthy control person. Comparisons of two different flow cytometers in two different laboratories.

Transmission electron microscopy

In the suspensions intended for PMP analysis and prepared from PRP we observed no platelets (*n*=4). The suspensions from non-stimulated PRP contained mainly intact round submicron particles in a size range of 0.1–0.3 μm (Fig. 11A). In samples prepared from PRP stimulated with collagen the particles observed seemed to be somewhat larger than those in samples from non-stimulated PRP (range 0.5−0.7 μm, Fig. 11 B, C).

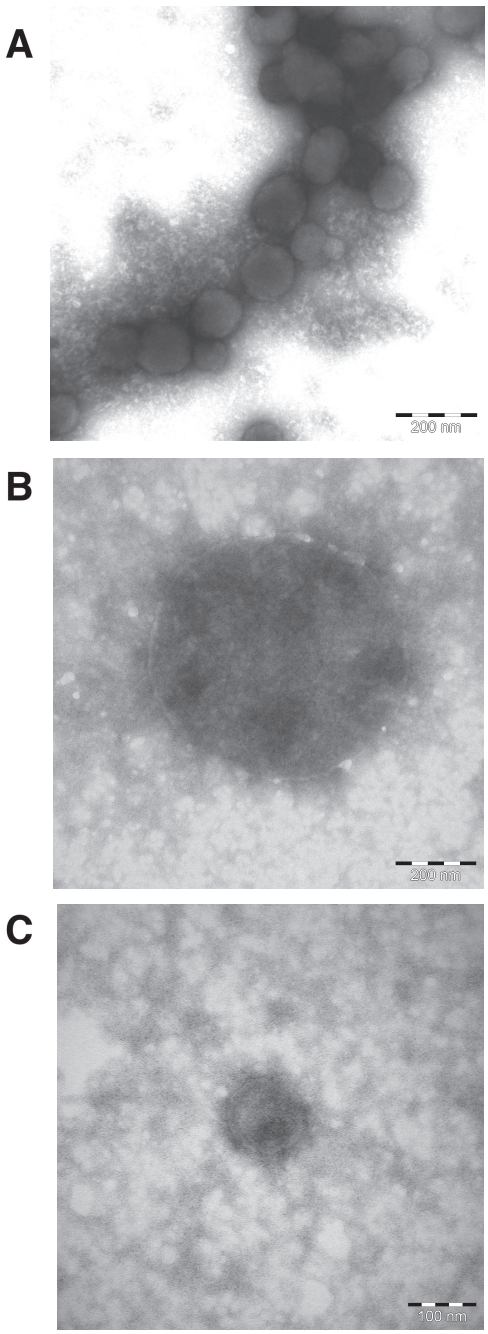

Figure 11. A: Particles in a PMP-enriched pellet obtained from non-stimulated PRP. B: A particle observed in a PMP-enriched pellet obtained from collagen-stimulated PRP. C: A particle observed in a PMP-enriched pellet from non-stimulated PRP

PMP in patients with type 1 diabetes mellitus and in healthy controls

To validate our method further, we measured the expression of CD62P and CD142 on PMP in patients with type 1 diabetes (n = 24) and healthy controls (n = 18). The patients had significantly higher exposure (MESF values) of CD62P (4498 ± 785 compared with healthy controls, 2405 ± 311; $p < 0.0001$) and CD142 (2282 ± 374 compared with healthy controls, 1069 ± 242; $p < 0.0001$) on PMP. The proportion of positive events for phalloidin in plasma from healthy controls was 8.9 ± 2.6% compared with 10.3 ± 3.8% in plasma from patients with type 1 diabetes (p = 0.14 between groups).

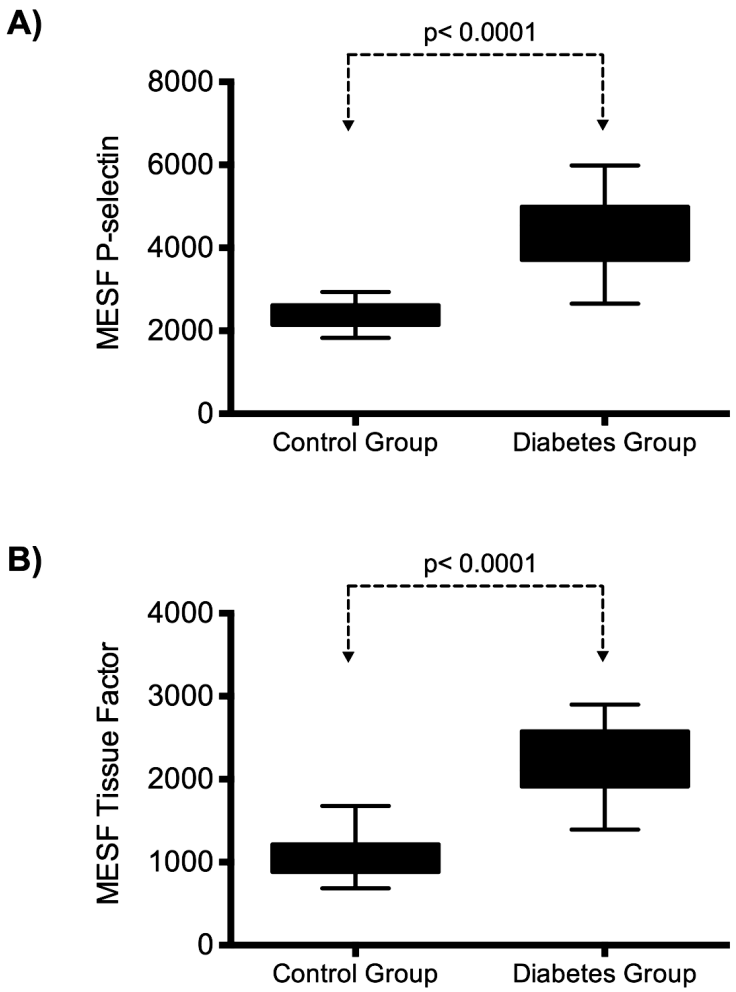

Figure 12. PMP exposing CD62P (A) and CD142 (B) in plasma samples from patients with type 1 diabetes (n = 24) and healthy control subjects (n = 18). Data are shown as MESF values.

Discussion

We describe a quantitive flow cytometric assay for measuring PMP and their expression of some specific markers. Expression of these markers were measured as MFI and calculated and presented as MESF values. With this approach we obtained more reproducible data, compared with counting the number of particles per volume unit. Using this flow cytometric assay we found that patients with type 1 diabetes had PMP with approximately twice as much CD62P and CD142 exposure as PMP from healthy controls matched for age and sex.

We also found that platelet fragments/broken platelets can be detected with FITC-labeled phalloidin. This peptide binds to the intracellular contractile protein f-actin, and is not cell-permeable [110,111]. When destroyed, platelets can expose cytoskeleton proteins like f-actin and thus bind phalloidin. Detection of phalloidin-positive particles could be used for quality control of plasma samples intended for PMP measurements. This approach could be used to ensure adequate pre-analytical handling, and to exclude the possibility that cell fragments present in the sample could disturb MP measurements.

The sample preparation procedure used in Paper I was very similar to the one previously described by Pereira et al. [92]. A common way to detect MP is to label them with annexin V (also used by Pereira et al.). Annexins are a group of proteins generally found in eukaryotic cells, and annexin V is known to bind to PS. Annexin V has been used as a general marker for MP, although this approach has been questioned [22,38]. Relatively early in our method development we encountered unspecific binding of annexin V and therefore we abandoned this labeling technique. For detection of PMP we used CD42a, a platelet-specific protein (GPIX), and moreover we assessed exposure of CD62P or CD142. By measuring only the amount of exposure (MESF) instead of concentration of particles, the variability of the method was significantly reduced. This may be of special importance when older cytometers (like BD FACScan) are used, since they have lower sensitivity for detection of small particles. Another advantage with the MESF protocol is that very similar data can be obtained in different laboratories with different cytometers.

In order to validate the MESF protocol in a clinical setting we analyzed plasma samples collected from patients with type 1 diabetes and from healthy controls. Patients with type 1 diabetes had significantly higher exposure of CD62P and CD142 on PMP compared with healthy controls. Similar results have been shown in patients with type 2 diabetes, and support the concept of diabetes being associated with increased platelet activation [87,88].

5.2 PAPER II

Laboratory investigations

Atorvastatin treatment significantly reduced levels of total cholesterol, LDL-cholesterol and triglycerides, whereas HDL-cholesterol levels did not change (Table 9). Aspirin treatment also significantly reduced the excretion of 11-dehydro TxB_2 into urine and levels remained stable during the randomized treatments ($p = 0.3$ between treatments).

Table 9. Measurements during atorvastatin treatment and placebo. Data are presented as mean ± SD.

	Baseline	Placebo	Statins	*p*
Total cholesterol (mmol/L)	7.3 ± 1.0	7.0 ± 1.1	4.1 ± 0.8	< 0.001
LDL-cholesterol (mmol/L)	5.0 ± 0.8	4.8 ± 0.8	2.2 ± 0.6	< 0.001
HDL-cholesterol (mmol/L)	1.3 ± 0.3	1.3 ± 0.4	1.4 ± 0.4	0.3
Triglycerides (mmol/L)	2.1 ± 1.2	2.0 ± 1.3	1.4 ± 0.6	< 0.001
Platelet count (10^9/L)	286 ± 54	286 ± 54	284 ± 56	0.8
11-dehydro TxB_2 (ng/mmol creatinine)	75.6 ± 33.4	16.3 ± 6.5	15.0 ± 4.6	< 0.001*

**) placebo or statin treatment vs baseline. All other p-values refer to comparisons between statin treatment and placebo.*

Thrombin generation

Thrombin generation *in vivo* measured as F1+2, decreased during atorvastatin therapy (from 1.25 [95% CI, 1.07, 1.39] nM with placebo to 1.06 [0.90, 1.31] nM during atorvastatin treatment; $p < 0.01$). In addition, the thrombin generation potential in plasma assessed as ETP was significantly lower during atorvastatin treatment compared with placebo (1684 ± 286 *vs.* 1883 ± 282 nM × min; $p < 0.001$). The peak thrombin concentration attained was reduced (328 ± 42 during atorvastatin treatment *vs.* 347 ± 54 nM during placebo, $p < 0.02$), as was the time to peak thrombin concentration (5.0 ± 0.6 *vs.* 5.2 ± 0.7 min, $p < 0.02$). No change was seen in lag time (2.51 ± 0.40 *vs.* 2.55 ± 0.42 min, $p = 0.7$).

Platelet derived microparticles

Exposure of CD61, CD62P and CD142 was significantly reduced during atorvastatin treatment compared with placebo ($p < 0.001$). However, no effect was seen on PS expression measured by annexin V or lactadherin binding (Fig. 13A-B). Roughly 10% of PMP were positive for phalloidin, indicating acceptable sample handling and sample quality.

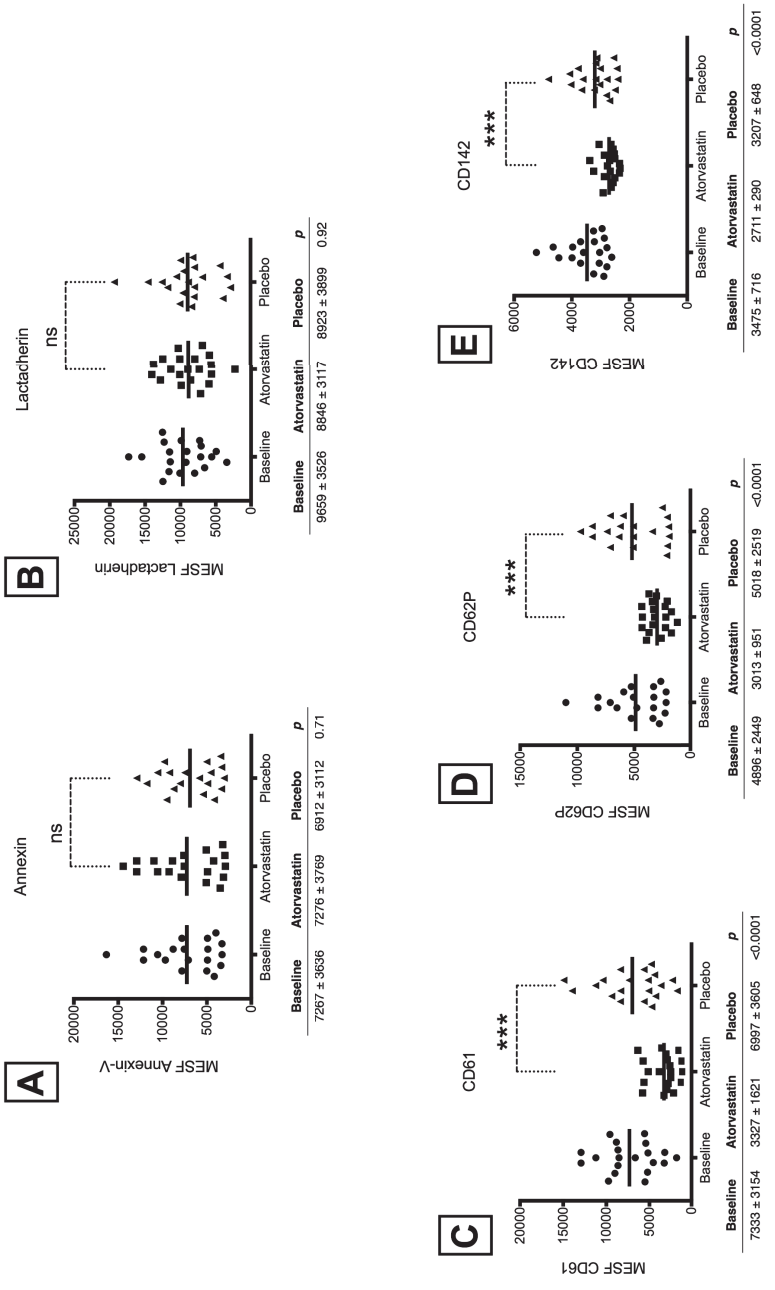

Figure 13. The effect of atorvastatin on phosphatidylserine expression, i.e. annexin V or lactadherin binding (A and B), and on CD61, CD62P and CD142 exposure on PMP (i.e. $CD42a^{+}$ MP) (C–E).

Influence of microparticles on thrombin generation

As both thrombin generation *in vivo* and *in vitro,* and CD142 exposure on PMP, were reduced during atorvastatin treatment, further experiments were performed to investigate the possible influence of MP on thrombin generation. The results showed that when MP from an MP-enriched pellet were added to plasma all of the variables of the thrombogram were altered. ETP and peak thrombin increased significantly, and lag time and time to peak was significantly reduced (Table 10). To further investigate if PS or TF on MP contribute to this effect, MP were pre-incubated with either annexin V (22 μM) to block PS, or pre-incubated with an antibody against TF. In addition, to evaluate TF inhibition, we used monoclonal and polyclonal TF antibodies. Thrombin generation was initiated with 5 pM TF, and the effects of increasing concentrations of the antibodies were measured against a positive control (i.e. no TF blocking). At 40 ng/mL, both antibodies prolonged lag time by roughly 93% in relation to the positive control. The addition of mouse IgG1 had no effect in the CAT assay. The experiments described in Table 10 were performed with polyclonal anti-TF. The results showed that by blocking TF exposure with the antibody a slight but statistically significant decrease in thrombin generation was obtained. However, blocking PS with annexin V resulted in much greater inhibition of thrombin generation.

Table 10. The effect of MP on thrombin generation. Results are shown as mean ± SD ($n = 5$). Values of p were obtained via paired t-tests. **$p < 0.001$ *vs.* without MP, †$p < 0.01$ *vs.* with MP, ∞∞$p < 0.001$ *vs.* with MP.

	Without MP	**With MP**	**With TF antibody**	**With Annexin V**
ETP (nM*min)	929 ± 72	1438 ± 24 **	1219 ± 20.1 †	640 ± 92 ∞∞
Lagtime (min)	24.7 ± 3.2	8.4 ± 1.2 **	10.9 ± 0.7 †	21.9 ± 0.9 ∞∞
Peak (nM)	59.4 ± 11.8	135.2 ± 5.4 **	116.2 ± 0.9 †	55.42 ± 0.5 ∞∞
ttPeak (min)	36.7 ± 2.0	15.3 ± 2.0 **	16.3 ± 0.8 †	30.3 ± 1.3 ∞∞

Further testing showed that by pre-incubating MP with different concentrations of annexin V or lactadherin, we could see a concentration-dependent inhibition of thrombin generation with both compounds (Fig. 14). Notably, lactadherin is, as described previously (page 5), a protein that binds to PS with a higher sensitivity than annexin V [112].

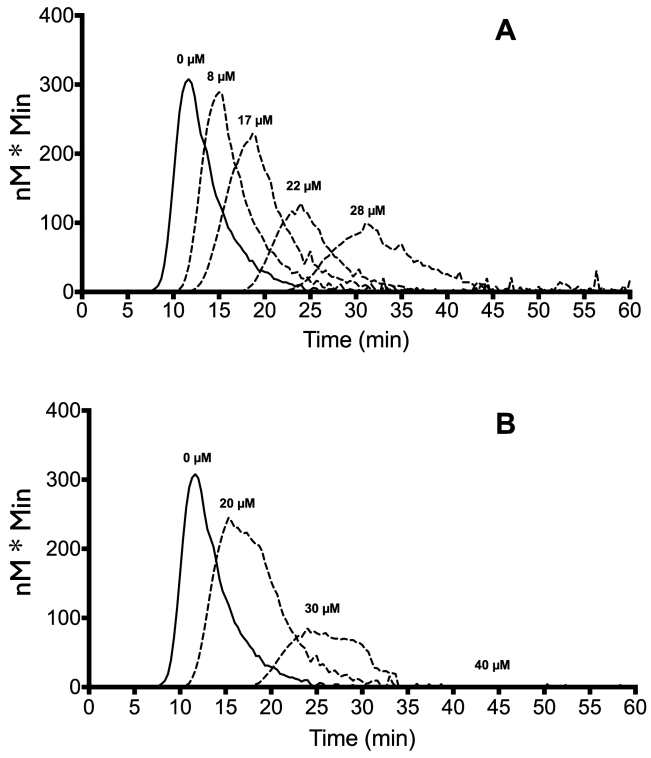

Figure 14. Effects of microparticles on thrombin generation (ETP) in the presence of increasing concentrations of annexin V (Panel A) and lactadherin (Panel B).

Discussion

We found significant reductions in exposure of CD61, CD142 and CD62P on PMP, whereas the amount of PS exposure on PMP, as measured by binding of annexin V or lactadherin, was not influenced by atorvastatin treatment. The reduction of CD61, CD142 and CD62P on PMP could indicate platelet-inhibiting effects of atorvastatin therapy. In the present study all patients were put on 320 mg aspirin daily after baseline sampling (see Fig. 5). This made it possible to assess whether or not aspirin treatment influenced thrombin generation or PMP, albeit in an uncontrolled and open fashion. Our data strongly suggest that aspirin has no significant effect on these variables in patients with PAOD.

We have previously observed similar reductions of exposure of CD61, CD142 and CD62P in patients with type 1 diabetes treated with 80 mg atorvastatin per day [113]. In Paper II (or in the study by Teherani et al [113]) we did not investigate the concentration of PMP and therefore we cannot say to what extent our findings reflect reduced PMP counts, reduced antigen exposure on PMP, or a combination of both. However, our interpretation (which is mainly based on data obtained from Paper III) is that antigen

expression grossly follows the number of MP. Whether or not statins inhibit platelet function is still considered controversial, and there are reports showing both inhibiting effects [114,115] and no effects [116]. Our data agree with a platelet-inhibiting effect of statin treatment. Notably, in line with our findings there is another study that shows reduction of CD61 (GPIIIa) expression on PMP from patients with type 2 diabetes treated with pravastatin [117].

In vitro experiments revealed pro-coagulant properties of MP, i.e. they increased thrombin generation in plasma samples. Our experimental data indicate that TF measured on MP has a small but statistically significant enhancing effect on thrombin generation. However, great inhibition of thrombin generation could be seen by blocking PS exposure by annexin V, indicating that PS exposed on MP membrane is quantitatively more important than TF. Nevertheless, our data strongly suggest that TF exposure together with the negative surface provided by MP represent mechanisms behind the pro-coagulant functions of MP. Statins may inhibit initiation of thrombin generation partly through a MP-dependent mechanism, but the main effect is most likely through other mechanisms, e.g. reduction of lipoprotein levels. Lipoproteins such as VLDL, and especially oxidized LDL, may be as pro-coagulant as MP in terms of supporting thrombin generation through PS exposure [118-120].

5.3 PAPER III

Measurement of endothelial derived microparticles

EMP were measured both as concentration and amount of expression (MESF protocol). Our data showed that EMP measured as lactadherin^{+} CD144^{+} particles or lactadherin^{+} CD144^{+} CD142^{+} particles were significantly higher during atorvastatin treatment compared with placebo (Figs. 15 A and B). Significantly higher values during atorvastatin treatment were also seen when using the MESF protocol (Figs. 15 C and D). As seen in the figure below, the MESF protocol seems to yield lower variability compared with the protocol that assesses concentration,

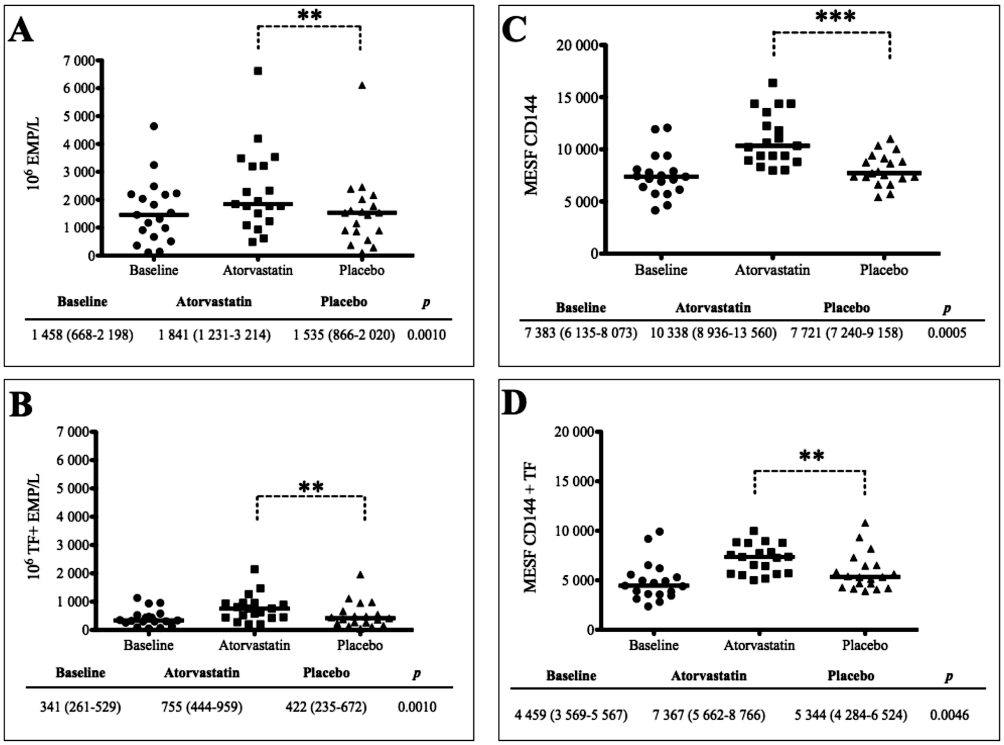

Figure 15. In panel A (CD144^{+}) and in panel B (CD144^{+} CD142^{+}) concentration of EMP are presented ($\times 10^{6}$ EMP/L). In panel C (CD144^{+}) and in panel D, (CD144^{+} CD142^{+}) EMP results are presented as the amount of expression (MESF values). Horizontal lines indicate medians. $^{**}p < 0.01$, $^{***}p < 0.001$.

Numbers Vs. amount of expression

A statistically significant correlation was observed between the MESF data and the number of EMP (Spearman's Rank Correlation, $R^2 = 0.35$, $p < 0.0001$; $n = 19$).

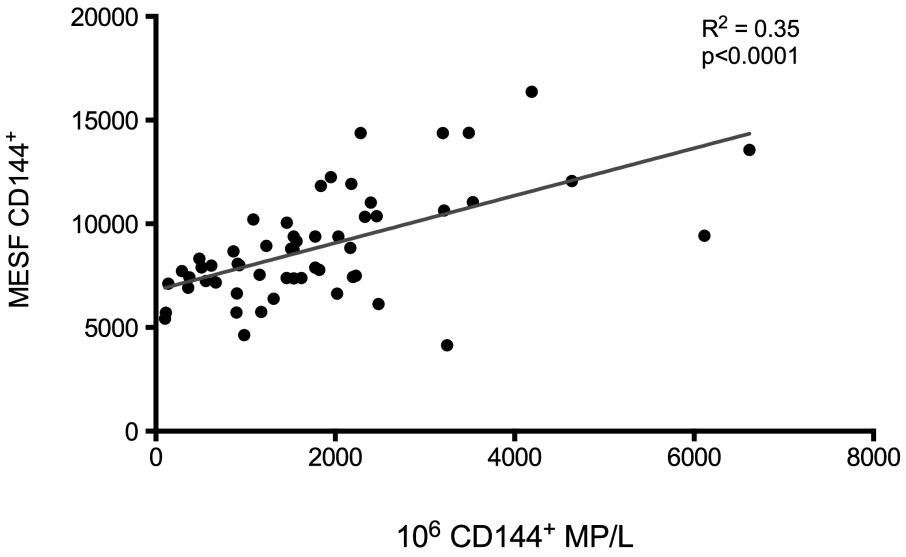

Figure 16. Endothelial microparticles during atorvastatin treatment measured as concentration or MESF values.

Discussion

In paper II we observed a *reduction* of CD61, CD62P and CD142 exposure on PMP in patients with PAOD during atorvastatin treatment. In contrast, in paper III we observed a significant *increase* in exposure of the endothelial-specific molecule CD144 (VE cadherin) on circulating EMP, as well as *increased* exposure of CD142 on $CD144^+$ EMP. Furthermore, the number of $CD144^+$ EMP and the number of $CD142^+$ $CD144^+$ EMP were significantly *increased*. Thus, during atorvastatin treatment, circulating EMP are *increased*, but the meaning of this finding is not clear. *In vitro* data do, however, support the idea that statins may increase the formation of EMP. In studies with human umbilical endothelial cells (HUVEC) incubated with simvastatin an increase in EMP has been observed [121]. Interestingly, it was reported that these EMP contained caspase-3, which is a pro-apoptotic enzyme [31]. The authors concluded that elevated release of caspase-3-containing EMP may prolong endothelial cell survival; in this way statin treatment may protect endothelial cells from apoptosis. Unfortunately, we did not measure any apoptotic markers in the present study, so we cannot study such mechanisms in more detail.

We compared two different methods of measuring MP; counting their numbers and measuring the amount of exposure of different antigens on MP (the MESF protocol). The data indicated that more exposure of a specific antigen (CD144) agrees with

higher numbers of EMP ($R^2 = 0.35$, Fig. 16), further supporting our (rather unexpected) finding of elevated numbers of EMP during statin treatment.

Taken together, our results demonstrate the complexity of statin treatment with respect to effects on MP. In Paper II, we demonstrated that treatment with 80 mg atorvastatin per day reduces some platelet markers and CD142 on PMP in patients with PAOD. In paper III we report that atorvastatin increases the exposure of CD144 and CD142 on EMP, as well as the number of circulating EMP. Interestingly, in Paper II we observed no changes in PS expression when MP (irrespective of cell origin) were labeled with annexin V or lactadherin. Further research is needed to gain full insight into statins and MP formation. Studies on MP should take into account the fact that circulating MP originate from different cell types which may respond differently to a treatment or an intervention, as shown in Papers II and III.

5.4 PAPER IV

The study presented in Paper IV was originally designed to investigate if inhaled nitric oxide together with glucocorticoids modulate the inflammatory response to LPS in normal human subjects. These data were negative, as reported previously [122].

However, LPS administration had expected effects, and around 60–90 minutes after LPS infusion the subjects felt varying degrees of flu-like symptoms (e.g. headache, fever and fatigue) and these symptoms lasted for about 3 to 4 hours. White blood cell counts and plasma concentrations of TNF-α, IL-6, 8 and 10 increased significantly over time, with normalization after 24 hours [122].

Microparticles

MP concentrations were determined by flow cytometry as in our previous studies. The percentage of particles in the MP gate positive for phalloidin was 10.6 ± 6.9 % (mean ± SD) which indicates acceptable sample handling. During LPS infusion the total number of MP (lactadherin$^+$ MP) and cell-specific MP (CD42a$^+$, CD62E$^+$ and CD14$^+$) increased significantly over time (Fig. 17, $p < 0.001$), but EMP defined by expression of CD144 (Ve-Cadherin) did not increase significantly (Fig. 17D). Further investigation of MP revealed HMGB1 exposed on CD14$^+$ MP, which also increased significantly over time (Fig. 18, $p < 0.001$).

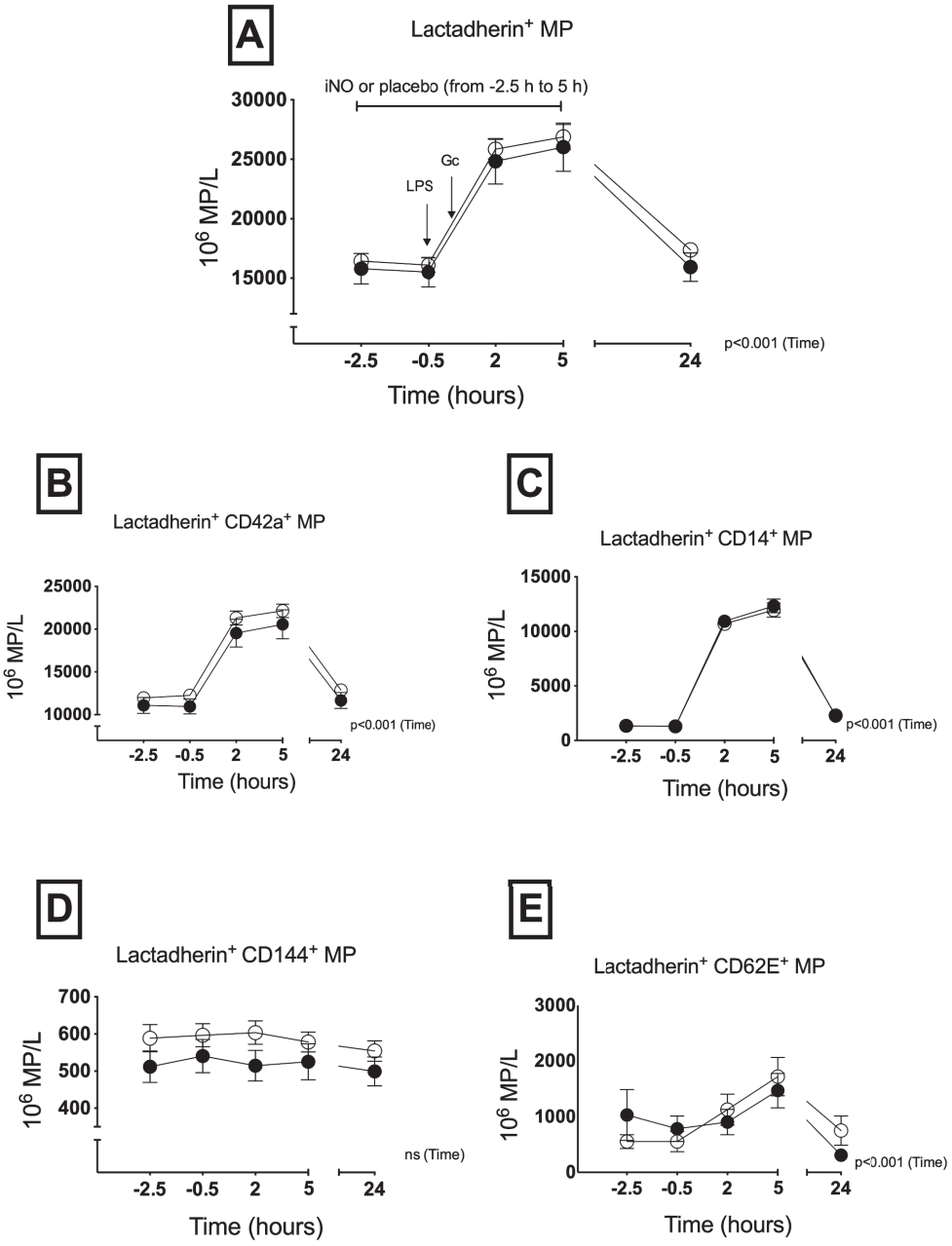

Figure 17. Number of circulating MP before and after LPS administration in healthy volunteers. LPS: lipopolysaccharide, Gc: glucocorticoids, iNO: inhaled nitric oxide.

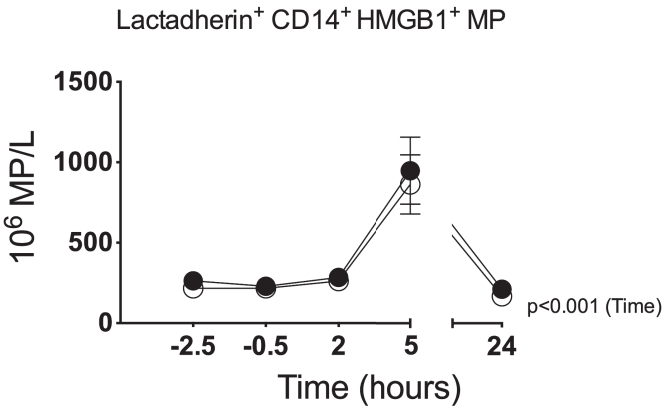

Figure 18. MMP exposing HMGB1 before and after LPS administration in healthy volunteers.

Nuclear content in microparticles

Labeling with SYTO 13 was used to measure the presence of DNA or RNA in MP. To further evaluate the origin of MP containing nuclear content, SYTO 13 was added together with labeled antibodies against CD41 or CD14. The results showed that the number of SYTO 13^+ MMP and PMP increased significantly over time (Figs. 19A, B and D, $p < 0.001$). Since HMGB1 is bound to DNA, we also investigated whether or not SYTO 13^+ MMP or PMP could expose HMGB1. Our findings indicated that both PMP and MMP expose HMGB1 following LPS infusion (Figs. 19C and E, $p < 0.001$).

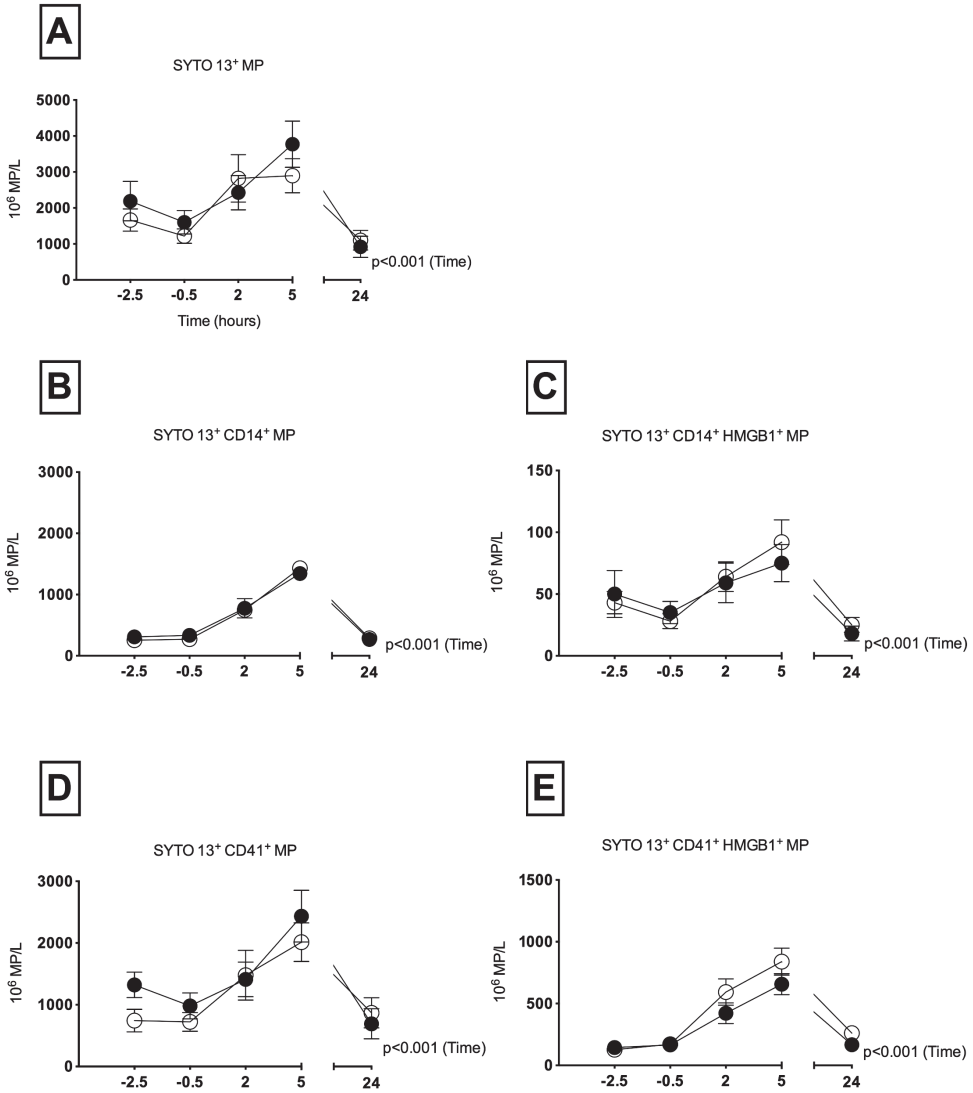

Figure 19. SYTO 13^+ MP, reflecting presence of nucleic acids of MP, before and after LPS administration in healthy volunteers.

Interestingly, we observed that the number of SYTO 13^+ PMP was higher than the number of SYTO 13^+ MMP. However, when SYTO 13 data were expressed as MFI, we found significantly higher levels of SYTO 13 binding in MMP than in PMP (Fig. 20A, $p < 0.001$), indicating a greater content of nucleic acids in MP derived from monocytes. This finding is not unexpected, since monocytes, in contrast to platelets, are nucleated cells and should contain more DNA and RNA. However, no significant differences were observed in SYTO 13^+ $HMGB1^+$ exposure (expressed as MFI) between MMP and PMP (Fig. 20B, $p = 0.9$).

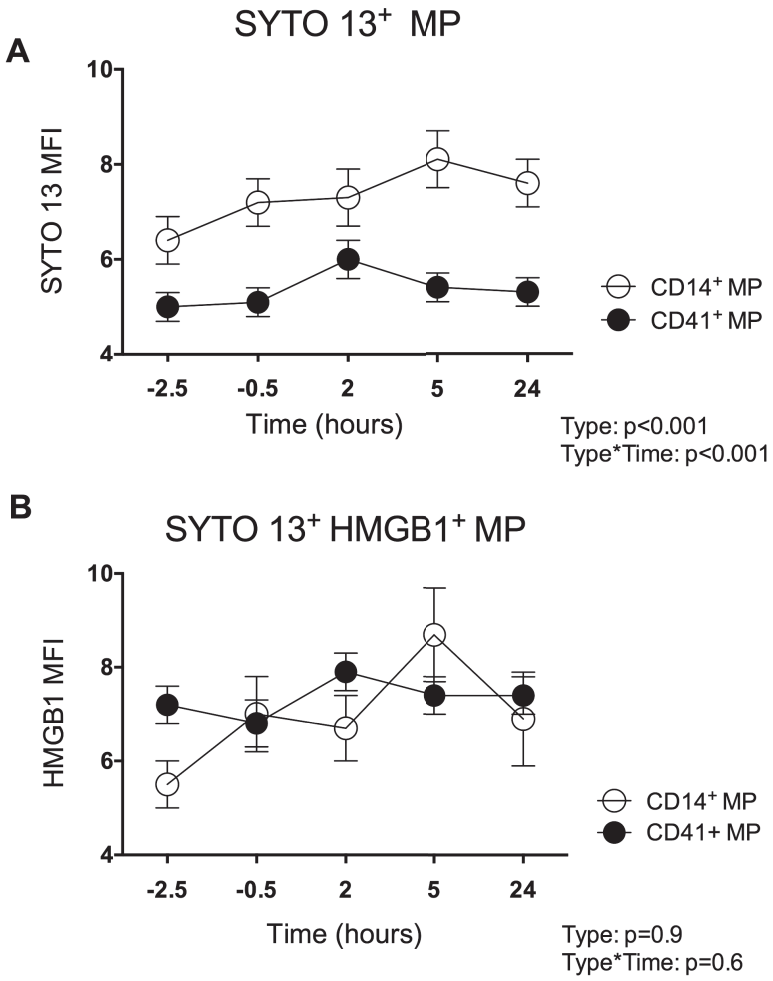

Figure 20. Mean fluorescence intensity (MFI) of SYTO 13 binding (A) and SYTO 13 binding and HMGB1 exposure (B) in platelet (CD41+) and monocyte (CD14+) microparticles.

Discussion

We demonstrated MP formation *in vivo* when healthy volunteers were exposed to LPS in the presence of intravenous hydrocortisone. We found significant increases in numbers of different MP (of platelet, monocyte and endothelial origin) and also increases in MP containing DNA and/or RNA. Furthermore, we demonstrated that MP released during LPS administration expose HMGB1 – an intranuclear protein which has been identified as a potent pro-inflammatory molecule when present extracellularly [75,77].

The increase in MP (irrespective of origin) is expected, since LPS stimulation has been shown to activate platelets, leukocytes and endothelial cells [123,124]. In this study we measured the number of circulating EMP by using two different monoclonal antibodies, which were directed against CD144 and CD62E. We did, however, observe a significant increase only in $CD62E^+$ MP. This is largely in accordance with the results of a previous study, which showed activation of the vascular endothelium following LPS administration, as reflected by elevated plasma levels of von Willebrand factor antigen [125]. Regarding CD144 it should be noted that this is a large molecule (VE-cadherin) considered to be an endothelial cell-specific marker but less sensitive in detecting activation of vascular endothelial cells [126].

We assessed SYTO 13 binding in MP, in order to provide additional information regarding their content of nuclear components. SYTO 13 is a cell-permeable dye which binds to both DNA and RNA with high fluorescent yield, although it has preference for double-stranded DNA. Previous studies have demonstrated SYTO13 binding in MP generated from both apoptotic and necrotic cells [25,127]. Significant increases in SYTO 13^+ and SYTO 13^+ $HMGB1^+$ PMP and MMP, were observed after LPS administration. We observed higher numbers of SYTO 13^+ and STYO 13^+ $HMGB1^+$ PMP compared with MMP (Fig. 18). When the SYTO 13 data were expressed as MFI (Fig. 19), we found significantly higher levels of SYTO 13 binding in MMP compared with PMP, which was expected, as MMP, being derived from nucleated cells, should contain more DNA.

No significant differences were observed with regard to MFI values between SYTO 13^+ $HMGB1^+$ MMP *vs.* SYTO 13^+ $HMGB1^+$ PMP (Fig 20B). Since HMGB1 could bind directly to PS exposed on cells or MP [76], we believe that the higher number of circulating SYTO 13^+ $HMGB1^+$ PMP could be due to higher concentrations of circulating PMP compared with MMP (i.e. more circulating PS^+ PMP compared with circulating PS^+ MMP, Fig. 17).

In summary, we found that LPS can increase circulating levels of MP and influence their phenotype and content of nuclear components.

6 GENERAL DISCUSSION

Numbers of circulating MP are elevated in a variety of diseases such as cardiovascular diseases [82,128], neuro-inflammatory and autoimmune disorders [96,98,129], infectious diseases [23,130], cancer [131], and perhaps also in psychiatric diseases [7]. Thus, MP are potential biomarkers in various clinical settings but studies on MP are associated with methodological issues.

6.1 FLOW CYTOMETRIC MEASUREMENT OF MICROPARTICLES

There are several methods described in the literature for detection of MP, and among these, flow cytometry is most commonly used [132]. Flow cytometry is a reliable method to characterize cells by size and antigen expression, but there are several limitations, which need to be discussed.

Instrument limitations

MP are generally described as particles of 0.1−1.0 μm, but as described in Paper I, MP measured by TEM were mainly between 0.1–0.4 μm. Particles of this size are too small to be detected by most flow cytometers, since the laser beam that hits by-passing vesicles in the flow chamber has a wavelength of 488 nm. This means that the analysis is performed below the resolution of the instrument, which is a limitation [133]. According to nanoparticle tracking analysis and AFM measurements, flow cytometry greatly underestimates the concentration of particles (roughly 1000-fold) [103]. However, the new generation of flow cytometers has increased FS sensitivity. This has been achieved by increasing the angle at which FS light is collected (1–19° in Beckman Coulter Gallios™, and 1–70° in Apogee A50 instrument). Another possibility to increase sensitivity, especially in older instruments, is to select the SS detector, which has an even larger collection angle (roughly between 30−150°) [29,101]. The impact of such refinements on sensitivity of flow cytometry remains, however, to be determined in more detail.

Another limitation is the flow chamber of the flow cytometer. Most instruments are manufactured for measurement of cells, for example leukocytes. The design of the flow chamber forces the cells to pass through the laser beam one by one, a procedure which improves “counting events”. However, if smaller particles are to be measured (e.g. MP), a phenomenon called “swarm detection” may occur. “Swarm detection” is when multiple smaller particles pass through the chamber and the laser beam, and are counted as a single event [101]. This phenomenon frequently leads to increased variability when the concentrations of MP are to be assessed. To avoid this we described a flow cytometric approach where the MFI of a marker bound to MP that

pass the laser beam is assessed (Paper I). Notably, the flow cytometer used in Paper I and partly in Paper II, a BD FACScan instrument, lacked the sensitivity for measuring MP based on FS threshold, as indicated by relatively high intra- and interassay CVs. By choosing fluorescence as threshold, we managed to decrease the variability. The MFI results were then presented as MESF values using fluorescent beads to create a "calibration plot" (see Fig. 7, page 21). Data expressed as MFI only are dependent on the instrument and not comparable between platforms, whereas MFI results presented as MESF values enable data comparison between different instruments and laboratories [134-136]. As described earlier, newer flow cytometers such as the Beckman Coulter Gallios™ (used in Papers II–IV) do have improved sensitivity, but could still suffer from "swarm detection".

Labeling limitations

The "total" MP population, i.e. all MP irrespective of cellular origin, is commonly labeled with annexin V, a protein that binds to PS. However, as described previously, the annexin V labeling technique may have inherent problems and may be less sensitive to partial or low amounts of PS exposure[29]. Also, as described above, it seems that not all MP expose PS [22]. We abandoned annexin V in the experimental work in Paper I and instead we measured PMP according to their ability to express the platelet-specific receptor CD42a. Later on in the project we noted publications by Dasgupta on lactadherin (Dasgupta 2006). This protein is an opsonin released from activated macrophages which binds to cells undergoing apoptosis and promotes their engulfment [38,137]. The binding of lactadherin to PS is not calcium-dependent and a more sensitive marker of PS [38]. We therefore began to use lactadherin to determine PS exposure in the work that followed (Papers II–IV). Annexin V, however, was reintroduced in Paper II, in order to rule out the possibility of missing any treatment effect by using only lactadherin. Additionally, we wanted to convince readers, as lactadherin binding was not a common technique used to determine PS exposure.

As of today, there is still no agreed general and standardized protocol for the assessment of MP. Depending on the MP antigens investigated, results and interpretations could differ. As observed in Paper IV, EMP measured with CD144 or CD62E displayed conflicting results. During LPS administration no changes could be seen in CD144-positive EMP ("LPS infusion does not influence the vascular endothelium"), but a significant increase was seen in CD62E-positive EMP ("LPS infusion activates the vascular endothelium"). Furthermore, as shown in Papers II and III, the same pharmacological treatment (statin therapy) could lead to different responses in MP patterns from different cells; PMP showed decreased exposure of CD142 whereas EMP increased their exposure of CD142 and there was an increase in $CD142^+$ EMP concentration. Thus, interpretation of data should always be carried out with consideration of the antibodies used and the MP phenotypes that are measured. Circulating microparticles are heterogenic entities, and data obtained with one protocol does certainly not apply to "all microparticles".

6.2 PRE-ANALYTICAL VARIABLES

Pre-analytical handling of plasma samples, such as blood collection, centrifugation and freezing, is important for MP analysis and truly remains a challenge, especially in clinical research.

Blood sampling

All sampling performed in Papers I–IV was performed according to standardized hemostasis laboratory protocols. Blood was always collected in vacutainer tubes containing 0.129 M sodium citrate, using 21-gauge needles, with no or minimal use of a tourniquet. Although the effect of needle diameter on MP formation is not known, smaller needles could in theory lead to platelet activation and destruction of red blood cells due to shear stress, all of which could affect MP analysis and the data obtained.

Sodium citrate is the most commonly used anticoagulant, but there are others who use ethylenediaminetetraacetic acid (EDTA) or citrate-theophylline-adenosine-dipyridamole (CTAD) [138]. Platelet activation during blood collection is an important pre-analytical factor, since it will lead to PMP formation. Comparison of anticoagulants with respect to MP concentrations has demonstrated a false-positive increase in PMP counts in EDTA tubes [139]. Interestingly, platelets collected in EDTA tubes showed increased CD62P expression after 30 minutes compared with citrate or CTAD, indicating some kind of "EDTA-induced platelet activation" [140]. Moreover, platelets in EDTA tubes undergo shape change and there is an irreversible separation of the dimeric GPIIb/IIIa receptor in this medium [141]. Samples collected in CTAD or sodium citrate tubes showed similar MP concentrations, supporting the use of both these anticoagulants when it comes to blood sampling for MP measurements [139].

To minimize platelet activation during blood sampling, all samples in Papers I–IV were immediately centrifuged. When samples were stored for 24 hours at room temperature we observed an increase in $CD42a^+$ $phalloidin^+$ events (Paper I, Table 7). Thus, in samples that are not centrifuged immediately after blood collection platelet fragments may be generated. In accordance with our results, Connor et al. reported that the concentration of PS^+ MP increased in sodium citrate tubes left at room temperature for 30 minutes [142]. It may very well be that the increase in PS^+ MP observed by Connor et al. could represent generation of platelet fragments falsely detected as MP, as observed in Paper I (Paper I, Table 7).

Centrifugation and freezing

In Paper I we investigated the possible effects of different centrifugation speeds prior to sample storage. We showed that removal of platelets from plasma by centrifugation is important to reduce false-positive results. In order to quantify "false positive" PMP we proposed to label samples with phalloidin. This is a cyclic peptide

which binds to f-actin and stains platelet fragments, discriminating these from intact PMP. An acceptable level of "platelet fragment contamination" was set to be roughly 10% phalloidin exposure. Results showed that samples frozen as PFP yielded the lowest phalloidin-positive events upon thawing. This centrifugation protocol has also been recommended by several other groups [138,143,144].

In Papers I–III blood samples were centrifuged at 15 °C and in Paper IV at 4 °C to separate blood cells from plasma. The effect of cold centrifugation on MP is not known and this could therefore be a limitation in Paper IV. Sample handling was, however, standardized and all samples were handled in an identical manner throughout the study.

Freezing and thawing a sample containing MP could lead not only to morphological changes but also to a (false) increase in numbers of MP. When fresh PPP samples were compared with paired frozen/thawed PPP samples we found significantly higher concentrations of PS^+ MP as well as exposure of PS, with respect to lactadherin and annexin V binding (Table 11). In the same experiment we also measured phalloidin levels and, as seen in Table 11, there were significantly higher phalloidin levels in frozen/thawed PPP samples. Samples centrifuged as PPP most likely contain some platelets. Upon freezing/thawing, these platelets will become platelet fragments, thus causing a false-positive increase and exposure of PS^+ MP. Our interpretation is in line with that proposed by Ayers et al., who found a significant increase in annexin V^+ MP and PMP in frozen/thawed samples [145]. It is also possible that more PS is exposed on MP during the freezing/thawing procedure. Thus, frozen and fresh samples should not be directly compared.

Table 11. Phosphatidylserine (PS) exposure on MP labeled with lactadherin or annexin V in fresh *vs.* frozen/thawed PPP. Data are shown both as MFI and as numbers of MP (± SD). Measurements were performed in fresh PPP and paired frozen/thawed samples from 10 healthy individuals. Frozen samples were centrifuged and frozen at -80 °C for 2 days, then thawed and analyzed. Phalloidin levels were also assessed. *p*-values obtained with paired t-test.

	Fresh PPP	**Frozen/thawed PPP**	***p***
Lactadherin (10^6MP/L)	6289 ± 285	8010 ± 414	< 0.0001
Lactadherin MFI	19.4 ± 1.2	21.4 ± 2.0	< 0.05
Annexin V (10^6MP/L)	3631 ± 843	5757 ± 1228	< 0.001
Annexin V MFI	6.2 ± 0.5	11.2 ± 0.9	< 0.0001
Phalloidin (%)	3.2%	5.9%	< 0.0001

In Paper I and II, we studied MP based on antigen exposure. To do so, our flow cytometric protocol was based on “fluorescence” and not on “size”. To ensure that we were only measuring PMP and not platelets (since both expose the same antigens), we needed to prepare our samples as MP-enriched pellets. To obtain MP-enriched

pellets we chose a centrifugation protocol originally presented by Pereira et al., where they achieved a recovery rate of over 95% of MP originally presented in PFP [92]. Our own pre-analytical validation by TEM displayed homogeneous sample preparations which included vesicles in the size range of 0.1−0.3 μm. No platelets could be seen, indicating acceptable centrifugation procedures. Also, as indicated by phalloidin data, the pre-analytical procedures presented in Papers I and II were adequate (around 10% phalloidin$^+$).

In Paper III and IV we modified our pre-analytical sample preparation to better suit the new flow cytometry protocol, which was based on FS *vs.* SS (i.e. size and complexity). Since we were able to distinguish cells and vesicles down to 0.2−0.3 μm with acceptable resolution, there was no need separate MP from other cells by high-speed centrifugation. Instead, measurements could be performed directly in the supernatant (after two centrifugations steps). This new centrifugation protocol also showed low phalloidin levels (again around 10% phalloidin$^+$ particles), indicating acceptable sample preparation.

In Paper I we describe how samples stored as PFP were associated with the lowest phalloidin$^+$ events. However, all samples analyzed in Paper I–IV had been stored as PPP. Therefore, it cannot be ruled out that if these samples had been centrifuged and stored as PFP, we might have obtained even lower phalloidin levels. Nevertheless, our results indicate that despite this possible limitation, we could still show differences in MP patterns between patients (Paper I), effects of pharmacological treatment (Papers II & III) and significant *in vivo* formation and changes in phenotype and content of nuclear molecules on circulating MP in experimental inflammation (Paper IV).

Centrifugation is a common preparation procedure to obtain samples for MP analysis, but there are obvious limitations with this procedure, i.e. loss of MP due to centrifugation force [145,146]. According to Chandler et al., PRP centrifuged at 1500 × *g* for 20 minutes resulted in 95% removal of platelets, but also a loss of 79% of PMP (Annexin$^+$ CD41$^+$). A second centrifugation led to 99% removal of platelets and a further 58% reduction of PMP. Alternative methods have been described where MP isolation is performed by means of a combination of centrifugation and filtration [147,148]. Briefly, PFP is obtained by double centrifugation and loaded into a 96-well 0.2 μm filtration plate to remove MP. Another technique involves the use of larger filters (0.4 μm or 0.08−0.4 μm) to remove platelets and other cells and thus let MP pass through the filters [149]. Unfortunately no data are available on recovery using these techniques, but since centrifugation is used initially there might still be loss of MP. A new and interesting approach is to label MP with target-specific magnetic nanoparticles and detect them in a miniaturized nuclear magnetic resonance system [150]. Isolated MP from glioblastoma cells have been labeled with magnetic nanoparticles via CD63. During detection, only microparticles bound to magnetic nanoparticles were measured. This methodology is not widely available, but it places

an interesting twist on MP isolation for future research.

6.3 MICROPARTICLES AND THROMBIN FORMATION

Procoagulant MP are present in several diseases associated with a thromboembolic tendency, such as acute coronary syndrome [151], systemic lupus erythematosus [92] and traumatic brain injury [152]. This procoagulant effect is thought to be related to MP exposure of PS alone or in combination with TF [11,28,153].

Phosphatidylserine

In vitro experiments (Paper II) revealed that by blocking PS on MP with annexin V or lactadherin we observed strong and concentration-dependent inhibition of thrombin formation. Thus, we concluded that the effect of MP on thrombin formation is mainly through PS exposure. Interestingly, flow cytometric data on PS exposure measured as lactadherin- or annexin V^+ MP showed no changes in PS exposure (i.e. "all PS^+MP") during atorvastatin treatment. In Paper III we demonstrated that atorvastatin (80 mg daily) *increased* EMP, measured as lactadherin$^+$ CD144$^+$ or lactadherin$^+$ CD144$^+$ CD142$^+$, thus demonstrating both a decrease and an increase of PS^+ MP by atorvastatin., This may explain why no changes in "total PS^+MP" could be seen in PS exposure between atorvastatin and placebo treatment in Paper II.

Tissue factor

Using flow cytometry we could detect TF exposure on PMP (Papers I & II). The main source of intravascular TF is considered to be monocytes [48], but TF could also be transferred to platelets and to PMP from monocytes [154]. In Paper II we observed that TF present on MP had a small but significant effect on thrombin generation in vitro, as presented in the table below.

Table 12. The effect of MP on thrombin generation. Results are shown as mean ± SD (n = 5). Values of p were obtained by paired t-tests. **$p < 0.001$ *vs.* without MP, †$p < 0.01$ *vs.* with MP, ∞∞$p < 0.001$ *vs.* with MP.

	Without MP	With MP	With TF antibody	With Annexin V
ETP (nM*min)	929 ± 72	1438 ± 24 **	1219 ± 20.1 †	640 ± 92 ∞∞
Lagtime (min)	24.7 ± 3.2	8.4 ± 1.2 **	10.9 ± 0.7 †	21.9 ± 0.9 ∞∞
Peak (nM)	59.4 ± 11.8	135.2 ± 5.4 **	116.2 ± 0.9 †	55.42 ± 0.5 ∞∞
ttPeak (min)	36.7 ± 2.0	15.3 ± 2.0 **	16.3 ± 0.8 †	30.3 ± 1.3 ∞∞

By blocking TF on MP by the addition of antibodies, the lag time and time to peak were significantly delayed. Peak thrombin was also significantly decreased. To be confident that we achieved proper blocking of TF we tested both monoclonal and

polyclonal antibodies against TF. Both antibodies showed a concentration-dependent increase in lag time when measured in the CAT assay. TF exposure on MP is still a debated issue, especially as regards whether or not it is "de-encrypted" (active) or "encrypted" (inactive) [155-159]. Activated monocytes express mostly encrypted TF; only 10–15% of the TF is de-encrypted [154]. One reason may be that de-encrypted TF needs PS to become more active [160], while others have discussed the role of protein disulfide isomerase (PDI) [161]. PDI disables the bond between Cys^{186} and Cys^{209} located within TF, thus causing TF to become encrypted (i.e. inactive). On the other hand, oxidation of these two cysteins increases TF activity [162]. Our results indicate a slight but significant TF activity on MP from patients with PAOD, in agreement with other findings where TF presented on MP can initiate formation of small amounts of thrombin [63,158].

Taken together, the reduction of thrombin formation *in vivo* (F1+2 data) and *in vitro* (CAT data on plasma samples from patients) during atorvastatin treatment could only to a small extent be due to MP-mediated effects. In our experimental set-up we showed that PS on MP – much more than TF on MP – were mechanisms that enhanced thrombin generation afforded by MP (Table 12). Notably, "all PS^+ MP" were not reduced by atorvastatin compared to placebo. One mechanism more likely to be the important one is the effects of atorvastatin on plasma lipoproteins. Indeed, lipoproteins such as VLDL and oxidized LDL, offer the same negatively charged surface as MP and therefore could enhance thrombin formation [118-120]. A significant reduction in lipoprotein plasma levels, which occurs during high-dose statin therapy, could thus be the (most important) mechanism behind decreased thrombin generation during atorvastatin treatment in our study.

6.4 STATIN TREATMENT AND MICROPARTICLES

Statins are hypolipidemic drugs which decrease plasma cholesterol concentrations by inhibiting hydroxy-methylglutaryl coenzyme A (HMG-CoA). By inhibiting HMG-CoA, cholesterol synthesis is blocked and a reduction of LDL-cholesterol levels is achieved. Statins were first developed in order to improve the lipid profile but have subsequently been shown to exert a variety of beneficial "pleiotropic" effects, particularly relevant to cardiovascular disease [163]. Beneficial effects of statin treatment in a large clinical trial were first described in the Scandinavian Simvastatin Survival study, which showed a clear-cut reduction in mortality in patients with coronary heart disease treated with simvastatin [164]. Since then, numerous studies have shown that statins have beneficial effects and protect against atherothrombotic complications [163,165], but the exact mechanisms through which these pharmacological agents reduce cardiovascular complications are still controversial.

The effects of statin treatment beyond their lipid-lowering effects have thus been discussed [166], and in Papers II and III we investigated the effects of atorvastatin on thrombin generation, PMP (Paper II) and later EMP (Paper III). Patients with an established atherothrombotic disease, i.e. peripheral arterial occlusive disease (PAOD), were investigated.

Statins and platelet derived microparticles

Our finding that atorvastatin reduced CD61, CD62P and CD142 on PMP is in line with the results of a previous study presented by our group, where patients with type 1 diabetes received 80 mg atorvastatin per day. This study was also a double-blind placebo-controlled study and, as in Paper II, we saw a significant reduction of CD61, CD62P and CD142 exposure during atorvastatin treatment [113]. The results of these two studies validate our method (MESF protocol) and extend information on the effect of atorvastatin on PMP in two different diseases. To understand the effect of statin treatment on PMP, the effects on platelets have to be considered. The reduction of PMP release during statin treatment could be due, for example, to reduced cholesterol content in the platelet cell membrane, altering signaling capabilities of the platelets [167]. More recently, it was shown that patients with metabolic syndrome receiving 1 of the 6 statins (atorvastatin, fluvastatin, lovastatin, pravastatin, rosuvastatin, or simvastatin, 10 patients per treatment) showed a significant reduction of PAR-1 receptors on platelet surfaces [168]. Lower numbers of PAR-1 receptors on platelets may result in less reactivity to thrombin, and reduced formation of PMP. Another possible mechanism is reduced formation of thromboxane A_2. This compound is produced by activated platelets and amplifies activation and recruitment of additional platelets [169]. However, in Paper II all patients were on aspirin, so it is unlikely that statin-induced inhibition of thromboxane formation was mechanistically involved.

It should be pointed out that no statistically significant correlations were seen between reductions in lipid levels and reductions in expression of CD61, CD62P or CD142 on PMP following atorvastatin treatment, in line with a mechanism of action independent of the lipid-lowering effects of statins.

Despite the fact that the mechanisms are unclear, we draw the conclusion that atorvastatin treatment inhibits platelet activation and thereby reduces PMP formation.

Statins and endothelial derived microparticles

The increase in circulating EMP presented in Paper III was somewhat surprising to us considering the clear-cut PMP-inhibiting effects of atorvastatin observed in Paper II. Few studies have concerned investigation of the effect of statin treatment on EMP [170]. Tramontano et al. reported inhibition of MP release when human coronary artery endothelial cells (HCAEC) were activated with TNFα in the presence of fluvastatin [171]. No data on the effect of fluvastatin only were reported, and the reduction in MP

release during TNFα stimulation was only seen in $CD105^+$ (endoglin) and $CD51^+$ (vitronectin) MP, whereas no effect was seen on $CD62E^+$ (E-selectin) or $CD31^+$ (PECAM-1) MP [171]. Furthermore, no data on $CD144^+$ MP were reported. Diamant et al. incubated HUVEC with simvastatin and observed an increased release of annexin V^+ MP [121]. Further experiments showed that MP contain caspase-3, and the authors suggested that MP containing caspase-3 were released to protect endothelial cells from apoptosis. On the other hand, caspase-3 activation leads to apoptosis [31], and thus increased EMP release during simvastatin treatment may be claimed to be due to apoptosis. In addition, it has been shown that HUVEC are more prone to apoptosis compared with HCAEC [172], which would fit with the idea that the effect of simvastatin on HUVEC reflects increased apoptosis. There are, to our knowledge, no *in vivo* data in humans on the effects of statin treatment on circulating EMP [170] (except for our Paper III), and the data from the above- mentioned *in vitro* studies are of little or no value as regards interpretation of data from our *in vivo* study. Nevertheless, our data as such are strong, as they were obtained in a randomized double-blind placebo-controlled study and with two different flow cytometric approaches which yielded virtually identical results. Subpopulations of MP (PMP *vs.* EMP) seem to behave differently during atorvastatin treatment in patients with PAOD, and further research is needed to understand these mechanisms better.

6.5 FORMATION OF MICROPARTICLES IN VIVO

Bacterial endotoxin (LPS) is a pro-inflammatory compound which leads to strong activation of immune competent cells by forming complexes with CD14, MD2 and TLR4, which in turn downstream leads to activation and release of various cytokines [173]. Platelets, which lack CD14, can still be activated by LPS, since plasma contains CD14 (either soluble or bound to MP) in sufficient concentrations to present LPS to platelet TLR4 [174]. In Paper IV, experimental inflammation induced by administration of LPS led to a clear-cut increase in the formation of MMP and PMP, reflecting activation of their parental cells, monocytes and platelets. EMP also increased, but to a lesser extent, and an elevation was only seen for $CD62^+$ EMP (i.e. those exposing E-selectin), and not for EMP defined by CD144 exposure. Perhaps this is due to the different functions of these two molecules, CD144 being involved in the junction of endothelial cells, whereas CD62E is an adhesion molecule responsive to inflammatory stimuli and primarily with a luminal location on the endothelium capturing rolling leukocytes. We propose that $CD62E^+$ EMP should be further tested as biomarker of inflammatory activity of the vascular endothelium.

Beside causing "cell activation" [123,124] LPS could also lead to apoptosis [175], which is a process involving DNA fragmentation and blebbing (MP formation). We labeled MP with SYTO 13 in order to see if they contained DNA/RNA and we found a significant increase in circulating $SYTO^+$ MP originating from monocytes following LPS

infusion. We expanded our assay to additionally include HMGB1 on MMP and found the same pattern, i.e. an increase in circulating $SYTO13^+$ $HMGB1^+$ MMP. As HMGB1 is a DNA-binding protein, and thought to be released into the extracellular space during apoptosis [75], one interpretation is that these circulating MMP are markers of an apoptotic response to inflammation. Interestingly, platelets lack a nucleus but still contain mitochondrial DNA and RNA, as well as HMGB1, and this latter molecule is exposed on the platelet surface upon platelet activation [76]. We observed increased circulating $SYTO^+$ $HMGB1^+$ PMP in response to LPS. Thus, the platelets seem to respond in a similar manner as monocytes when challenged with LPS, i.e. with formation of blebs exposing HMGB1.

Of note, once HMGB1 exits the cell it can function as a potent cytokine affecting various cells such as macrophages, dendritic cells and neutrophils, thus further activating the immune system [176] and perpetuating inflammation. HMGB1 can probably bind to most MP exposing PS, as shown in experiments with activated platelets as described earlier [76], and since MP regardless of origin could expose PS, they could offer an additional binding site for extracellular HMGB1 and act as vectors of inflammation. It should be remembered, however, that HMGB1 can have different redox states, and this will determine its biological activity. There are, to our knowledge, no available methods to distinguish between these two redox states. We cannot say to what degree HMGB1 bound to MP and detected by our assay, has any biological activity.

7 CONCLUSIONS

Conclusions from this thesis are that:

• Quantification of platelet-derived microparticles expressed as MESF values (Molecules of Equivalent Soluble Fluorochrome) gives greater reproducibility than quantifying the number and concentration of platelet microparticles. Results expressed as MESF values give comparable results between two different flow cytometers at two different laboratories. Using this MESF protocol we found twice as much CD62P and CD142 exposure on platelet derived microparticles in patients with type-1 diabetes compared with healthy controls.

• Pre-analytical sample handling is important. Centrifugation is a critical step as it removes cells, platelets and cell debris prior to freezing. Adequate centrifugation rate and time have to be employed, and the sample intended for analysis of microparticles should be frozen as platelet-/cell-free plasma.

• Quality control of frozen/thawed plasma samples can be performed through the use of labeled phalloidin. This fungal peptide detects and binds to f-actin attached to cell membrane fragments which may disturb microparticle analysis. We thus propose the use of phalloidin labeling for quality control when measuring microparticles in studies on frozen/thawed plasma samples.

• Pharmacological treatment with the statin atorvastatin reduces exposure of CD61, CD62P and CD142 on platelet derived microparticles in patients with established atherothrombotic disease. Unexpectedly, the same treatment increases the number of circulating $CD144^+$ and $CD144^+$ $CD142^+$ endothelial derived microparticles, as well as the exposure of these molecules on the microparticles. The reason behind this discrepancy, i.e. reduced platelet derived but increased endothelial derived microparticles, is unknown. Considering the complexity of microparticle formation, *in vivo* studies should include careful assessment of the source and phenotype of the microparticles investigated.

• Atorvastatin treatment reduces thrombin generation in plasma *in vivo* and in plasma samples following the addition of tissue factor (TF). The addition of microparticles from patients with atherothrombotic disease to plasma increases thrombin generation. This is largely due to the presence of negatively charged surfaces (mainly phosphatidylserine; PS) provided by microparticles, but TF exposed on the microparticles may also contribute. Circulating microparticles exposing PS, irrespective of cellular origin, are not reduced during atorvastatin therapy. Thus it is unlikely that the “antithrombin”-like effect of atorvastatin is mediated by changes in circulating microparticles.

• Inflammation elicited by administration of lipopolysaccharide significantly increases the number of circulating platelet, monocyte and endothelial derived microparticles in healthy volunteers. Furthermore, the numbers of platelet and monocyte derived microparticles exposing the alarmin HMGB1 are significantly increased. These microparticles also contain nuclear matter, as measured by SYTO 13, a cell-permeable dye which binds to DNA and RNA. Together, these results demonstrate effects of lipopolysaccharide on number and phenotype of microparticles in the blood and suggest that HMGB1 exposure on microparticles represents a useful biomarker for translocation of nuclear molecules during inflammation.

8 FUTURE PERSPECTIVE

Microparticles, initially described as “cell-dust”, clearly deserve to be tested more thoroughly as biomarkers; they may be used to assess cell activity, disease development and progression, and treatment effects. However, there is a need for standardization regarding both pre-analytical and analytical variables. In many settings we still have limited information on the functions and morphological characteristics of microparticles. If microparticles are *markers* or *makers* is likely dependent on the clinical setting, the type of microparticle investigated, the disease, and many other factors that need to be defined.

Future research should thus emphasize on better characterization of microparticles from other types of vesicles, by focus on further refinement and validation of microparticle detection. Recently developed methods such as atomic force microscopy and nanoparticle tracking analysis are likely to provide more accurate measurement of microparticles compared with conventional flow cytometry. However, these new methods are not yet as user-friendly nor available as flow cytometry. It is to be hoped that in the near future these methodological investigations will result in standardized protocols for measurement and phenotyping of microparticles. With proper and well validated *in vitro* and *in vivo* studies, microparticles may prove to be useful biomarkers of great value in medicine.

9 SVENSK SAMMANFATTNING

Mikropartiklar är små blåsor som avknoppas från celler som t ex vita blodkroppar eller blodplättar (trombocyter), vid aktivering eller celldöd. De förekommer fritt cirkulerande i blodet men finns även i ryggmärgsvätskan. Dessa mikropartiklar kan innehålla DNA eller RNA samt bära äggviteämnen (proteiner) och utöva effekter och/eller samtala med andra celler eller biologiska system. De kan ha viktiga funktioner vid t ex. blodlevring och inflammation, och kan ha betydelse för olika sjukdomars utveckling. Att studera funktionen hos mikropartiklar kan dock vara svårt och metoder för detta behöver utvecklas och utvärderas.

I denna avhandling har vi därför utvecklat och utvärderat metoder som kan användas för att öka vår förståelse kring mikropartiklar och dessa betydelse, samt hur de påverkas av blodfettsänkande läkemedel (statiner) och experimentellt utlöst inflammation.

I **studie I** presenterar vi en s.k. flödescytometrisk analys som baseras på att vi mäter proteiner som finns på ytan av varje mikropartikel. Vår metod visade sig vara mer pålitlig jämfört med mer traditionell metodik som baseras på antal mikropartiklar per volymsenhet. Detta beror på att en flödescytometer egentligen är byggd för att mäta större celler, t.ex. röda eller vita blodceller. Ett problem som identifierades tidigt var att metoden analyserade trasiga celler i provet som ”riktiga” mikropartiklar. Detta introducerade en osäkerhet i metoden (cell-fragment och mikropartiklar förväxlades). För att eliminera detta problem använde vi ett svamp-protein, falloidin. Detta är ett litet protein från lömsk flugsvamp som binder till proteiner som normalt inte är exponerade på en cellyta. Därigenom kunde vi skilja mellan fragment av celler och mikropartiklar i vår metod. Med vår metod kunde vi visa att patienter med diabetes har dubbelt så mycket av en särskild typ av mikropartiklar som friska individer. Dessa partiklar kan ha betydelse för blodproppsbildning och inflammation.

I **studie II** och **III** undersökte vi hur mikropartiklar påverkas av behandling med ett blodfettsänkande läkmedel, atorvastatin. Nitton patienter med fönstertittarsjuka (Claudicatio intermittens) undersöktes i en studie där de blev behandlade med atorvastatin eller sockerpiller (placebo). Vi fann i **studie II** att mikropartiklar från blodplättar sjönk under statinbehandling. Förvånande kunde vi också se att mikropartiklar från de celler som täcker blodkärlens insida (endotelet) ökade under statinbehandling (**studie III**). En minskning av antalet mikropartiklar från blodplättar vid atorvastatin behandling kan tänkas vara en orsak till varför detta läkemedel minskar insjuknandet i hjärtinfarkt. Hur vi skall tolka ökningen av mikropartiklar från kärlväggen under samma behandling är f.n. oklart.

Vi undersökte vidare hur en experimentellt utlöst inflammation påverkar mikropartikelbildningen i blodet (**studie IV**). Detta gick till så att friska försökspersoner fick endotoxin (ämne som finns på ytan av bakterier) injicerat i blodet. Endotoxin utlöser kortvariga ”influensaliknande” symtom hos försökspersonen. Under denna intervention fann vi kraftigt förhöjda mikropartiklar från både kärlvägg, vita blodkroppar och blodplättar. Vi fann också att vissa av dessa mikropartiklar uttryckte särskilda molekyler av betydelse för inflammation.

Sammanfattningsvis, har vi beskrivit en metod för att mäta mikropartiklar i blod. Vi finner att förekomsten av mikropartiklar i blodet kan påverkas med blodfettsänkande läkemedel. Kraftfull inflammation påverkar också mikropartiklar i blodet, och dessa kan bära för inflammationen viktiga molekyler. För att vidare förstå betydelsen av mikropartiklar och de molekyler som partiklarna bär behövs mer forskning. Vår idé är att mikropartiklar i framtiden ska kunna fungera som s.k. biomarkörer, och kunna användas för att mäta sjukdomsaktivitet, för prognostik samt för att följa effekten av olika typer av behandling. Detta kan på sikt komma många patienter till nytta och positivt påverka framtida hälso- och sjukvård.

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