Microparticles Derived from Stimulation of Human Umbilical Endothelium

Dissertation submitted by Elzette le Roux in accordance with the requirements for the degree MMedSc.

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DECLARATION

This dissertation is submitted for obtaining an MMedSc degree in Molecular Biology. I declare that this dissertation and study was submitted and performed independently by myself and has not been previously submitted or published for any other degree at the University of the Free State. I also declare that there were no conflicts of interests throughout the performance of the study. The copyright of this dissertation belongs to the University of the Free State.

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Elzette le Roux

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"The most inconsequential to man can have the most momentous impact. It is the small things in life that are often underestimated, overlooked or disregarded that make the biggest differences."

"Humans habitually envisage themselves as part of the bigger things in life. It is the bigger things in life that are sometimes very small..."

"Indeed, You have made my days as handbreadths,
And my age is as nothing before You;
Certainly every man at his best state is but vapor. Selah"

Psalm 39:5 (King James Bible)

CONTENTS:

ABBREVIATION LIST

ADAMTS-13	A Disintegrin and Metalloprotease with Thrombospondin Type 1 Motif Number 13
ADAMTS-13:Ag	ADAMTS-13 Antigen
APC	Activated Protein C
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
DAF	Decay-Accelerating factor
DNA	Deoxyribonucleic Acid
eNOS	Endothelial Nitric Oxide Synthase
EPC	Endothelial Protein C
EPCR	Endothelial Protein C Receptor
ER	Endoplasmic Reticulum
ERG	E-Twenty Six (ETS) Related Gene
FIX	Factor IX
FVa	Activated Factor V
FVII	Factor VII
FVIIa	Activated Factor VII
FVIII	Factor VIII
FVIIIa	Activated Factor VIII
FX	Factor X
FXa	Activated Factor X
FBS	Foetal Bovine Serum
HRP	Horseradish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells

ICAM-1	Intercellular Cell Adhesion Molecule-1
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible Nitric Oxide Synthase
JNK	C-Jun NH2 Terminal Kinase
LSGS	Low Serum Growth Supplement
MAPK	Mitogen-Activated Protein Kinase
mRNA	Messenger RNA
miRNA	Micro RNA
MMP	Matrix Metalloproteinases
NF-κβ	Nuclear Factor κβ
NO	Nitric Oxide
OPD	Ortho-phynylenediamine
P13K/AKT	Phosphatidylinositol-3-Kinase and Protein Kinase B
PAI	Plasminogen Activator Inhibitor
PAR	Protease-Activated Receptor
PBS	Phosphate Buffered Saline
PC	Phosphtidylcholine
PE	Phosphatidylethanolamine
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PMT	Photomultiplier Tube
PS	Phosphatidylserine
RIP-1	Receptor-Interacting Protein
rRNA	Ribosomal RNA
RNA	Ribonucleic Acid

ROCK	Rho-Associated Coiled-Coil Forming Kinase
ROS	Reactive Oxygen Species
S	Sphingomyelin
STAT	Signal Transducers and Activators of Transcription
TAFI	Thrombin-Activatable Fibrinolysis Inhibitor
TGA	Thrombin Generation Assay
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TM	Thrombomodulin
TNF-α	Tumour Necrosis Factor Alpha
TRADD	Tumour Necrosis Factor Receptor-1 Associated Death Domain Protein
TRAF	Tumour Necrosis Factor Receptor-Associated Factor
TRAIL	Tumour Necrosis Factor-α-Related Apoptosis-Inducing Ligand
TRAIL-R	TRAIL Receptor
TTP	Thrombotic Thrombocytopenic Purpura
ULVWF	Ultra-Large Von Willebrand Factor
uPA	Urokinase-Type Plasminogen Activator
uPAR	Urokinase-Type Plasminogen Activator Receptor
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VWF	Von Willebrand Factor
VWF:Ag	VWF Antigen

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1 INTRODUCTION

Microparticles are currently a very novel and exciting field of haemostasis research and may be implemented as a treatment or as diagnostic agents in the near future. There is still a lot to be learned about the role of microparticles in inflammatory and thrombotic disorders. Microparticles are formed from a variety of cells. Endothelial dysfunction is proposed as one of the main triggers of aberrant endothelial microparticle formation.

Conditions such as inflammation and thrombosis alter endothelial microparticle formation. Therefore, inflammatory or cardiovascular disorders such as sepsis, atherosclerosis and thrombotic thrombocytopenic purpura may also alter microparticle formation. It is unfortunately not yet clear whether these endothelial microparticles are the consequence or the cause of these disorders.

The processes of inflammation and thrombosis are closely related. Inflammatory cytokines and coagulation stimuli activate endothelial microparticle formation. They also influence protein secretion of endothelial cells and of endothelial microparticles. A protein that plays a role in both these conditions is von Willebrand factor (VWF), the endothelium's first defence against bleeding. Endothelial microparticles can carry VWF. The composition and function of the microparticles that form can vary according to the concentration and differences in stimuli on the cell.

In order to understand the role of endothelial microparticles in inflammation and thrombosis better, we designed a study to investigate the effect of the cytokines interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor alpha (TNF-α) and coagulation stimuli, tissue factor and thrombin (also different combinations thereof) on endothelial microparticle formation and on the secretion of VWF and its regulating protease, ADAMTS-13 (A Disintegrin and Metalloprotease with Thrombospondin Type 1 Motif Number 13) by endothelial microparticles.

2 LITERATURE REVIEW

2.1 Introduction to microparticles

Microparticles were first described in 1967 by Wolf as “platelet dust”. Today, microparticles can be defined as vesicles formed by the incarceration and release of plasma membranes due to internal cellular processes. The diameter of microparticles range between 100 nm and 1 µm (Dignat-George & Boulanger, 2011). While there are no concise definitions for microparticles, cell-released particles with a diameter of >1,5 µm that are formed during the later stages of apoptosis are described as apoptotic bodies and particles between 40 nm and 100 nm are known as exosomes. Cells create and store these exosomes to release upon stimuli or sometimes without activation (Boulanger *et al.,* 2006). In this chapter, the origin of microparticles will be described and thereafter the roles of microparticles in inflammation and thrombosis.

2.2 Origin of microparticles

2.2a Sources of microparticles

Microparticles originate from apoptotic, inflamed, thrombotic, activated, injured or senescent platelets, red blood cells, white blood cells, endothelial cells, and smooth muscle cells (Burnier *et al.,* 2009, Orozco & Lewis*.,* 2010). The originating cell of a microparticle determines the membrane antigen composition of the microparticle (Dignat-George, 2008, Nomura *et al.,* 2008, Peterson *et al.,* 2008). Environmental conditions in and around the cell of origin may also contribute to the composition of microparticles formed (Benameur *et al.*, 2009, Dignat-George & Boulanger, 2011).

2.2b Conditions for microparticle formation

Microparticles are formed from activated cells in both normal and pathophysiological states (Navasiolava *et al.,* 2010, Dignat-George & Boulanger, 2011). Factors that are responsible for a difference in characteristics and number of microparticle formation include shear stress, pathophysiological conditions, inflammatory and coagulatory environments. For example, increased numbers of prothrombotic microparticles are formed under inflammatory and coagulatory conditions (Freyssinet & Toti, 2010, Ramkhelawon *et al.,* 2009). Antithrombotic

agents (Sulodexide, Enoxaparin and protein C), on the other hand, stimulate the release of microparticles rich in anticoagulant proteins. These microparticles also contain reduced amounts of the thrombotic tissue factor (TF) (Pérez-Casal *et al.,* 2005, Pérez-Casal *et al.,* 2011, Adiguzel *et al.,* 2009). Microparticle formation can also occur without cellular stimulation, for example, during cell death (Burnier *et al.,* 2009, Dignat-George & Boulanger, 2011). The next section describes how microparticles are formed.

2.2c Microparticle formation

Microparticle formation is induced by the process of membrane remodelling. Microparticles are formed by a flip (blebbing) in the cell membrane. The precise mechanism of microparticle formation is still being debated (Combes *et al.,* 1999, Shet, 2008, Morel *et al.,* 2011, Zhang *et al*., 2011). Studying membrane remodelling brought about important discoveries in microparticle formation. Before membrane reorganisation, the inner membrane layer of cells usually consists of phosphatidylethanolamine (PE) and phosphatidylserine (PS) and the outer membrane layer of phosphatidylcholine (PC) and sphingomyelin (S) among other phospholipids (Seigneuret *et al.,* 1984). Figures 1a and 1b illustrate the process of microparticle formation. The three-lipid transport proteins play an important part in transportation of phospholipids across the bilayer of the cell membrane. These are the calcium-dependent scramblase (in- and outward transportation), the phosphatidylserine (PS) specific Mg-ATP dependent flippase/aminophospholipid translocase (inward transportation) and the Mg-ATP dependent floppase (outward transportation, Manno *et al.,* 2002, Daleke, 2003, Boulanger *et al.*, 2006, Doeuvre *et al*., 2009, Smith, 2009). Throughout the literature these proteins are referred to as scramblase, flippase, and floppase.

Under normal resting conditions, phospholipids within eukaryotic cell membranes are asymmetrically distributed (Manno *et al.,* 2002). Activation signals on cells cause an increase in the release of calcium from the endoplasmic reticulum (ER). This leads to structural changes of the membrane (Hugel *et al.*, 2005, Chironi *et al.,* 2009). The increase of intracellular calcium activates the scramblase and floppase enzymes and downregulates the flippase enzyme activity. This leads again to a loss in membrane asymmetry (Daleke, 2003, Hugel *et al.*, 2005, Doeuvre *et al*., 2009, Smith, 2009, Bevers & Williamson, 2010). Elevated intracellular calcium levels, calcium influx and calcium released from the inside of the cell further results in the release of cystein proteases (Fox *et al.*, 1991, Piccin *et al.,* 2007). Cystein proteases, such as calpain, activates integrins which leads to modification and reorganisation of actin filaments and membrane blebbing that subsequently leads to

microparticle formation (Fox *et al.,* 1991, Cunningham, 1995, Huot *et al.,* 1998, Combes *et al.,* 1999, Keller *et al.,* 2002, , Osborne, 2004, Wang *et al.,* 2005, Biro, 2008).

After the processes of membrane remodelling and blebbing, the once negatively charged inside layer that contained amino phospholipids like PS and PE is then exposed to the external environment due to the activity of phospholipid transport proteins scramblase, flippase and floppase activity. The negatively charged membrane layer and the composition of these microparticles that are formed suggest some important functions thereof (Urbanija *et al.,* 2007). This mechanism is proposed for platelet and erythrocyte microparticle formation (Weerheim *et al.,* 2002, Nguyen, 2010). This specific membrane remodelling mechanism for endothelial microparticle formation is still unclear (Leroyer *et al.,* 2010, Dignat-George & Boulanger, 2011).

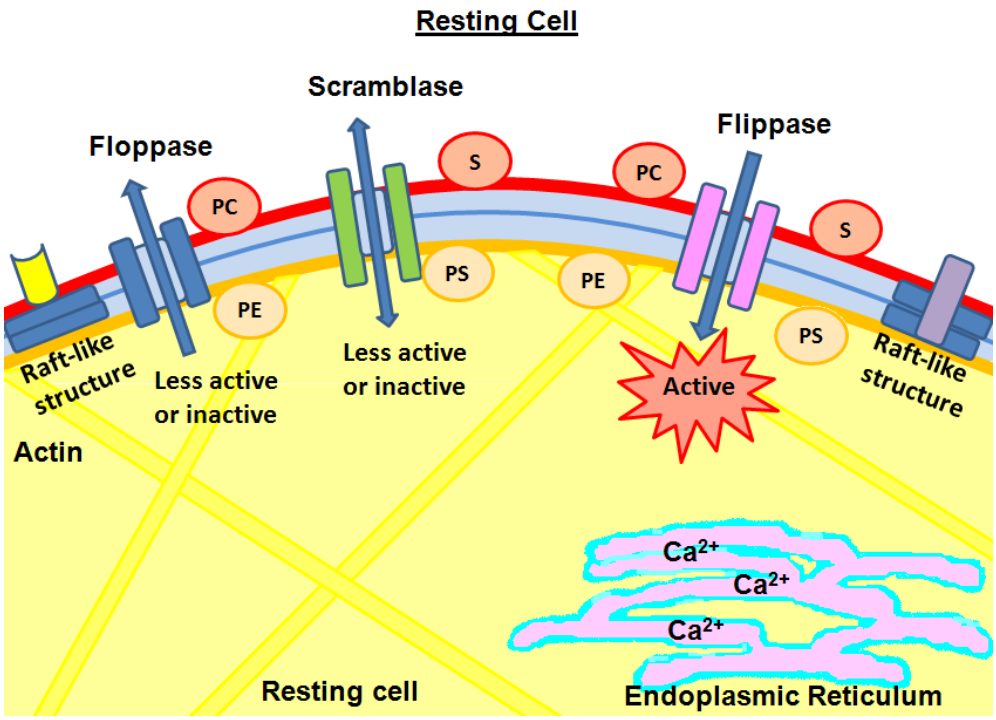

Figure 1a An illustration of a resting cell undergoing membrane remodelling in order to form a microparticle

Phosphatidylcholine (PC) and Sphingomyelin (S) in a resting cell are usually found predominantly on the outer membrane layer, whereas phosphatidylserine (PS) and phosphatidylethanolamine (PE) are more commonly found on the inner membrane layer. Flippase is only one of the lipid transport proteins involved in retaining membrane asymmetry by inward transportation of PS.

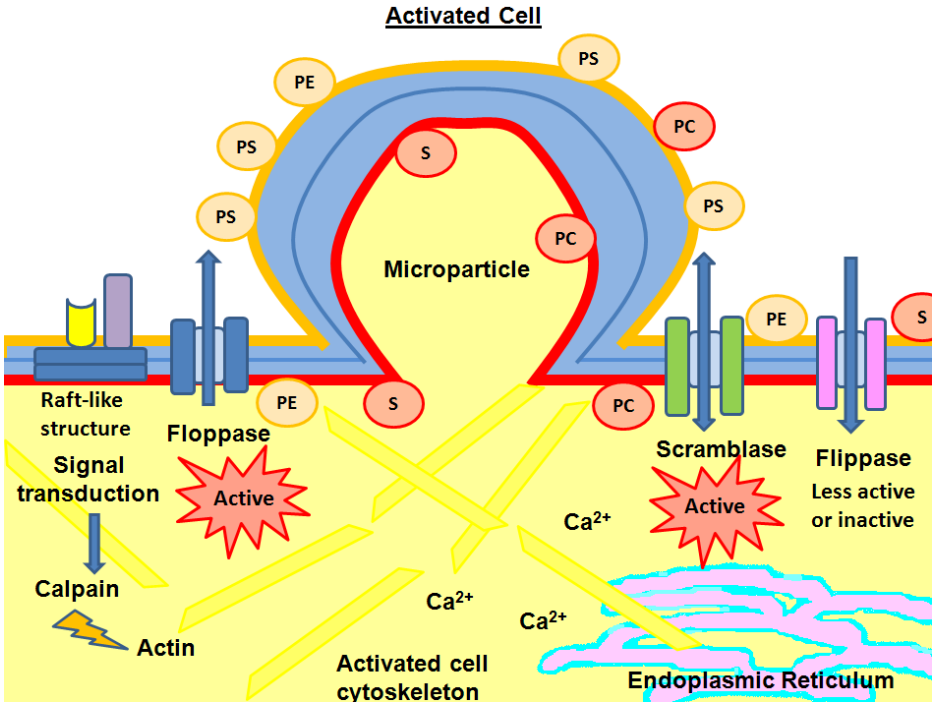

Figure 1b An illustration of an activated cell undergoing membrane remodelling and microparticle formation

After membrane remodelling the inner membrane that was rich in negatively charged PS is now on the outside and membrane asymmetry is lost by the working of the transport proteins, flippase, floppase, and scramblase. Calcium influx from the ER and increased intracellular calcium levels are induced, which subsequently activates calpain. This protein mediates cleavage and modification of the actin filaments and rearrangement of the cytoskeleton, which leads to blebbing of the cell membrane and subsequent microparticle formation. Raft-like structures on the membrane are specific organisations of lipids in a certain domain of a cell or microparticle and are involved in signalling (Adapted from Hugel *et al.,* 2005, López *et al.,* 2005 ,and Piccin *et al.,* 2007)

2.2d Endothelial microparticle formation upon stimulation

Stimulation of cells is the most common cause of microparticle formation (Freyssinet & Toti, 2010). Thrombin is a potent stimulator of cells and is also the key enzyme in blood coagulation. It stimulates the phosphorylation of the enzyme focal adhesion kinase p125 that is responsible for membrane remodelling and detachment (Schaphorst *et al.,* 1997). Besides this, thrombin also activates the Rho-kinase enzymes (ROCK-II – Rho-Associated Coiled-Coil Forming Kinase – 2, Sapet *et al.,* 2006). These enzymes have pleiotropic properties that may cause cell death or cell survival under different circumstances. Cellular interactions between different participating cell types and substances in the cellular environment cause differential expression and regulation of ROCK enzymes. Rho-Associated Coiled-Coil Forming Kinases (ROCK) can induce cell survival mechanisms.

ROCK can also initiate apoptosis via both caspase dependent or independent ways in endothelial microparticle formation (Street & Bryan, 2011).

TNF-α also stimulates the formation of human umbilical vein endothelial microparticles. Caspases and calpain play important parts in this process. They are involved in the activation of Rho-kinase enzymes during apoptosis (Petrache *et al.,* 2001, Tramontano *et al.,* 2004, Lippens *et al.,* 2005, Sapet *et al.*, 2006, Gonçalves *et al.,* 2009). Calpain also activates caspases in this process and induces apoptosis (Vindis *et al.,* 2005, Smith & Schnellmann, 2012). For example, high numbers of microparticles derived from human umbilical vein endothelial cells (HUVEC) are formed by caspase 3-mediated apoptosis (Hussein, 2008).

Shear stress may have different influences on endothelial microparticle formation (Ramkhelawon *et al.,* 2009). Shear stress mediates the crosslinking of α-actin and F-actin, which can lead to both hardening and softening of the cytoskeleton (Xu *et al.,* 2000). The role that shear stress plays in endothelial microparticle formation is still unknown. Calpain also participates in cytoskeletal changes, which can lead to endothelial cell shape changes and the formation of microparticles (Osborne, 2004, Boulanger *et al.*, 2006, Flaumenhaft, 2006, Piccin *et al.,* 2007, Chironi *et al.*, 2009, Ramkhelawon *et al.,* 2009). Shear stress can also activate calpain that is involved in membrane remodelling (Kang *et al.,* 2011).

One of the most important functions of this exposure of negatively charged PS is its interaction with antithrombotic proteins like thrombomodulin (TM) and protein C and pro-coagulatory factors like factor VII (FVII). Hereby PS can catalyse coagulation and suppress or promote thrombosis (Chattergee *et al.,* 2010, Danese *et al.,* 2010). Microparticle membrane composition specifically influences microparticle clearance. There is still much to be learned about microparticle contents and clearance (Woywodt *et al.,* 2008, Serda *et al*., 2009, Gregory & Pound, 2011).

2.2e Contents of endothelial microparticles

Microparticles express a variety of proteins. Endothelial microparticles contain inflammatory proteins such as platelet endothelial cell adhesion molecule-1 (PECAM-1, Hussein *et al.,* 2003), vascular cell adhesion molecule-1 (VCAM-1, Vince *et al.,* 2009), intercellular cell adhesion molecule-1 (ICAM-1, Simoncini *et al.,* 2009) and E-selectin (Yong *et al.,* 2013). It also express the TM receptor (Combes *et al.,* 1999) as well as tissue factor (TF, Shet *et al.,* 2003), von Willebrand factor (VWF, [a]Jimenez *et al.,* 2003), and activated protein C (APC,

Pérez-Casal *et al.,* 2009). Endothelial microparticles also contain matrix metalloproteinases (MMP) that may regulate angiogenesis (Taraboletti *et al.,* 2002, Pisetsky *et al.* 2011). It also contains proteins that regulate endothelial function such as endothelial nitric oxide synthase (eNOS , Deregibus *et al.,* 2007, Dignat-George & Boulanger, 2011), vascular endothelial growth factor (VEGF, Leroyer *et al.,* 2009), endoglin (Bakouboula *et al.,* 2007), and reactive oxygen species (ROS, Deregibus *et al.,* 2007). Microparticles are also responsible for expression of increased amounts of the vasodilatory factors, inducible nitric oxide synthase (iNOS), and endothelin after infusion into chicken lungs (Hamal *et al.,* 2009, Ait-Oufella *et al.,* 2010). Nitric oxide (NO) with its vasodilatory properties plays an imperative part in vascular haemostasis. It is also involved in the pathophysiology of inflammatory disorders with increasing shear and blockage of arterioles (Hamal *et al.,* 2009, Ait-Oufella *et al.,* 2010).

Other important factors that are expressed by endothelial microparticles include endoglin and VEGF. These factors mediate the signalling for proliferation and angiogenesis respectively (Bakouboula *et al.,* 2007, Leroyer *et al.,* 2009). Table 1 is a summary of different proteins or factors that are expressed by endothelial microparticles in different processes and shows the endothelial microparticle-derived proteins and factors involved in normal functioning of endothelial cells.

Table 1 A summary of proteins/factors expressed by endothelial-derived microparticles and its association with different cellular processes

(Adapted from Leroyer *et al.,* 2010 and Dignat-George & Boulanger, 2011)

Process mediated by endothelial microparticle-derived protein	Endothelial function	Adhesion	Remodelling and/or fibrinolysis	Coagu-lation	Cell survival
Protein carried by endothelial microparticles	eNOS ROS Endoglin VEGF	PECAM-1 VCAM-1 ICAM-1 E-Selectin	uPAR uPA MMP T-cadherin VE-cadherin	TF TM EPC PS VWF	APC EPCR

Endothelial microparticles contain nuclear material such as mRNA for transcription factors like OCT-4 (Ratajczak *et al.,* 2006). Endothelial microparticles can also carry specific

subsets of mRNA, which can be transfered to human microvascular endothelial cells in order to induce expression of eNOS and activation of the phosphatidylinositol-3-kinase (P13K/Akt) signalling pathway. This process activates and mediates angiogenesis (Deregibus *et al.,* 2007, Leroyer *et al.,* 2009). Microparticles also contain rRNA, DNA (Pisetsky *et al.,* 2011), and specific subtypes of microRNAs (miRNA), which differs from the miRNA expression of the parent cell. These miRNAs may be involved in gene regulation (Diehl *et al.,* 2012). It has been shown that miRNA from HUVEC mediates angiogenic pathways and suppresses the regulatory proteins of VEGF in diabetes mellitus type 2 patients. This evidence illustrates the probability that endothelial microparticles play an important part in regulation of protein expression of cells by miRNA transfer (Zampetaki *et al.,* 2010).

2.3 The role of endothelial microparticles in inflammation

Inflammatory responses are usually initiated by proteins called cytokines. Chemokines, cytokines, and tumour necrosis factors (TNF) and other proteins are all classified as cytokines. The word cytokine is self-explanatory [*cyto* (cell) and *kinin* (hormones)] and literally means cellular hormones, a term that was first used by Stanley Cohen in 1974 (Tayal & Kalra, 2008). Cytokines interact with or activate cells and cause inflammatory responses. Cytokines are regulatory pleiotropic proteins (Tayal & Kalra, 2008, Sprague & Khalil, 2009). TNF-α, IL-6 and IL-8 are examples of cytokines with inflammatory and thrombotic properties (Bernardo *et al.,* 2004, Maya *et al.,* 2008, Sprague & Khalil, 2009, Montoro-García *et al.,* 2011). These three cytokines were therefore used in this study.

The next sections explain the possible role that endothelial microparticles play in inflammation and thrombosis. The different proteins that may play a part in endothelial microparticle-induced inflammation and thrombosis are shown in Figure 2.

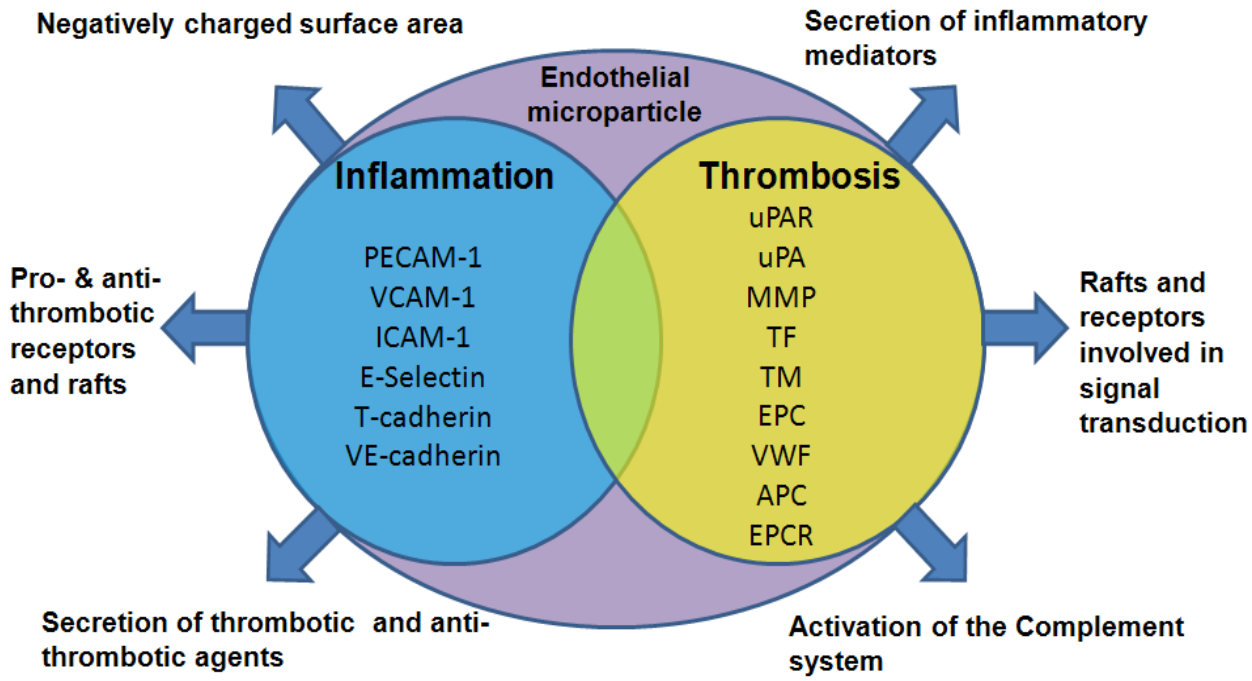

Figure 2 The role of endothelial microparticles in inflammatory and coagulatory processes and the different factors involved

Endothelial microparticles express adhesion molecules, the key factors involved in inflammation. These include E-selectin, VCAM-1 and ICAM-1. These adhesion molecules mediate the recruitment and activation of granulocytes, monocytes and lymphocytes during inflammatory processes (Perrot-Applanat *et al.,* 2011). These factors also increase neutrophil adhesion to endothelial cells and the rolling and transmigration of leukocytes with endothelial cells (Suarez *et al.,* 2010, Rossaint *et al.,* 2011). The expression of these adhesion molecules on the surface of endothelial cells are increased upon activation of the endothelium. TNF-α, viruses, and various antibodies activate endothelial cells (Shen *et al.,* 1997, Lawson & Wolf, 2009). The adhesion protein ICAM-1 mediates adhesion of leukocytes to endothelial cells and increases intracellular calcium levels. This has implications for microparticle formation. The increased intracellular calcium levels together with the increased adhesion potential of cells and microparticles lead to amplified inflammatory or coagulatory responses (Etienne-Manneville *et al.,* 2000, Lawson & Wolf, 2009, Leroyer *et al.,* 2010). Adhesion molecules like VCAM-1 and ICAM-1 are also involved in the pathophysiology of inflammatory-related atherosclerosis (El-Solh *et al.,* 2002).

PECAM-1 (CD31) is referred to as a “scaffold” protein of endothelial cells and is a marker for vascular integrity (Müller *et al.,* 2002). Increased PECAM-1 expression on the endothelium

inhibits apoptosis. During apoptosis PECAM-1 is cleaved into two parts, namely soluble PECAM-1, and a PECAM-1 cytoplasmic tail. The soluble form of PECAM-1 in the circulation inhibits inflammation and trans-endothelial migration and is thus anti-inflammatory. The remaining cleaved PECAM-1 cytoplasmic tail exhibits inflammatory properties (Ilan & Madri, 2003, O'Brien *et al.,* 2003, Privatsky *et al.,* 2010). PECAM-1 therefore has inflammatory and anti-inflammatory properties.

Compared to the binding characteristics of inflammatory markers, E-selectin is used as a reliable marker to detect inflammation in the endothelium under shear stress conditions (Jefferson *et al.,* 2010). TNF-α increases E-selectin expression on HUVEC. E-selectin expression is also increased in sepsis (Fina *et al.,* 1990, Sun *et al.,* 2013).

Microparticles can also secrete inflammatory mediators, such as chemokines and cytokines like interleukin-1β (IL-1β), or induce secretion of these factors in other cells. For example, cultured human microvascular dermal endothelial-derived microparticles induce IL-6 and IL-8 secretion in plasma of cytoid dendritic cells (Ardoin *et al*., 2007, Angelot *et al.* 2009, Mause & Weber, 2010,). Cytokines also serve as an activation signal for increased microparticle formation and increase the binding activity of platelets to endothelial cells by increasing the expression of certain selectins on endothelial cells (Bernardo *et al.,* 2004, Karahan *et al.*, 2005, Boulanger *et al*., 2006).

Endothelial microparticles also express cadherins, which are proteins involved in adhesion and signal transduction pathways. Examples of them are T-cadherin and VE-cadherin. Both T-cadherin and VE-cadherin are involved in signalling of proliferation and angiogenesis. T-cadherin is also involved in protection of endothelial cells by signalling for cell survival before oxidative stress-induced apoptosis and VE-cadherin plays an important part in cell permeability (Koga *et al.,* 2005, Vestweber, 2008, Philipova *et al.,* 2009, Philipova *et al.,* 2011).

Microparticles can also induce inflammatory responses via activation of the complement system (Distler *et al*., 2006). All these processes amplify inflammatory responses (Ardoin *et al*., 2007, Mause & Weber, 2010). The three cytokines that were used in this study were IL-6, IL-8, and TNF-α. The roles thereof in inflammation, thrombosis, and microparticle formation are discussed in the next 3 sections.

2.3a The role of interleukin-6 in inflammation, thrombosis and microparticle formation

The cytokine IL-6 causes differentiation of B and T cells (Finck *et al.,* 1994). The effect of IL-6 is dependent on its binding to its receptor. The IL-6 receptor is either membrane-bound or soluble (Jones *et al.,* 2001, Fonseca *et al.,* 2009).

After activation of inflammation, IL-6 acts as an amplifier of the inflammatory response by its ability to increase expression of adhesion molecules like ICAM-1 and VCAM-1 in stimulated endothelial cells (Wassmer *et al.,* 2011). IL-6 is also proposed as a marker for acute inflammatory responses since it is markedly increased in patients with high risk of myocardial infarction (Fonseca *et al.,* 2009). Another example of this is the increased IL-6 levels in patients with cerebral malaria (Wassmer *et al.,* 2011). IL-6 levels also correlate with the amount of endothelial microparticles in healthy individuals ([b]Chirinos *et al.,* 2005, Curtis *et al.,* 2009).

IL-6 furthermore amplifies thrombotic processes under certain circumstances. This may be due to the ability of receptor-bound IL-6 to increase ultra-large von Willebrand factor (ULVWF) expression in HUVEC (Bernardo *et al.,* 2004). IL-6 needs to bind to its soluble receptor in order to activate the process of cell signalling (Scheller & Rose-John, 2006; Barnes *et al.,* 2011). Despite the fact that IL-6 does not always induce an increase in VWF in endothelial cells, it has the ability to inhibit the disentegrin-like and metalloprotease with thrombospondin type 1 motif number 13 (ADAMTS-13), the protease involved in regulating the size of ULVWF multimers in whole blood (Bernardo *et al.,* 2004).

The inflammatory, anti-inflammatory and cytoprotective effects of IL-6 are also not yet clear (Waxman *et al.,* 2003). IL-6 may have different dose-dependent properties. The amount of glycoprotein receptors on the cell, to which IL-6 in combination with its receptor bind, may also determine the cellular response (Kallen, 2002). IL-6 thus may play a part in inducing increased levels of VWF. This reaction is mediated by increased activity of STAT-3 (a signal transducer and activator of transcription). In a study with IL-6 knockout mice it was proven that VWF positive cells were decreased (Gertz *et al.,* 2012).

Mice treated with IL-6 also showed increased FVIIIa activity (Mutlu *et al.,* 2007). Treatment of endothelial cells with IL-6 not only increases alternatively spliced TF (a soluble form of TF) but also decreases tissue factor pathway inhibitor (TFPI, Szotowski *et al.,* 2005). Both IL-6 and IL-8 induced an increase in monocytic TF expression (Neumann *et al.,* 1997). TF and

IL-6 are also useful markers in the prognosis of thrombotic events and the outcome for patients with congestive heart failure (Chin *et al.,* 2003). IL-6 induces acute inflammatory responses and is commonly elevated in disorders like acute kidney injury and unstable angina (Heinrich *et al.,* 1990, Nechemia-Arbely *et al.,* 2007, Awan *et al.,* 2008, Dennen *et al.,* 2010).

2.3b The role of interleukin-8 in inflammation, thrombosis and microparticle formation

IL-8, also known as neutrophil chemotactic factor, is a potent recruiter of granulocytes and monocytes to sites of inflammation (Yoshimura *et al.,* 1987, Apostolakis *et al.,* 2009). IL-8 is either bound to endothelial cells during inflammation or circulates in a soluble form. IL-8 is further known for its involvement in leukocyte-endothelial-mediated inflammatory responses. This chemokine mediates the rolling and adhesion of leukocytes to endothelial cells, a process that is supported by VWF (Chauhan *et al.,* 2008). Moreover, the ERG (E-twenty six (ETS) related gene) involved in regulating the transcription of VWF, ICAM-2 and VE-cadherin genes also mediates IL-8-induced neutrophil adhesion. In this process ERG induces inhibition of inflammation by suppressing the genes involved. The ERG expression is thus decreased in inflammatory conditions (Yuan *et al.,* 2009).

IL-8 is only co-localised with VWF in Weibel-Palade bodies after stimulation of HUVEC with inflammatory stimuli like interleukin-1 (IL-1), IL-1β, TNF-α and lipopolysaccharide (Utgaard *et al.*, 1998, Wolff *et al.,* 1998, Schraufstatter *et al.,* 2001). This chemokine is present in unstimulated HUVEC Weibel-Palade bodies (Wolff *et al.,* 1998). IL-8, unlike other cytokines, is known for its prolonged survival and functionality and is stable for days or weeks after an inflammatory response (Apostolakis *et al.,* 2009). When HUVEC are stimulated by various interleukins, IL-8 is immediately released from the Golgi, even after the stimulus had been retracted. IL-8 is stored in Weibel-Palade bodies, but is not secreted from these bodies until the cells are stimulated for a second time. In this way, the Weibel-Palade bodies may enable instant secretion of IL-8 and VWF upon the second stimuli as needed in inflammation (Wolff *et al,* 1998). IL-8 is further involved in different signalling reactions between cells. For example, IL-8 is involved in leukocyte-endothelial and neutrophil-endothelial interactions under shear stress conditions. Activated endothelial cells express adhesion molecules like E-selectin, P-selectin and ICAM-1, which are necessary for leukocyte and neutrophil adhesion. IL-8 forms tighter adhesions between leukocytes and/or neutrophils and endothelial cells by increasing the avidity (through activation of integrins) of the bonds between these cells (Lorenzon *et al.,* 1998, Furuta *et al.,* 2000, DiVietro *et al.,* 2001,

McIntyre *et al.,* 2003). IL-8 also increases the expression of the adhesion molecules E-selectin and ICAM-1 in HUVEC (Yoon *et al.,* 2010). It thus recruits and activates monocytes, granulocytes and neutrophils involved in inflammatory responses (Apostolakis *et al.,* 2009).

Overall, conditions of increased stress, like endurance running and oxidative stress, not only increase the expression of inflammatory cytokines but also the production of ROS. IL-8, TNF-α, and IL-6 also induce an increase in ROS. ROS are involved in acute immune responses and therefore high ROS levels and IL-8 levels are associated with endothelial dysfunction and increased permeability (Fehrenbach *et al.,* 2000, Lum & Roebuck, 2001, Gallová *et al.,* 2004, Laskowska *et al.,* 2007). Increased permeability of endothelial cells may be a contributing factor to an increase in intracellular calcium and possibly to microparticle formation (Liu & Schnellmann, 2003, Laskowska *et al.,* 2007). No mechanism for IL-8-induced microparticle formation has been described.

IL-8 increases the permeability of endothelial cells by activating vascular endothelial growth factor receptors (VEGFR – independent of VEGF – Van Nieuw Amerongen *et al.,* 1998, Liu & Schnellmann, 2003, Petreaca *et al.,* 2007). IL-8 also plays an important role in membrane remodelling and induces apoptosis via signalling that leads to caspase activation. The overall apoptotic and inflammatory mechanisms of IL-8-derived microparticle formation are still unknown (Govindaraju *et al.,* 2006, Gangadharan *et al.,* 2010).

The effect of IL-8 on endothelial cells influences the haemostatic process in different ways. Numerous studies illustrate that increased IL-8 levels may be associated with an increase in VWF levels (Velzing-Aarts *et al.,* 2002, Xiaoyong *et al.,* 2002, Bernardo *et al.* 2004, Qian *et al.,* 2011). Stimulation of HUVEC with IL-8 increased the expression of ULVWF significantly (Bernardo *et al.,* 2004). The effect of high IL-8 concentrations on ADAMTS-13 *in vitro* is still not known. In studies on pre-eclamptic patients with increased IL-8 levels, it was found that ADAMTS-13 levels were decreased (Sharma *et al.,* 2007, Stepanian *et al.,* 2011).

2.3c The role of tumour necrosis factor alpha in inflammation, thrombosis and microparticle formation

TNF-α was the first agent used to mediate HUVEC microparticle formation. These microparticles had prothrombotic but also inflammatory potential (Combes *et al.,* 1999, Bradley, 2008). Increased TNF-α levels and microparticle levels have been found in sepsis, atherosclerosis, and rheumatoid arthiritis as well as in thrombotic thrombocytopenic purpura

(TTP, [a]Jimenez *et al.,* 2003, Montoro-García *et al.,* 2011). Endothelial dysfunction and inflammation also correlate with increased TNF-α expression (Zhang *et al.,* 2010).

The inflammatory response of TNF-α is well described and involves its binding to Tumour Necrosis Factor Receptor 1 (TNFR-1) or Tumour Necrosis Factor Receptor 2 (TNFR-2). Tumour Necrosis Factor Receptor 1 activates nuclear factor κβ (NF-κβ) while TNFR-2 activates either Activator Protein-1 (AP-1) or NF-κβ (Pober, 2002). The exact mechanism whereby TNF-α induces endothelial microparticle formation in these inflammatory responses is still unknown (Dignat-George & Boulanger, 2011). Protease-activated receptor-2 (PAR-2) activation induces TF expressing endothelial microparticle release upon TNF-α stimulation (PAR-2, Collier & Ettelaie, 2011). TNF-α also mediates inflammatory endothelial microparticle formation through the P38 mitogen-activated protein kinase (MAPK) pathway (Curtis *et al.,* 2009).

More than 70 proteins are expressed by microparticles from TNF-α-stimulated cells. Some of these proteins may induce inflammatory and pro-coagulatory responses. Interestingly, copine-3 is representative of the the copine family of proteins found in endothelial microparticles (Creutz *et al.,* 1998, Peterson *et al.,* 2008). VWF is one of the proteins that are modulated by TNF-α. TNF-α stimulates the release of ULVWF from endothelial cells. It also induces an increase in the release of the high molecular weight VWF (Bernardo *et al*., 2004). In a study done by Cao *et al.,* (2008) TNF-α did not necessarily affect ULVWF secretion but it caused a reduction in the synthesis of regulatory protease ADAMTS-13. Nevertheless, stimulation by TNF-α still causes a prothrombotic state.

Another mechanism by which TNF-α induces prothrombotic responses is to stimulate endothelial cells to increase TF expression. This was proved in murine cardiomyocytic cells. Microparticles from cardiomyocytic cells were also able to diffuse through the neighbouring endothelial cell layer to carry out its prothrombotic process, possibly by inhibiting the transcription of TM (Conway & Rosenburg, 1988, Zhao *et al.,* 2005, Antoniak *et al.,* 2009). TNF-α-induced microparticles are mostly procoagulatory and inflammatory (Curtis *et al.,* 2009).

Endothelial microparticles formed by stimulation of TNF-α also express antithrombotic proteins such as TFPI, and the externalised PS on microparticles also supports protein C and S complex formation (Kushak *et al.,* 2005, Morel *et al.,* 2009).

The regulation of clot formation regarding endothelial microparticles is not yet clear. There is also no concrete evidence for ADAMTS-13 (VWF's regulating protease) expression in endothelial microparticles to date (Kitchens *et al.,* 2011).

2.4 Endothelial microparticles in haemostasis and thrombosis

Endothelial microparticles are associated with bleeding disorders such as Scott's and Castaman's disease. In these disorders, decreased numbers and/or defective microparticle formation leads to haemorrhage. These disorders are however very rare (Burnier *et al.*, 2009). Microparticles are more commonly known for their procoagulatory nature. For example, increased micoparticle numbers were found in venous thrombo-embolisms, atherosclerosis and acute coronary syndromes (Leroyer *et al.,* 2010). Microparticles can be favourable as well as detrimental as carriers of important pro- or anticoagulatory factors, depending on the presiding conditions or stimuli on the originating cell (Martinez *et al.,* 2011, Tushuizen *et al.,* 2011).

Patients with aortic stenosis have increased amounts of endothelial microparticles. These microparticles may give rise to the inflammatory responses of this disorder (Diehl *et al.,* 2008). These patients also present with increased thrombin formation and low levels of ULVWF multimers. This may be caused by increased cleavage of ULVWF multimers in high shear stress areas in the aorta. There is currently no evidence for the role of ADAMTS-13 in this phenomenon (Dong *et al.,* 2002, Natorska *et al.,* 2011).

Microparticles can also be antithrombotic in nature since they express protein C on their surface (Pérez-Casal *et al.*, 2005). Endothelial protein C (EPC) is mainly involved in FVa and FVIIIa inactivation in the coagulation cascade (Kalafatis *et al.,* 1996, Hockin *et al.,* 1997). Protein C expressed by these microparticles was also more protected from degradation by metalloproteases than soluble protein C and was able to inactivate FVa. It is important to note that protein C expression on endothelial microparticles was only demonstrated after stimulation of HUVEC with APC (Pérez-Casal *et al,* 2005). The endothelial microparticle-associated protein C–APC complex also first needs to bind to protease activated receptor-1 (PAR-1) in order to activate an anti-inflammatory and anti-apoptotic response (Pérez-Casal *et al.,* 2009). Protein C activation is dependent on TM activation by thrombin in order to activate the protein C–EPCR complex (Stearns-Kurosawa *et al.,* 1996). This indicates how pro- and antithrombotic mechanisms are interrelated and possibly mediated by microparticles.

Endothelial microparticles not only contribute to antithrombotic processes but also to fibrinolytic processes. Endothelial microparticles formed by TNF-α stimulated human microvascular endothelial cells express urokinase-type plasminogen activator (uPA) as well as its receptor, uPAR. These factors mediate the plasminogen conversion to plasmin on microparticle surfaces (Lacroix *et al.,* 2007). This ultimately leads to fibrinolysis. uPA and uPAR do not only play a part in fibrinolysis but they also play a part in migration and angiogenesis. They activate MMPs that are involved in the breakdown of extracellular matrix to allow migration of endothelial cells in order to form tube-shaped structures in angiogenesis (Lacroix *et al.,* 2007, Montoro-García *et al.,* 2011). In this way endothelial microparticles induce and regulate angiogenesis and fibrinolysis by the same receptor (Montoro-García *et al.,* 2011).

VWF bearing endothelial microparticles has great potential to improve the current therapy in patents with von Willebrand disease. Microparticles formed *in vitro* by desmopressin stimulation reduce the time of initial thrombin generation and improve platelet aggregation in the plasma of type 1 and type 3 von Willebrand disease patients (Trummer *et al.*, 2011). Endothelial microparticles may also be a potential reliable marker to facilitate diagnoses in disorders of vasculitis and organ transplant rejection. However, more studies are necessary to verify this (Erdbruegger *et al.,* 2008, Brodsky *et al.,* 2012). Endothelial microparticles may be a promising marker for the diagnosis of vascular endothelial disorders in the future.

The following two sections describe the effect of coagulation stimuli, TF, and thrombin on endothelial microparticle formation, and the last section mentions the disorders associated with increased microparticle counts.

2.4a The role of tissue factor in inflammation, thrombosis and microparticle formation

TF is a very important receptor in coagulation. It initiates the coagulation cascade by forming a complex with (factor FVII) FVII. This complex activates factor IX (FIX) and factor X (FX) which subsequently cause thrombin formation. Not only is TF involved in coagulation but it also mediates inflammation (DelGiudice & White, 2009). Cytokines and pathogens stimulate endothelial cells, which may lead to TF expression (Kirchhofer *et al.,* 1994, Szotowski *et al.,* 2005, Schouten *et al.,* 2008, Van der Poll, 2008).

Increased TF expression results in thrombosis. It is therefore not surprising that pathological conditions like diabetes, sepsis, and various vascular disorders, such as myocardial

infarction and sickle-cell disease, show increased TF levels (Dignat-George, 2008, Morel *et al.*, 2008, Schouten *et al.,* 2005, Chu, 2011).

Endothelial microparticles from TNF-α-stimulated HUVEC increased TF expression after 4 to 6 hours (Combes *et al.*, 1999). The expressed microparticle TF could lead to FX activation (Kushak *et al.,* 2005). TF expressed by HUVEC derived microparticles is also able to generate thrombin *in vitro* (Hussein *et al.,* 2008).

Protease-activated receptors (PARs) may be an important link between signalling reactions of inflammation and thrombosis (Van der Poll, 2008). The TF-FVIIa-FXa complex activates PAR-1 and PAR-2 in HUVEC. This results in cell proliferation, cell survival and expression of proteins, growth factors, cytokines and chemokines. Activation of PAR-2 also leads to increased VWF levels (Langer *et al.,* 1999). The process of TF-mediated signalling in this respect is still unclear (Riewald & Ruf, 2001).

There is a correlation between FVIIa and VWF in diabetic patients. TF may therefore play a role in inducing VWF expression in these patients (Kario *et al.,* 1995). VWF bearing endothelial microparticles was increased in TTP patients and may be one of the factors contributing to the thrombotic tendencies in these patients ([a]Jimenez *et al.,* 2003).

2.4b The role of thrombin in inflammation, thrombosis and microparticle formation.

Thrombin is a well known pleiotropic protease with pro- and antithrombotic as well as pro- and anti-inflammatory properties (Esmon, 2005, Esmon *et al.,* 2006). Its function in coagulation include the activation of coagulation factors V, FVIII, FXI, FXIII and the conversion of fibrinogen into fibrin (Ferry & Morrison, 1947, Hill-Eubanks *et al.*, 1989, Gailani & Broze, 1991, Brummel *et al.,* 2002). The prothrombotic properties of thrombin have been well investigated. Microparticles thus provide a prothrombotic PS-positive surface area that enhances this property. The amount of PS expressed by microparticles correlates with the amount of thrombin generated in coronary artery calcification (Jayachandran *et al.,* 2008).

Also favourable to the prothrombotic nature of thrombin is its ability to be antifibrinolytic. Thrombin can activate and increase the gene expression of the antifibrinolytic plasminogen activator inhibitor-1 (PAI-1) that usually protects the forming or already formed fibrin clot (Erickson *et al.,* 1985, Martorell *et al.*, 2008, Siller-Matula *et al.,* 2011). It is not yet known whether endothelial microparticles are able to express PAI-1. It has been suggested that

endothelial microparticles may inhibit fibrinolysis in a fibrin-dependent way (Howes *et al.,* 2008). More research on the involvement of endothelial microparticles in the inhibition of fibrinolysis is needed.

Thrombin also activates thrombin-activatable fibrinolysis inhibitor (TAFI). This protein induces a modification of fibrin and plasminogen receptor proteins and it influences the plasmin activity, fibrin interactions and ultimately leads to regulation of fibrinolysis (Nesheim, 2003, Binette *et al.,* 2007, Okumura *et al.,* 2009). The thrombin-TM complex is thought to be one of the principle activators of TAFI (Refer to Figure 3, Binette *et al.,* 2007, Okumura *et al.,* 2009). This complex regulates fibrinolysis by activating TAFI that subsequently regulates the conversion of plasminogen into plasmin. Elevated TAFI levels in plasma also gives an indication of endothelial cell injury (Małyszko *et al.,* 2004). Figure 3 illustrates the effect of the thrombin-TM complex on the coagulation and fibrinolytic cascades.

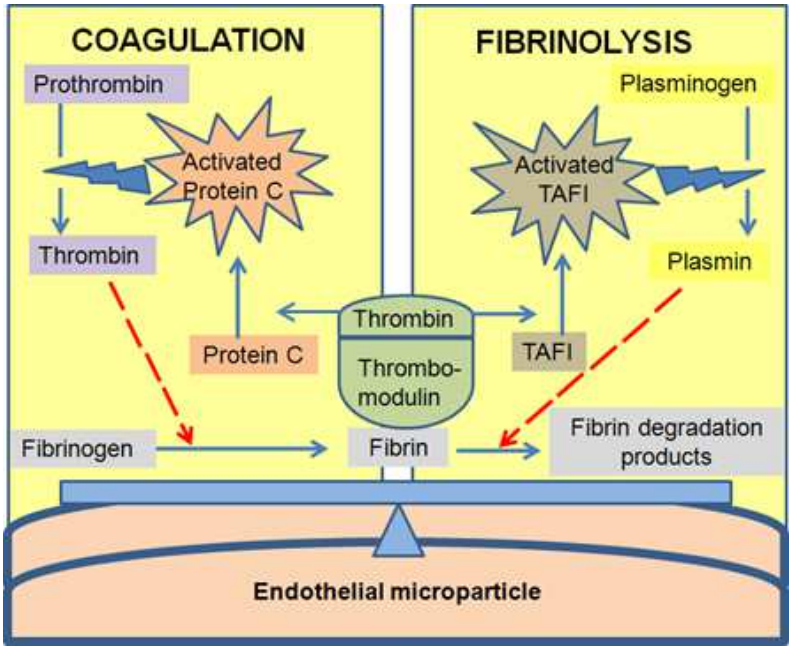

Figure 3 The effect of thrombin/thrombomodulin on the coagulation and fibrinolytic cascades

In coagulation, the thrombin-TM complex activates protein C that inhibits the conversion of prothrombin to thrombin and so leads to decreased fibrin formation from fibrinogen. In fibrinolysis the thrombin-TM complex activates TAFI, which prevents the conversion of plasminogen to plasmin to prevent fibrin breakdown (Adapted from Nesheim, 2003).

It is important to notice that the concentration of thrombin that is used to stimulate endothelial cells and the expression of different thrombin receptors may determine whether a prothrombotic or antithrombotic effect will take place (Levi *et al.,* 2004). It was suggested that rapid infusion of a single high dose of thrombin in animals exerts the prothrombotic properties of thrombin. This was probably caused by injury to the vessel wall. Slow

systematic infusions of relatively high doses of thrombin led to the induction of the anticoagulant and fibrinolytic properties. This was most likely caused by the intact endothelium (Siller-Matula *et al.,* 2011). Thrombin is also an important mediator of increased TF gene expression. Interestingly, this process is proposed to be mediated through the PAR-1 and RhoA/Rho (ROCK) pathway (Liu *et al.,* 2004, Martorell *et al.,* 2008).

Thrombin also has fibrinolytic and anticoagulatory properties involving its ability to activate protein C via the EPCR expressed on endothelial microparticles (Esmon, 1987, Pérez-Casal *et al.,* 2009, Siller-Matula *et al.,* 2011).

Thrombin is one of the most important activators of PARs and activates PAR-1, PAR-3, and PAR-4 (Coughlin, 2000). There is also evidence that thrombin activates PAR-2 (Lindington *et al.,* 2005). These PARs play an important part in many disorders, such as atherosclerosis, hypertension, cancer, central nervous system inflammation, neurodegeneration, nerve injury, arthritis, hepatitis, ischemia, inflammatory myopathy, and pre-eclampsia among others (Martorell *et al.,* 2008). Inflammatory stimuli (including TNF-α) upregulates some of these PARs in human coronary artery endothelial cells (Hamilton *et al.,* 2001). Even though PARs are extensively studied, the exact mechanisms of these PARs and how these receptors are "switched off" after thrombin stimulation are still unclear. Different concentrations of thrombin may explain the enhanced or sometimes decreased effects of PAR-mediated mechanisms (Coughlin, 2000). PARs, but specifically PAR-1, appears to be involved in microparticle formation in a HUVEC line (Sapet *et al.,* 2006). The mechanism by means of which thrombin induces microparticle formation may be as follows: Thrombin binds to PAR-1 to activate the small GTP-binding protein Rho and one of its effectors, cytoskeleton reorganising Rho-kinase (ROCK-II). Caspase-2 is necessary in this specific pathway which would usually occur in the early stages of apoptosis. However, the process of microparticle formation is not dependent on cell death. This is followed by, and dependent on, activation of NF-κβ which mediates microparticle formation. No thrombin-induced microparticles are formed after inhibition of ROCK-II (Sapet *et al.*, 2006, Leroyer *et al.,* 2010, Dignat-George & Boulanger, 2011). Other pathways that contribute to microparticle formation involves caspase-3 and ROCK-I activation during apoptosis, which subsequently leads to "blebbing" (Coleman *et al.,* 2001). During apoptosis-induced blebbing, the activity of Rho-kinases seems to be involved in the transport of nuclear material (DNA) to the area where microparticle formation takes place and possibly into microparticles and apoptotic bodies (Coleman *et al.,* 2001, Leroyer *et al.,* 2010).

Another process by which thrombin-induced endothelial microparticle formation occurs during the later stages of apoptosis is through TNF-α related apoptosis inducing ligand (TRAIL) and its receptor TRAIL-R2 (Simoncini *et al.,* 2009). When TRAIL binds with TRAIL-R2, three adaptor proteins, TNF receptor-1 associated death domain protein (TRADD), TNF Receptor Associated Factor-2 (TRAF-2) and Receptor Interacting Protein-1 (RIP-1) are recruited, which leads to NF-κβ activation. This process induces upregulation of inflammatory mediators ICAM-1 and amplifies the formation of prothrombotic endothelial microparticles (Simoncini *et al.,* 2009, Dignat-George & Boulanger, 2011).

Even though many of these mechanisms of thrombin-induced microparticle formation are understood, there is still much to be learned. The hypothesis that an increased number of microparticles formed upon thrombin stimuli is debatable, since Šimák *et al.* (2002) found that HUVEC stimulation with thrombin *in vitro* did not result in increased levels of microparticle formation.

In many thrombotic disorders endothelial microparticle numbers are increased. In pre-eclamptic patients, however, there is no significant increase in endothelial microparticles compared to normal pregnant and unpregnant individuals (Van Wijk *et al.,* 2002, Brodsky *et al.,* 2004). This was interesting, since pre-eclamptic individuals at risk for developing thrombosis (Van Walraven *et al.,* 2003).

It was shown in other studies that endothelial microparticle numbers are increased in disorders of increased thrombotic risk, such as cardiovascular disorders and renal failure (Van Wijk *et al.,* 2003, Martinez *et al.,* 2011). Microparticles proved to be a reliable marker to predict the outcome in these patients (Amabile *et al.*, 2009). Disorders with elevated endothelial microparticles and expected increased thrombin levels include acute disorders like angina, acute coronary syndrome, and acute myocardial infarction, but also sickle cell disease, lupus anticoagulant, TTP, and premature coronary artery calcification (Combes *et al,* 1999, Mallat *et al.,* 2000, Jimenez *et al.,* 2001, Simak *et al.,* 2004, Jayachandaran *et al.,* 2008).

Thrombin is commonly associated with increased VWF secretion and microparticle formation (Kim *et al.,* 2008, Van den Biggelaar *et al.,* 2008, Simoncini *et al.,* 2009, Dignat-George & Boulanger, 2011). Thrombin stimulation in endothelial cells may mediate VWF expression in a calcium- and calmodulin-dependent manner. A rise in calcium levels in endothelial cells seems to be associated with the increased VWF levels secretion upon stimulation with thrombin (Birch *et al.,* 1992, Fisher *et al.,* 2007). Observations of increased VWF levels after

thrombin stimulation in intact endothelium may be debated by the fact that VWF exocytosis in intact endothelium needs to be a regulated process (Zhou *et al.,* 2007, Valentijn *et al.,* 2010, Liu *et al.,* 2012).

Thrombin apparently influences VWF secretion, which involves the functioning of Weibel-Palade bodies. The function of Weibel-Palade bodies with regard to VWF synthesis is not yet fully understood (Metcalf *et al.,* 2011). Both slow and quick-release processes of VWF in HUVEC after thrombin stimulation have been described. The major pathway by which thrombin stimulates VWF release is via fusion of multiple Weibel-Palade bodies (Valentijn *et al.,* 2010). Additionally, after acute VWF release upon thrombin stimulation, there is a need to replenish the VWF stores, yet thrombin stimulation has no effect on VWF mRNA levels (Mayadas *et al.,* 1989, Richardson *et al.,* 1994, Cleator *et al.,* 2006). Thrombin-induced VWF secretion levels also seem to vary largely between *in vitro* and *in vivo* studies, both at different time measurements in different models and proposed mechanisms of secretion (Cleator & Vaughan*,* 2008, Fish *et al.,* 2007, Richardson *et al.,* 1994).

Another valid argument is that thrombin may also induce VWF release from factor VIII, yielding VWF more vulnerable to dissociation by ADAMTS-13 (Dong *et al.,* 2002, [a]Cao *et al.,* 2008). Thrombin is however involved in the proteolysis and inactivation of ADAMTS-13 (Crawley *et al.,* 2005). Thrombin-cleaved ADAMTS-13 appears to have an 8 times lower affinity for ULVWF. The metalloprotease was somehow protected in plasma from rapid inactivation (Lam *et al.,* 2007). There is also a correlation between ADAMTS-13 levels and the risk for myocardial infarction due to arterial thrombosis. This led to the hypothesis that not only increased VWF levels but also decreased ADAMTS-13 activity may be involved in prothrombotic processes (Chion *et al.,* 2007, Ruggeri, 2007).

A decrease in prothrombotic processes may be explained by decreased binding of VWF to its receptors due to thrombin stimulation (George *&* Torres, 1988, Englund *et al.,* 2001, Berny *et al.,* 2007). Interestingly, different forms of thrombin may lead to decreased binding of VWF to platelet glycoprotein Ib. A decrease in prothrombotic processes may be explained by decreased binding of VWF to its receptors due to thrombin stimulation (George *&* Torres, 1988, Englund *et al.,* 2001, Berny *et al.,* 2007). The increase in VWF levels after thrombin stimulation can therefore be debated and may be dependent on the time of measurement, type and state of the cells and Weibel-Palade bodies as well as the relative VWF binding affinity of the receptors (George *&* Torres, 1988, Richardson *et al.,* 1994, Crawley *et al.,* 2005, Cleator *et al.,* 2006). The effect of thrombin on VWF secretion in endothelial microparticles has not been studied. The last section summarizes the thrombotic disorders

associated with microparticles formation in order to stress the importance of microparticles in haemostatic disorders.

2.5 Microparticle-associated thrombotic disorders

Endothelial microparticles play an important part in thrombotic disorders where coagulation and inflammation are involved. Examples of such disorders are HIV-associated TTP, sickle cell disease, sepsis, coronary artery syndromes, stroke, deep vein thrombosis, and pulmonary embolisms (Piccin *et al.,* 2007, Meiring *et al.,* 2011).

In TTP patients, ADAMTS-13 levels are very low. Auto-antibodies against ADAMTS-13 were found in about half of the patients, while endothelial microparticle numbers were increased (Jimenez *et al.,* 2001, Shelat *et al.,* 2006).

Sickle cell disease is also an inflammatory and thrombotic disorder. In sickle cell disease, adhesion molecules (like VCAM-1, ICAM-1 and E-selectin) on circulating endothelial cells are increased. These adhesion molecules are markers for inflammation (Johnson & Telen, 2008, Kato *et al.,* 2005). In high mortality-risk sickle cell disease patients there is also a correlation between adhesion molecules and endothelial dysfunction (Kato *et al.,* 2005). These patients also have increased endothelial microparticles (Burnier *et al.,* 2009).

Inflammatory adhesion molecules, such as ICAM-1 and VCAM-1, are also increased in patients with coronary artery disease (Givtaj *et al.,* 2010). E-selectin positive microparticles are associated with coronary artery disorders (Lee *et al.,* 2012). The number of TF expressing microparticles may predict outcome in coronary artery disease (Shet *et al.,* 2003, Koga *et al.,* 2005, Morange *et al.,* 2007). Increased VWF levels may also predict the outcome in patients with vascular dysfunction and coronary artery syndrome (Paulinska *et al.,* 2009).

VWF levels may also be a good predictor of thrombosis and endothelial dysfunction. The VWF levels were significantly correlated with infarct size and were elevated in patients with stroke (Sato *et al.,* 2006). Not only was increased expression of the cytokines IL-6 and TNF-α present in patients with acute ischemic stroke, but also increased endothelial microparticle numbers (Simak *et al.,* 2006).

Endothelial microparticles were increased in patients with deep vein thrombosis and venous thrombo-embolisms ([a]Chirinos *et al.,* 2005). IL-6 and IL-8 are elevated in patients with a

further risk of deep vein thrombosis (Fox & Kahn, 2005). The association between these inflammatory markers and deep vein thrombosis is not yet clear. Similar to inflammatory markers, VWF and FVIII are also associated with an increased risk of venous thrombo-embolism (Tsai *et al.,* 2002).

Sepsis is an inflammatory and thrombotic disorder that occurs in response to an infection from pathogens or other foreign material. This disorder involves the activation of the innate immune responses while IL-6 induces an increase in TF. TNF-α impairs the antithrombotic process leading to the prothrombotic state encountered in sepsis (Cinel & Dellinger, 2007). TF bearing endothelial microparticles are better indicators of prothrombotic activity in a sepsis model than PS on endothelial cells (Wang *et al.*, 2009).

Since both coagulation and inflammatory proteins are involved in microparticle formation, it is clear that the processes of inflammation and thrombosis are interrelated. In order to gain a better understanding of the role of microparticles in these disorders, the effect of inflammatory agents and coagulation stimuli and the combined effect of inflammatory and thrombotic stimuli on the function and numbers of microparticles have to be determined. The role of inflammatory agents (IL-6, IL-8 and TNF-α) and coagulation stimuli (TF and thrombin) and also the combinations thereof on HUVEC microparticle formation and content is still unknown.

3 AIM AND OBJECTIVES

The aim and first objective of this study was to determine the *in vitro* effect of stimulation on HUVEC-derived microparticle formation by the cytokines IL-6, IL-8, TNF-α, and the coagulation stimuli TF, thrombin, and combinations of cytokines and coagulation stimuli. The contribution of microparticles to haemostasis was also investigated, but with specific reference to VWF. The second objective was to determine the relative number of microparticles upon stimulation. The third objective was to determine the contribution of microparticles to the thrombin that is generated by HUVEC.

This study will help us to gain a better understanding of the mechanisms that are involved in the stimulation of microparticle formation that may contribute to thrombotic and inflammatory disorders.

4 MATERIALS AND METHODOLOGY

4.1 Procedure outline

HUVEC were cultured under optimal conditions and stimulated by inflammatory (TNF-α, IL-6, IL-8) (Gibco) and thrombotic stimuli (thrombin and TF). The HUVEC were treated three times with a stimulus and measurements were obtained in duplicate. Unfortunately, due to the availablility of recombinant IL-8, HUVEC were only treated two times with IL-8 and combinations thereof. Thus each treated sample has an untreated control. Microparticle thrombin generation and quantification from IL-8 was also not possible. The cells were cultured at the University of the Free State, Department of Haematology and Cell Biology in a class 2 cell culture laboratory. The HUVEC line was selected because of its availability. It was subjected to a shear flow of 2,5 dyne/cm^2 on an orbital shaker for an hour (Pearce *et al.,* 1996). Microparticles were isolated using a microparticle filtration unit (Technoclone, Vienna, Austria, EU). The treated and untreated samples were stored in a temperature-controlled freezer at -80 °C and thawed in a water bath at 32 °C before the different assays were done. The assays were done at the University of the Free State, Specialised Haemostasis Laboratory of the Department of Haematology and Cell Biology, University of the Free State. Flow cytometric analyses were done on a Beckman Coulter Gallios flow cytometer using CD-31 FITC antibody (specific to endothelial cells and apoptosis) and a normal IgG 1 FITC conjugated isotype control as well as CD62E PE conjugated antibody (an activation marker) with its isotype control IgG2 κ (Biolegend). This was done in order to assess the amount of endothelial microparticles formed. Megamix beads (BioCytex, Marseille, France), as well as Flowcount beads (Beckman Coulter, Miami, Florida), were used for quantification of microparticles. The flow cytometry assay was done at the Department of Haematology of the University of Witwatersrand. The samples were transported on dry ice. A microparticle-dependent thrombin-generation assay (Technoclone, Vienna, Austria, EU) was done to determine the extent to which endothelial microparticles participate in thrombin formation. ELISA assays were done to quantify VWF and ADAMTS-13 levels in microparticles. These assays were done at the University of the Free State in the Specialised Haemostasis Laboratory. The Ethics Committee of the Faculty of Health Sciences approved this project (ETOVS number: 74/09).

4.2 Cell Cultures

4.2a Coating of Petri Dishes

Round Petri dishes of 21.1cm^2 (Cat no: 93060, Seperations, Techno Plastic Products, Trasadingen, Switzerland, EU) were coated with Fibronectin (10 µg/ml in Dulbecco's phosphate-buffered saline (PBS) without $CaCl_2$ and $MgCl_2$, Gibco, Grand Island, New York, USA) before culturing. Round Petri dishes were used, since shear had to be applied to the cells using a rotary shaker. Fibronectin is needed to facilitate attachment of HUVEC cells to the culture surface area. The flasks were coated using 500 µL of fibronectin per flask to cover the surface areas completely. The flasks were incubated overnight at 37 °C, after which the unbound fibrobectin was discarded and the Petri dishes rinsed with 1ml of PBS.

4.2b Cell culturing

Medium 200 with phenol red (Cascade Biologics Invitrogen Corporation, Portland, Oregon, USA) was first supplemented with low serum growth supplement (LSGS) using the LSGS kit (Gibco, Carlsbad, Califonia, USA). The supplemented medium with the kit contains 2% foetal bovine serum (FBS), 1 µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor, 10 µg/ml heparin and 0,2% gentamicin/amphotericin.

HUVEC were used for the study, since these are cells that express VWF and ADAMTS-13 and are mostly used in this type of studies (Combes *et al.,* 1999, [a]Jimenez *et al.,* 2003, [b]Jimenez *et al.,* 2003, Jy *et al.,* 2005, Turner *et al.,* 2006) The C-003-5C HUVEC cell line (Cascade Biologics Invitrogen Corporation, Portland, Oregon, USA) was thawed from storage in liquid nitrogen according to the instructions of the manufacturer. Seventy percent ethanol (99,9% ethanol, Saarchem, Krugersdorp, Gauteng, South-Africa, diluted in double distilled water) was used to sterilise gloves, all equipment-, reagent bottle and working surfaces. Cell culturing was done in a laminar flow culture cabinet under sterile conditions. Cells were counted on a haemocytometer (Marienfeld Fuchs-Rosenthal, Lauda-königshofen, Germany, EU) by using 10 µl cells from the initial vial with 3 µl 0,4% Trypan blue stain (Gibco, Grand island, New York, USA). The initial 1 ml vial contained approximately 5 x 10^5 cells. The cells were diluted in 40 ml supplemented medium 200 and then divided into 8 Petri dishes containing 5 ml each. These Petri dishes each contained approximately 2,5 x 10^3 viable cells/ml. After thoroughly mixing, only 3 ml of the 5 ml cells that were diluted in medium were transferred to the fibronectin-treated Petri dishes under sterile conditions.

Cells were evenly distributed by gentle shaking and were cultured from the recommended concentration at approximately ≥80% confluency. The endothelial cells were cultured at 37 °C with 5% CO_2 at 95% humidity in a NAPCO water-jacketed CO_2 incubator (Thermo Fisher Scientific, Shandong, China).

4.2c Subculturing

Cells were only subcultured after 6 days (Cascade Biologics Invitrogen Corporation, Portland, Oregon, USA) to obtain approximately 80% confluency. Thereafter they were subcultured for 4 passages before being used for experimental purposes. This is because HUVEC also have the potential to change phenotype and protein expression with prolonged cultivation time (Takahashi *et al.,* 1990, Peters *et al.,* 2009)

An inverted Nikon transmission light microscope was used to observe the cells. Cells were counted and set to 2.5 x 10^3 cells /mL. Subculturing was done with sterile recombinant bovine trypsin with 0,01% EDTA in PBS (pH 7,4) to detatch the cells. Trypsin/EDTA (Cascade Biologics, Portland, Oregon) was used to neutralise trypsin. All expended medium was removed and Trypsin/EDTA (4 ml per dish) was added to detach the cells. The dishes were swirled to ensure coverage of the entire surface area before removing 3 ml. The cells were left for approximately 2 minutes in the remaining 1 ml of Trypsin/EDTA to ensure detachment of all cells. The detached cells were then transferred to 15 ml conical tubes and the trypsin was neutralised with 6 ml of Trypsin neutraliser (0,5% newborn bovine serum in calcium and magnesium-free PBS, pH 7,4, Cascade Biologics, Portland, Oregon, USA). The suspension was centrifuged at 180 x g for 7 minutes, the supernatant discarded and the cell pellet resuspended in medium 200 to obtain approximately 2,5 x 10^3 cells/ml. The cells were only treated with thrombotic or coagulation activators and combinations of stimuli after the fourth passage to obtain enough flasks needed for the study.

4.3 Stimulation of endothelial cells

Flasks with 80% confluent endothelial cells were stimulated in round Petri dishes (21,1cm^2 growth surface) using the following concentration of inflammatory agents: recombinant human TNF-α [5,71 pM, (100 ng/ml), TNF-α, Gibco, Camarillo, California, USA], recombinant human IL-6 [4.69 pM, (100 ng/ml IL-6), Gibco, Camarillo, California, USA] and recombinant human IL-8 [11.24 pM, (100 ng/ml), NAP-1, Gibco, Camarillo, California, USA]. The following coagulation stimuli were used: Bovine Thrombin (2 U/ml or 10 nM, Instrumental Laboratory, Lexington, Massachusetts, USA) and recombinant TF (2 U/ml or

0.02 nM Recomboplastin HemosIL, 2G, (20ml), Instrumentation Laboratory, Bedford, Massachusetts, USA). All the final dilutions were made in supplemented Medium 200. TNF-α was first suspended in sterile double distilled water to a concentration of 100 µg/ml, IL-6 (5 µg) in 100 nM acetic acid (Saarchem) to 100 µg/ml and IL-8 (25 µg) was reconstituted in sterile double distilled water to a concentration of 100 µg/ml. Further dilutions were made in supplemented medium. Cell cultures were handled in duplicate, in which one of the dishes received 100 ng/ml inflammatory stimulus and the other was diluent without stimulus. All dilutions were made up to a final volume of 3 ml. Coagulation stimuli thrombin and TF were diluted in supplemented medium to 2 U/ml and inflammatory stimuli TNF-α, IL-6 and IL-8 in 100µg/ml. High concentrations of all agents were chosen to assess the effect of excessive inflammatory and coagulation stimuli on HUVEC microparticle formation. The following combinations were chosen to stimulate endothelial cells: the TF and TNF-α combination, as well as thrombin and TNF-α combination. The same concentrations of inflammatory agents and coagulation stimuli were used in the combinations as with the separate stimuli. The cytokine concentrations were based on the experiments done by Combes *et al.* (1999), Bernardo *et al.* (2004) and [b]Cao *et al.* (2008), where these cytokine concentrations were used in microparticle formation and subsequent VWF stimulation.

Due to the number of samples and the time it took to administer treatments, concentrations of control cells could not be adjusted just before treatment. To correct this, it is important to note that each treated flask had its own untreated control flask. Each treatment was administered and handled separately, each with its own control, before incubation. Dilutions were made up to a final volume of 3 ml per dish. The HUVEC that received these treatments as well as their controls were subjected to static conditions at 37 °C for 24 hours to stimulate microparticle generation. Each untreated control was subjected to the same conditions as its treated counterpart. After 24 hours a shear stress of approximately 2,5 dyne/cm^2 was applied for 60 minutes to all treated and untreated cells in order to induce the release of VWF multimers from microparticles, as observed under normal haemostatic flow conditions (Dong, 2005). A similar method was used by Pearce *et al.* (1996), in which shear stress was applied. Microparticles that formed during these treatments were then isolated using a Ceveron microparticle filtration unit. The filtration process uses a low protein-binding filter to isolate particles no larger than 2 µm in diameter. The microparticles were isolated from 200 µl of supernatant from the treated and non-treated endothelial cells as proposed by the Ceveron Microparticle filtration unit 500 protocol (Technoclone, Vienna, Austria, EU). The isolated microparticles were then diluted in 200 µL of PBS and used in subsequent experiments. Immediate analysis of samples was unfortunately not possible due to the large number of samples, the large number of assays to be done, as well as the

different locations for doing the tests. The microparticle samples were stored in a temperature-controlled freezer at -80 °C and thawed in a water bath at 32 °C before doing the different assays. The formed microparticles were quantified and its VWF and ADAMTS-13 levels were measured. The multimeric pattern of the microparticle-derived VWF was also determined.

4.4 Quantification of endothelial microparticles

Endothelial microparticles were quantified using a Beckman Coulter Gallios flow cytometer, Megamix beads (Biocytex, Marselle, France, EU) and Fluorocount fluorospheres (Beckman Coulter, Miami, Florida, USA). Megamix is a reagent that is used to standardize microparticle quantification by defining microparticle gate regions to exclude as much background and electronic noise events from captured data. This reagent contains a mixture of differently sized fluorescent beads of 0,5 µm, 0,9 µm and 3 µm and has been tested to quantify microparticles ranging in size from 0,1 µm to 1 µm (Biocytex, Marselle, France, EU). Microparticles were labeled with both monoclonal anti-CD31-FITC (a constitutively expressed apoptotic marker, Cascade Biologics Invitrogen Corporation, Portland, Oregon, USA) and monoclonal anti-CD62E-PE (an activation marker, BioLegend, Bar Hill, Cambridge, UK) antibodies. These antibodies were only used to stain particles with endothelial-specific apoptosis and/or activation markers for the purpose of counting the relative number of endothelial microparticles in the specific gated region. The percentage increase or decrease in the total number of microparticles from the untreated controls was also determined.

Fifty microlitre of microparticle sample was incubated with 10 µL of each anti-CD31-FITC and anti-CD62E-PE antibodies and 30 µL FlowCount fluorospheres (Beckman Coulter) in the dark for 30 minutes for the antibodies to bind to the microparticles. The Flowcount beads served as a calibrator for the number of microparticles in the region. The cytometry tube was filled up to 500 µL with PBS (Gibco Grand island, New York, USA). Megamix beads were used to set up the thresholds for acquisition to obtain the number of microparticles according to the size instruction of the Megamix beads. Megamix (500 µL) was added to a separate cytometry tube and vortexed. The threshold and acquisition settings were done according to the instructions of the manufacturer. The “megamix” gate was defined based on light scattering from 0,5 µm beads, which is defined by the Megamix as the lowest gate region for accurate measurement of microparticles. The upper gate was set to just above the 0,9 µm bead region, which yields microparticle results that are very

close to 1 µm and the 3 µm polysterene microspheres were used to define the region of interest.

A Fluorescent channel 1 (FL-1)/Side scatter dot plot was compiled. To define the gate regions, a rectangle was drawn around each bead region to indicate the regions for the particles of relative sizes that would fall in each region. Figure 4a illustrates the different bead regions for the 0,5 µm (A), 0,9 µM (B) and 3 µm (C) beads.

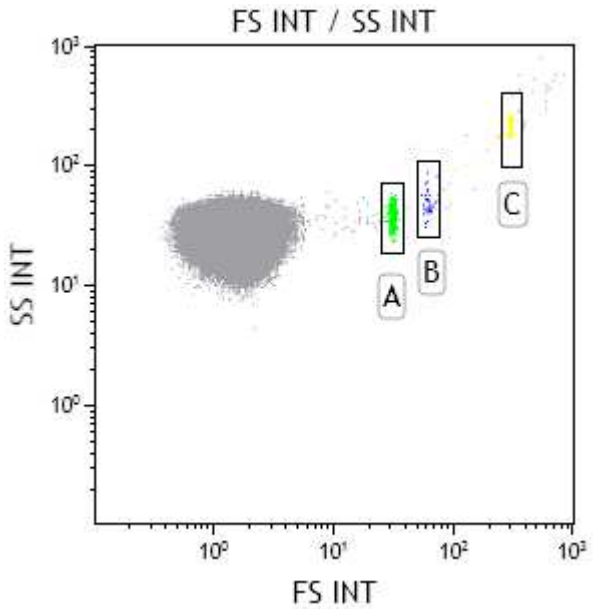

Figure 4a An FL-1 vs.SS dot plot to illustrate the different bead regions for 0,5 µm (A), 0,9 µm (B) and 3 µm (C) beads

The 3 µm beads were used to acquire a density dot plot and a histogram for the fluorescence channels, only to define the region of interest and to show approximate sizes of events outside of the gated microparticle bead region. Threshold 1 was set using a density dot plot and histogram of the 0,5 µm beads to exclude background and other contaminants. The 0,5 µm beads were used as the lower threshold of acquisition (Figure 4b). By setting the photomultiplier tube (PMT) voltage to approximately 50%, the lowest reliable and reproducible measurement of microparticles was done (Robert *et al.,* 2008).

The lower gate was set in the middle of the 0,5 µm peak, and the upper gate was set just above the size distribution of the 0,9 µm microspheres. The 0,9 µm beads were used as the upper threshold for microparticle quantification and the threshold was set to acquire to the end of the 0,9 µm bead threshold. This provides a threshold that is very close to 1 µm and

was used to measure microparticles between approximately 0,5 µm and 1 µm in diameter (Robert *et al.,* 2008).

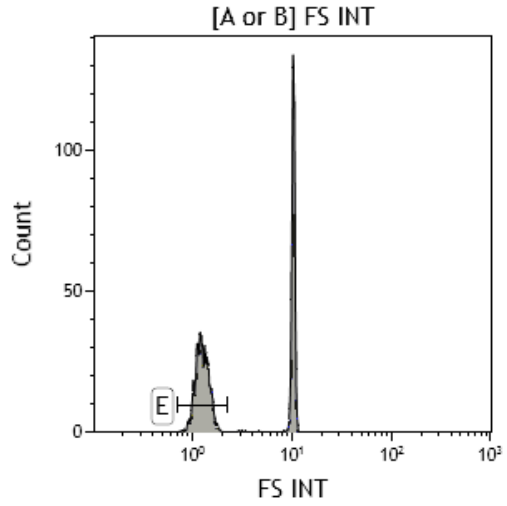

Figure 4b An illustration of the count histogram for 0,5 µm (E) beads

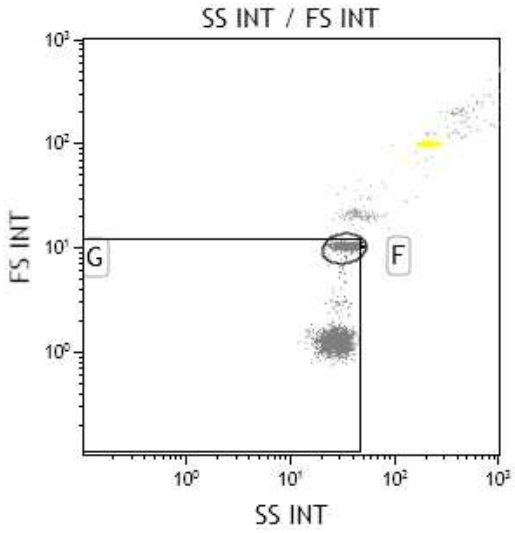

Figure 4c An illustration of the dot plot for the 0,9 µm (F) beads and the region of interest (G) for gating and microparticle quantification

Finally an acquisition dot plot for log forward scatter vs. log side scatter was set up using the 0,9 µm (F) beads as threshold to gate the microparticles of interest (Figure 4c). The samples were run on a low flow rate and set to acquire at least 10 000 events. The number of micropartices was determined by means of a simple calculation.

$$[\text{microparticle concentration}] = \frac{(\text{microparticle counts} \times \text{standard beads / microlitre})}{\text{standard beads counted}}$$

The relative percentage increase or decrease in microparticle numbers of the treated samples compared to the control samples was determined.

4.5 Von Willebrand factor antigen levels in microparticles

An enzyme-linked immune-adsorbent assay (ELISA) was used to determine the VWF concentration in the microparticle containing perfusates (Meiring *et al*., 2005). This assay was done by precoating an 96-well ELISA plate (Nunc Maxisorp, Rochester, New York, USA) with a polyclonal rabbit anti-human VWF antibody (DAKO, Glostrup, Denmark, EU). This antibody was diluted in PBS (11,68g NaCl; 9,44 g Na_2HPO_4; 5,28 g $NaH_2PO_4 \cdot 2H_2O$ in 2 l distilled water with pH 7,2) at a dilution of 1:6 000. One hundred microlitres was added per well and incubated at 4 °C for 24 hours in a humid environment. The plate was then washed three times by submerging the plate in a wash buffer containing PBS and 0,1% tween-20 solution (Merck, Schardt, Germany, EU) and then discarding the solution to wash away all unbound antibodies. After washing, the plate was blocked with 4% skimmed milk (Difco laboratories, Detroit, Michigan, USA) in PBS solution (200 µl/well) for 2 hours at room temperature to prevent unspecific binding.

A serial dilution of the sixth FVIII/VWF standard of the World Health Organisation (National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, UK) was made. One millilitre of a 1:10 dilution in 2% skimmed milk in PBS was added to the first tube. This tube was set as the 100% VWF standard. A dilution series was made for 50%, 25%, 12,5%, 6,25%, 3,125% and 1,5625% VWF respectively. A blank of only 2% skimmed milk in PBS was also used. A hundred microlitres of the standards were added in duplicate to a 96-well plate. Hundred microlitres of each of the treated and untreated microparticle samples were added to the wells in duplicate and incubated at 37 °C for 2 hours for optimal binding of the VWF to the coated antibody. Hemosil-calibrated plasma was used as positive control (Beckman Coulter, Miami, Florida, USA). The plate was washed 5 times to remove all unbound proteins. A second horseradish peroxidase-conjugated rabbit polycolonal anti-human VWF antibody (Dako, Glostrup, Denmark, EU) was diluted 1:8 000 in 2% skimmed milk/PBS solution. One hundred microlitres of this was added to each well and incubated at room temperature for an hour. This was followed by another 5 washing steps. Ortho-phenylenediamine (OPD, 0,1M Na_2HPO_4, 0,05M citric acid, 0,0005M OPD, and 0,0001M H_2O_2 made up to a total volume of 20 ml) was added to the wells (100 µl per well) as a substrate for HRP. The reaction was stopped after 30 minutes using 30 µl of 4M H_2SO_4 per well. The colour intensity of the wells was measured at 490 nm minus 630 nm absorbance with a Biokinetics BioTek Synergy HT reader (Analytical & Diagnostic Products, Winooski,

Vermont, USA). A standard cuve was constructed and the von Willebrand concentrations in the perfusate were calculated from the standard curve.

4.6 ADAMTS-13 antigen levels in microparticles

ADAMTS-13 levels were also measured with an ELISA. Because of the extremely low levels of ADAMTS-13 in the microparticles, 100 µl of the perfusates were all concentrated in a Savant SpeedVac SC110 (Instruments incorporated, Farmingdale, New York, USA) for 90 minutes. Due to the small volumes after concentration, absolute quantification of ADAMTS-13:Ag levels was not possible. Variances occurred in concentrations between samples and only reflect a relative measurement as measured from the untreated controls. A monoclonal mouse IgG anti-ADAMTS-13 antibody (1:1 000 in PBS, 100 µl per well, R&D Systems) was used to coat an ELISA plate overnight at 4 °C. The plate was then blocked at room temperature for 2 hours with 4% of bovine serum albumin (BSA in PBS, 200 µl per well). After blocking, the plate was washed 4 times with PBS/0,1%Tween-20. The same procedure was followed with standards used for VWF levels to prepare the ADAMTS-13 standard curve. It was assumed that ADAMTS-13 was 100% in normal pooled plasma, since standards for ADAMTS-13 was not readily available at the time. The microparticle perfusates were added to the wells. The perfusates were left to bind to with the antibody for 2 hours at 37 °C. The plate was washed once again. A biotynilated polyclonal rabbit IgG anti-ADAMTS-13 antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, California, USA) was added after washing and left for 1 hour to bind. Horseradish peroxidase- conjugated Streptavidin (1:2 000, Biolegend, San Diego, USA) was added and also incubated for an hour. Once again, OPD was used as a substrate for the streptavidin-conjugated HRP. Samples were immediately measured in the Biokinetics Biotek Synergy HT reader. ADAMTS-13 levels in the samples were calculated by using the standard curve. The results were expressed as the percentage ADAMTS-13.

4.7 Microparticle-dependent thrombin generation assay

Microparticle-dependent thrombin generation was done using the Technothrombin Microparticle kit from Technoclone (Vienna, Austria, EU). First, a calibrator curve was constructed using the standards supplied with the kit. Thrombin Generation Assay (TGA) buffer (Hepes-NaCl buffer containing 0,5% bovine serum albumin) provided in the kit was reconstituted in 3 ml of distilled water. Standards 1-4 were reconstituted TGA buffer at concentrations of 1:2, 1:4, 1:20 and 1:200 respectively. This was added in duplicate (40 µl) to the wells of a black ELISA plate. The TGA fluorogenic substrate (1 mM Z-G-G-R-AMC,

15 mM $CaCl_2$) included in the kit was reconstituted in 1,5 ml of distilled water and 50 µl substrate was added to these wells to start the kinetic reactions. The calibration reactions were measured with a BioTek Synergy HT reader at a temperature of 37 °C for 10 minutes at 30 second intervals at wavelengths of 360 nm excitation and 460 nm emission.

The perfusates containing microparticles (not filtered) and those without microparticles (filtered) were used in this assay. Forty microlitre of each sample was added to a 96-well ELISA plate according to a template setup on the Technothrombin software provided (Technoclone, Vienna, Austria, EU). Ten microlitres of TGA reagent C Low (low concentration of phospholipid micelles containing recombinant human TF, Technoclone, Viennea, Austria, EU) were added as well as 50 µl of the TGA substrate. Fifty microlitres of normal human plasma were added to all wells in order to supply the needed clotting factors for thrombin generation. The reaction started immediately after the addition of the substrate and fluorescence was measured using a wavelength of 360 nm excitation and 460 nm emission. The kinetic study was done at 37 °C for 120 minute measurements recorded at 1 minute intervals on a BioTek Synegy HT reader. Microparticle-mediated thrombin generation was calculated using the provided software. The amount of thrombin generated in the microparticle-rich perfusate was subtracted from the thrombin generated by the microparticle-poor filtrate to give the amount of thrombin generated by the microparticles.

4.8 Von Willebrand factor multimer analysis

VWF multimers were only visualised to confirm the presence of VWF in the endothelial-derived microparticles. The same concentrated samples that were used for the ADAMTS-13 assay were used for the multimeric analysis. Electrophoresis buffer (pH7.8,40 mMTris-acetate, 0.1% Sodium dodecyl sulphate (SDS), 1mM Ethylenediamine-tetra-acetic acid (EDTA) in 1L distilled water,from Saarchem) was prepared and stored at -4°C. A 0.65% agarose gel was prepared in electrophoresis buffer by melting the agarose in a microwave oven and swirling occasionally to dissolve all particles. This agarose solution was degassed and cooled to approximately 50°C and then poured into an electrophoresis apparatus with a 20 tooth comb. The gel was allowed to set at 4°C for 30 minutes to solidify. Prepared sample buffer (H_3PO_4, 0,01M Na_2HPO_4, 37 mM C_2H_4INO, 1% SDS topped up to 100 ml with distilled water, pH 7) was used to dilute treated and untreated samples at a ratio of 9:1 (sample: sample buffer). The total sample volume was 90 µl. Samples were then incubated in a waterbath at 37 °C for an hour, after which it was stained with 10 µl of 1% bromphenol blue (Sigma-Aldrich, St. Louis, Missouri, USA) and centrifuged at 14 000 rpm for 1 minute. The 20-tooth comb was removed and the gel was covered with electrophoresis buffer at 4

°C. Twelve microlitre of sample was then loaded into each well. Electrophoresis was done at 30 mAmps for 30 minutes, after which it was set to 50 mAmps for approximately 5 hours (Krizek & Rick, 2000).

A Western Blot was done as follows: Transfer buffer, (2,5 mM tris, 19,2 mM H_2NCH_2COOH,20% CH_3OH, 0.01% SDS in 2 l of water, pH 8,8) was prepared and a 0,45 µm polyvinylidene difluoride (PVDF, Pierce, Rockford, Illinois, USA) membrane was cut to be just smaller than the gel was used along with filter paper (Whatman 1 mm and 3 mm filter papers from Bio-rad, Hercules, Carlifornia, USA) to transfer the proteins from the gel to the membrane. After running for approximately 5 hours, the gel was soaked in transfer buffer for a further 30 minutes and rinsed while the PVDF membrane was placed in methanol for 2 minutes and then transferred into transfer buffer.

A Western blot sandwich was prepared by wetting the filter paper in transfer buffer and packing thick filter paper on the outside, thinner filter paper on the inside and the membrane and gel in the middle. The western blot apparatus was assembled in the -4 °C walk-in fridge and run overnight at 70 mAmps.

A blocking solution was prepared using 100 ml tris-buffered saline/tween-20 solution [0,1% tween-20 in TBS (0,05 M Tris, 0,1 M NaCl, filled up to 2 l with water, pH 7,4). Five percent of dehydrated skim milk (Difco laboratories, Detroit, Michigan, USA) was used as a blocking reagent. The membrane was transferred to the blocking solution and incubated for 75 minutes on a slow speed orbital shaker at room temperature and then washed three times with 50 ml TBS/tween for 1 minute. Another three washing steps with T-TBS of 7 minutes each followed.

The membrane was incubated for 75 minutes at room temperature in rabbit anti-human VWF/HRP antibody while shaking it gently (DAKO, Glostrup, Denmark, EU). A dilution of 1:33 in 100 ml TBS/tween was used. Three washing steps with 50 ml TBS/tween for 1 minute and 100 ml TBS/tween for 7 minutes respectively followed. Ten millilitre of each of the two luminescence ECL Western Blotting reagents 1 and 2 (Amersham Biosciences, Piscataway, New Jersey, USA) were combined and poured onto the membrane on a matt surface area and left for 1 minute. The membrane was transferred to a dark room and placed protein-side up on a Curix blue HC-S X-ray film (Afga, Thousand oaks, California, USA) in a cassette in the dark for 30 seconds. The X-ray was developed in G354 developing reagent (Afga, Kempton Park, Gauteng, RSA) until bands could clearly be observed and then fixed in G153 fixing reagent (Afga, Kempton Park, Gauteng, RSA).

Photos were taken of the X-ray films with a Sony Digital Mavica camera (Sony Corporation, Tokyo, Japan).

4.9 Statistical Analysis

Each treatment with its own culture flask and control was repeated three times. Means and standard deviations were calculated in Excel, using descriptive statistics for data analysis. The increase or decrease in VWF-, ADAMTS-13:Ag levels and microparticle numbers was calculated as the percentage change from the untreated sample. The paired t-test was used to determine whether the difference between untreated and treated sample values were statistically significant. A difference was considered statistically significant when $p < 0,05$.

5 RESULTS

5.1 Quantification of microparticles

The relative mean percentage change with standard deviations in the number of microparticles from untreated to treated HUVEC are illustrated in Figure 5, the significance as indicated by the p-values are given in Table 2 and addendum.

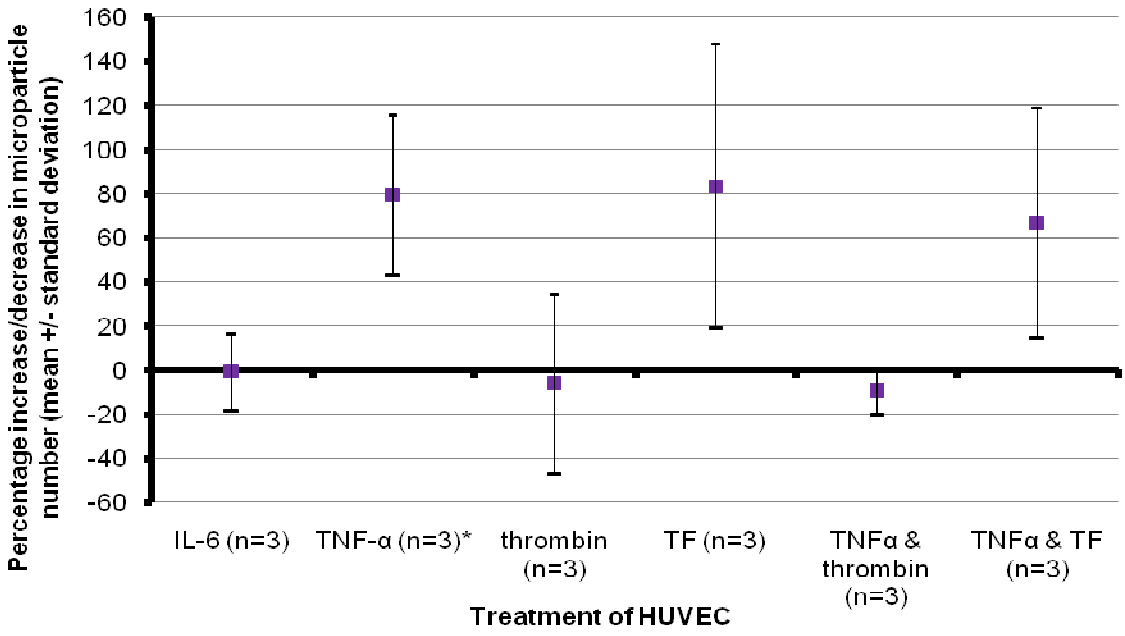

Figure 5 The percantage increase/decrease in relative microparticle numbers after different treatments of HUVEC (mean +/- standard deviation)

Table 2 Statistical significance of the mean relative increase/decrease (%) in microparticle numbers after the different treatments

	IL-6	TNF-α	Thrombin	TF	TNF-α & thrombin	TNF-α & TF
Mean	-0,9	79,4*	-6,2	83,6	-9,5	66,5
Probability value of the t-test	0,85	0,04*	0,62	0,07	0,26	0,10

Significant P-value indicated with *

IL-6, thrombin and the combination of TNF-α and thrombin decreased the microparticle numbers by 0,9%, 6% and 10% respectively. TNF-α, TF and the combination of these two

agents increased microparticle numbers by 79%, 84% and 67%. The only significant increase was found with TNF-α with the p-value of 0,04.

5.2 Endothelial microparticle Von Willebrand factor antigen levels

The percentage increase or decrease in microparticle VWF:Ag levels after the different treatments is shown in Figure 6 and the statistical significance is shown in Table 3. Refer to the addendum section for raw data and outliers.

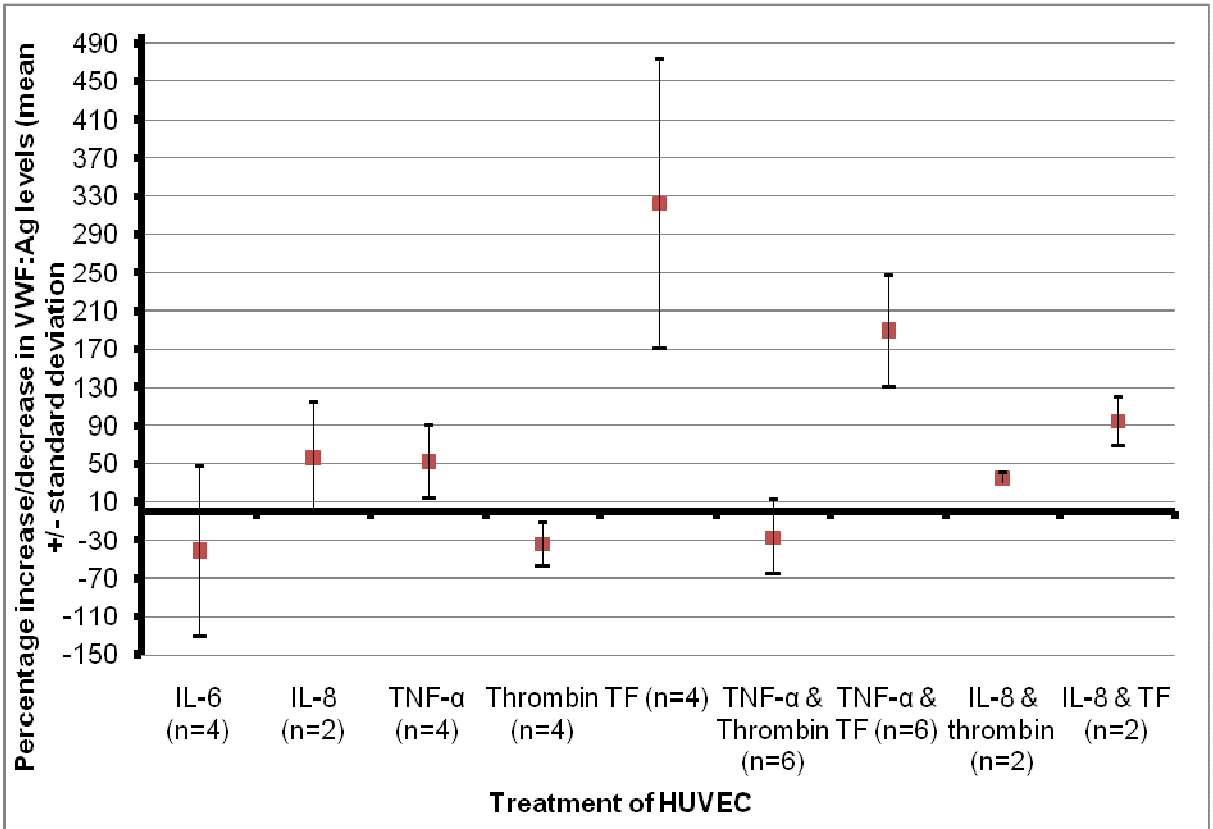

Figure 6 Percentage increase/decrease in microparticle VWF:Ag levels after different treatments of HUVEC (Mean +/- standard deviation)

Table 3 Statistical significance of the mean increase/decrease in microparticle Von Willebrand factor antigen levels after the different treatments

	IL-6	IL-8	TNF-α	Thrombin	TF	TNF-α & Thrombin	TNF-α & TF	IL-8 & Thrombin	IL-8 & TF
Mean	-42,1	56,6	51,8	-34,7	312,9*	-27,2	188,4*	35,2	94,1
Probability value of the t-test	0,18	0,35	0,05	0,22	0,02*	0,08	0,002*	0,05	0,07

significant P-value indicated with *

The cytokines, IL-8 and TNF-α increased the microparticle VWF:Ag levels by 57% and 52% (p=0,05) respectively. A decrease of 42% in microparticle VWF:Ag was observed with the IL-6 treatment. TF increased the microparticle VWF:Ag levels significantly by 313%. This was also the highest microparticle VWF:Ag increase observed among all treatments. Interestingly again, HUVEC treated with thrombin caused a decrease of 35% in VWF:Ag levels of microparticles. The TF and TNF-α combination increased microparticle VWF:Ag levels by a median of 188% (p=0,002), the second highest increase. The third highest increase of microparticle VWF:Ag levels was the IL-8 and TF combination treatment with a median of 94%. Thrombin in combination with IL-8 increased the microparticle VWF:Ag levels by a moderate 35% (p=0,05) and the combination of thrombin and TNF-α decreased microparticle VWF:Ag levels by 27%.

5.3 Endothelial microparticle ADAMTS-13 antigen levels

The ADAMTS-13 antigen (ADAMTS-13:Ag) levels in microparticles were extremely low (<40%). Figure 7 and Table 4 illustrate the mean percentage change in ADAMTS-13 levels detected in isolated microparticles and the statistical significance. IL-8 increased the microparticle ADAMTS-13:Ag levels by 16% and was not statistically significant. IL-6 did not have any significant effect on ADAMTS-13 levels and was increased by 7%. The TNF-α treatment decreased the microparticle ADAMTS-13 levels by 1%. This was also not statistically significant. TF also had no significant effect on the microparticle ADAMTS-13:Ag levels and decreased it by 3%. Thrombin treatment increased the microparticle ADAMTS-13 levels by 18%, which was not statistically significant. The combination of TNF-α and TF resulted in a statistically significant decrease (p=0,046) in microparticle ADAMTS-13:Ag levels with a mean value of 19%. The TNF-α and thrombin combination induced an increase of 12% in microparticle ADAMTS-13:Ag levels, which was again not statistically significant.

An increase of 34% in microparticle ADAMTS-13:Ag levels was observed with the IL-8 and thrombin combination, which was also not statistically significant. The mean percentage change in microparticle ADAMTS-13:Ag levels after the different treatments is shown in Figure 7 and its statistical significance in Table 4. Refer to the addendum for raw data and outliers.

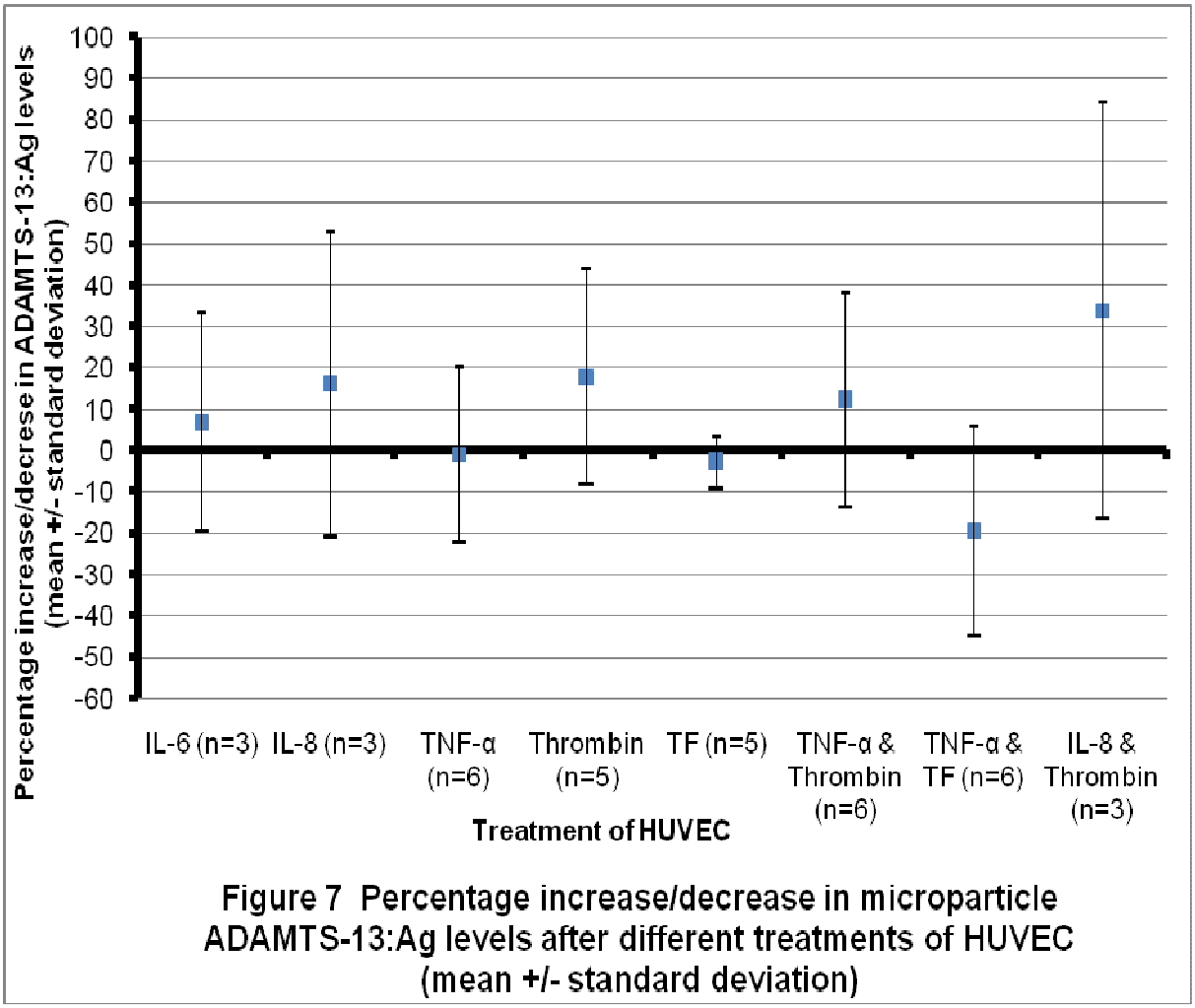

Figure 7 Percentage increase/decrease in microparticle ADAMTS-13:Ag levels after different treatments of HUVEC (mean +/- standard deviation)

Table 4 Statistical significance of the mean increase/decrease in microparticle ADAMTS-13 antigen levels after the different treatments

Percentage increase /decrease	IL-6	IL-8	TNF-α	Thrombin	TF	TNF-α & Thrombin	TNF-α & TF	IL-8 & thrombin
Mean	6,9	16	-1,1	17,8	-2,8*	12,2*	-19,4*	33,9
Probability value of the t-test	0,74	0,87	0,72	0,27	0,37*	0,39*	0,046*	0,18

significant P-value indicated with *

5.4 Thrombin generation of endothelial microparticles

The relative percentage contribution of the microparticles to thrombin generation is also shown in Table 5. Table 5 summarises the peak thrombin concentration as well as the area under the curve (AUC) or thrombin potential of the microparticles when the HUVEC were stimulated with inflammatory and/or coagulation stimuli. Figures 8a to 8d are examples of the HUVEC microparticle thrombin generation curves of microparticle-containing and microparticle-free plasma after inflammatory, thrombotic and combined stimuli.

Microparticles formed by TNF-α contributed to 78% in the total thrombin generation in the system. TF-induced endothelial microparticles contributed to 29% of the total thrombin generation. The combination of TNF-α and thrombin contributed to 21% towards microparticle formation induced by thrombin. The lowest contribution towards microparticle thrombin generation was TF in combination with TNF-α with a value of 12%. IL-6 and thrombin did not stimulate any microparticle-dependent thrombin generation.

Table 5 Peak thrombin concentrations and AUC of thrombin generated by endothelial microparticles after treatment with inflammatory and coagulation stimuli

Treatment of HUVEC	Peak thrombin without endothelial microparti-cles	Peak thrombin with endothelial microparti-cles	Thrombin generation without endothelial microparti-cles (AUC)	Thrombin generation with endothelial microparticles (AUC)	Percentage difference in microparticle-dependent thrombin generation (AUC in %)
TNF-α	18	347	490	2179	78
TNF-α & thrombin	191	339	1984	2514	21
TF	176	363	1717	2429	29
TF & TNF-α	125	317	1484	1684	12
IL-6	0	11	0	0	0
Thrombin	0	0	0	0	0

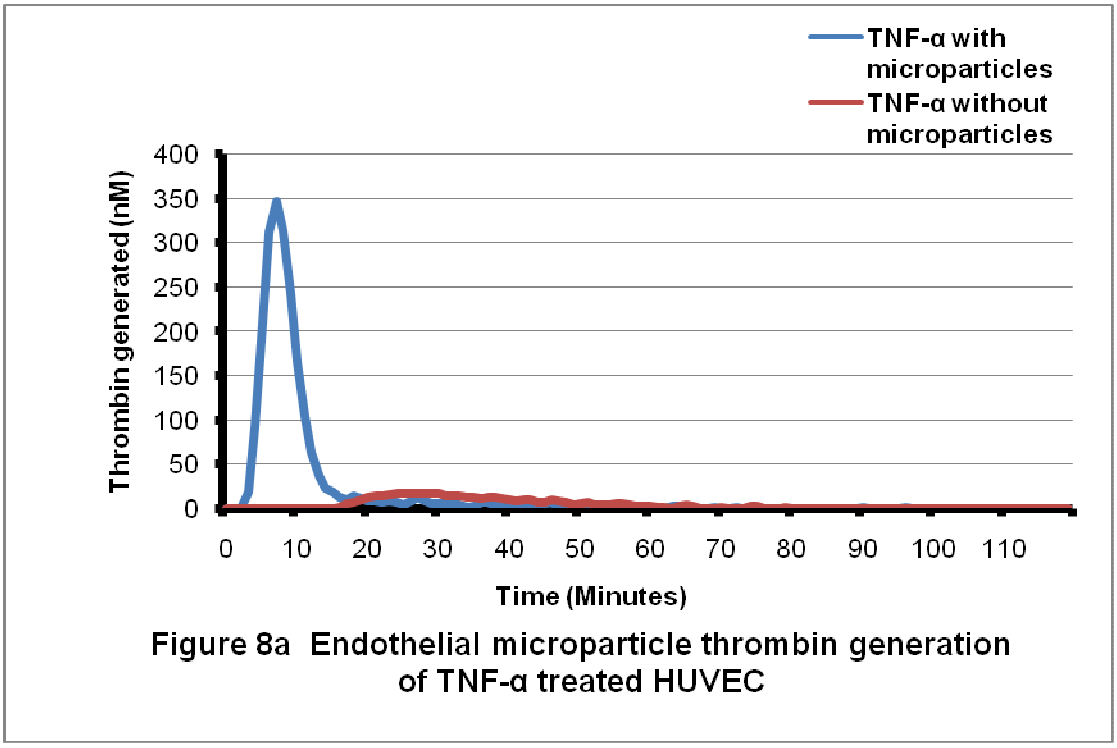

Figure 8a Endothelial microparticle thrombin generation of TNF-α treated HUVEC

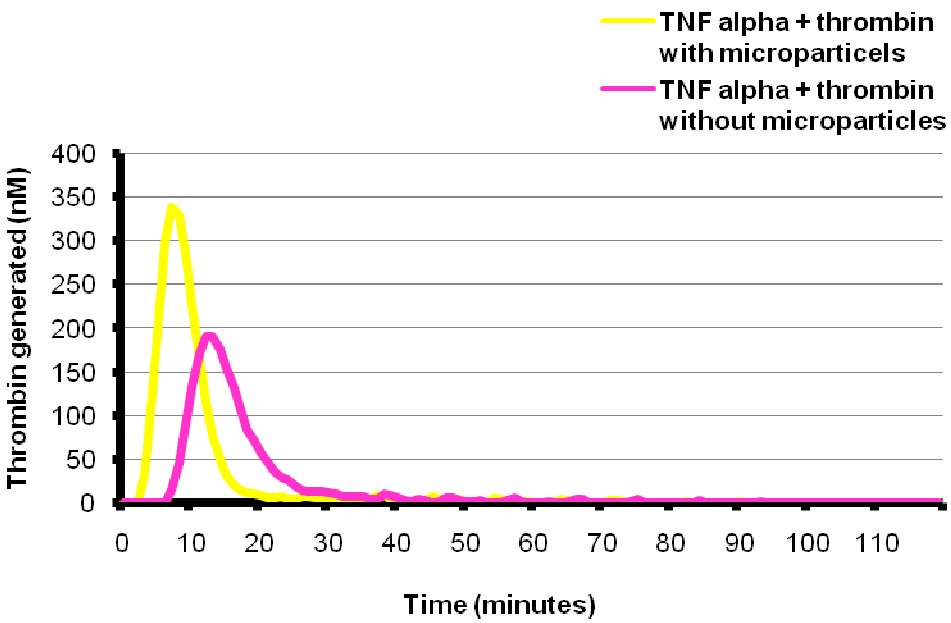

Fig 8b Endothelial microparticle thrombin generation of thrombin and TNF-α treated HUVEC

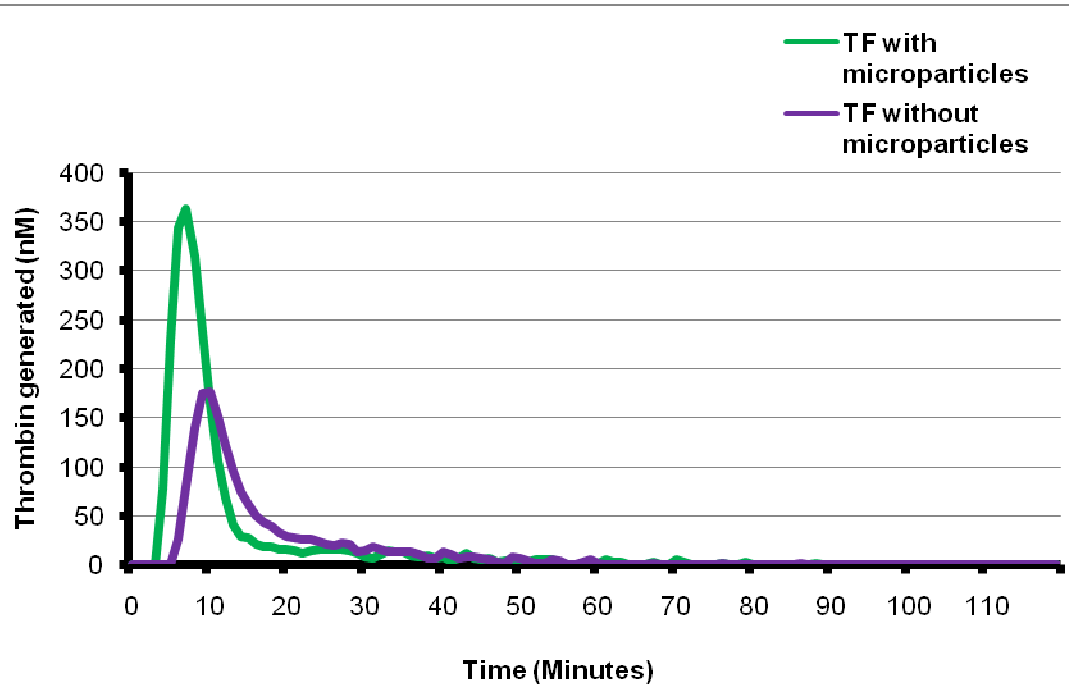

Fig 8c Endothelial microparticle thrombin generation of TF treated HUVEC

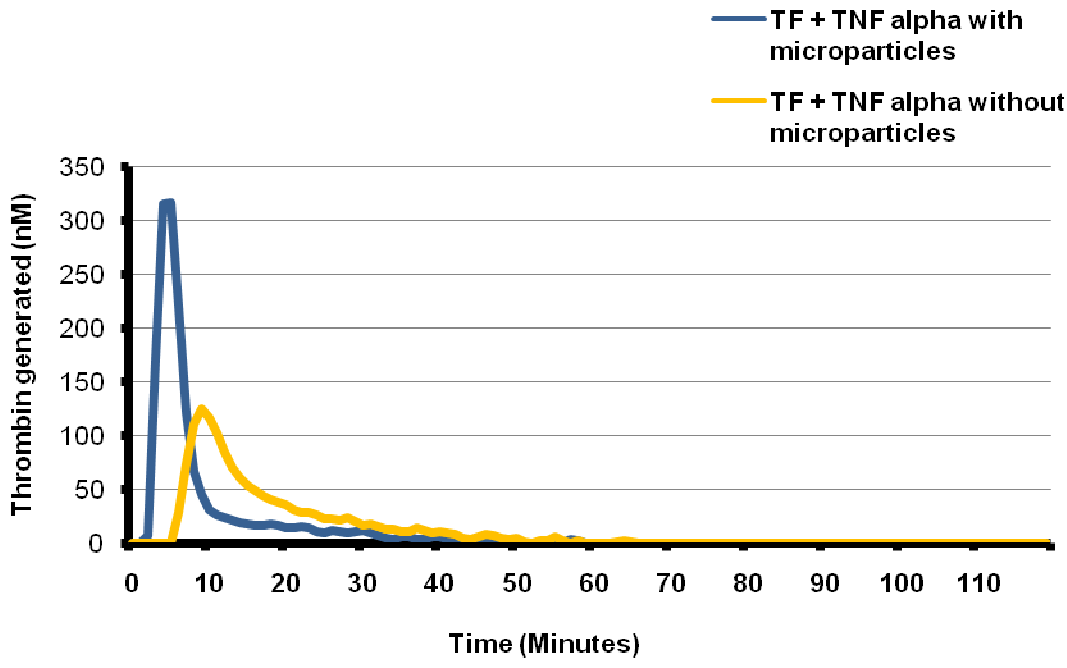

Fig 8d Endothelial microparticle thrombin generation of TNF-α and TF treated HUVEC

5.5 Multimeric analysis of endothelial microparticles

To confirm the presence of VWF in the microparticles and, where possible, assess the multimeric pattern, endothelial microparticle multimeric analysis was done. The samples were concentrated to 4 times the original volume due to low VWF:Ag levels in the samples. The VWF multimeric pattern of all individually stimulated and unstimulated samples are illustrated in Figures 9a and 9b. TNF-α and the combination of TNF-α and TF increased the density of the multimeric bands. This correlates with the VWF:Ag levels in the microparticles of these perfusates.

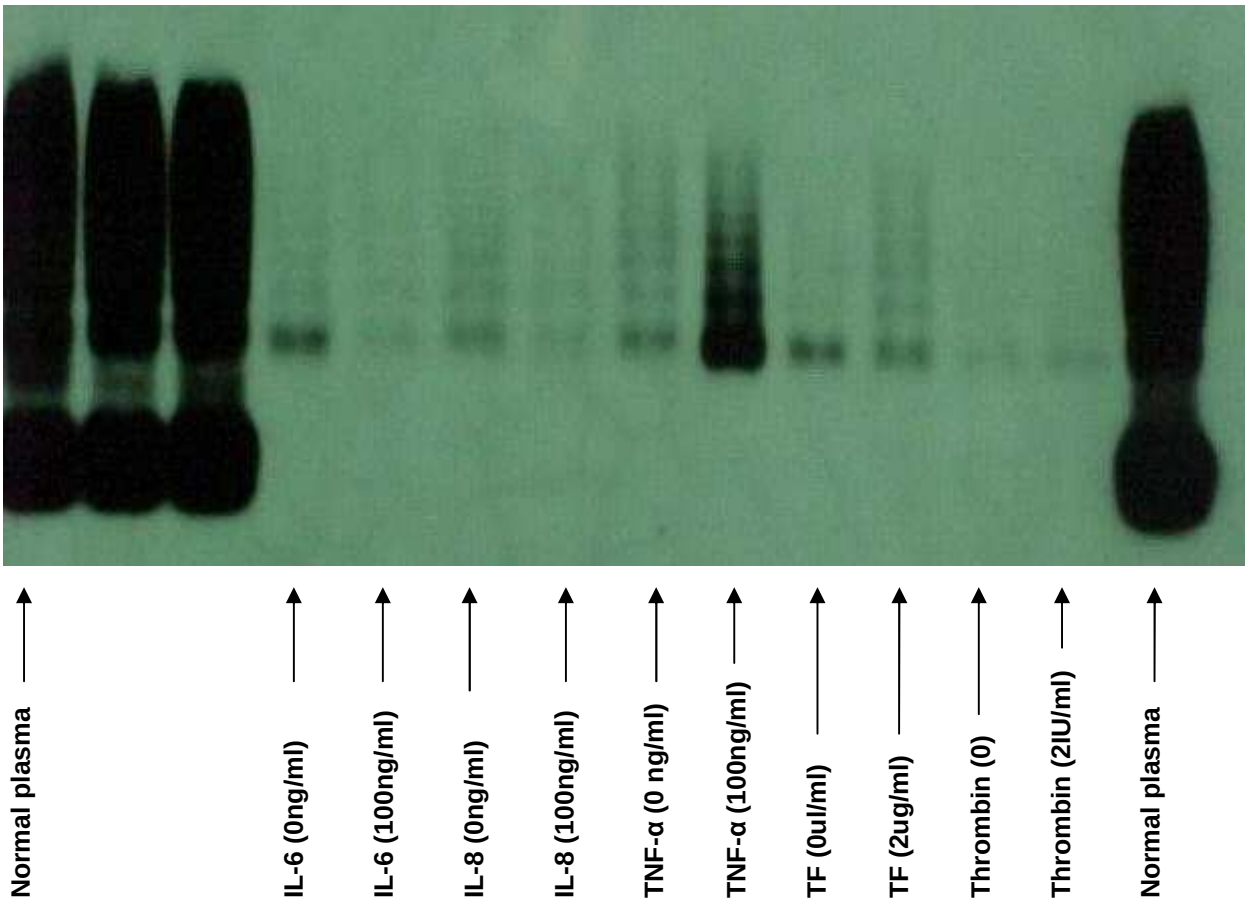

Figure 9a Endothelial microparticle VWF multimeric analysis after different treatments of HUVEC

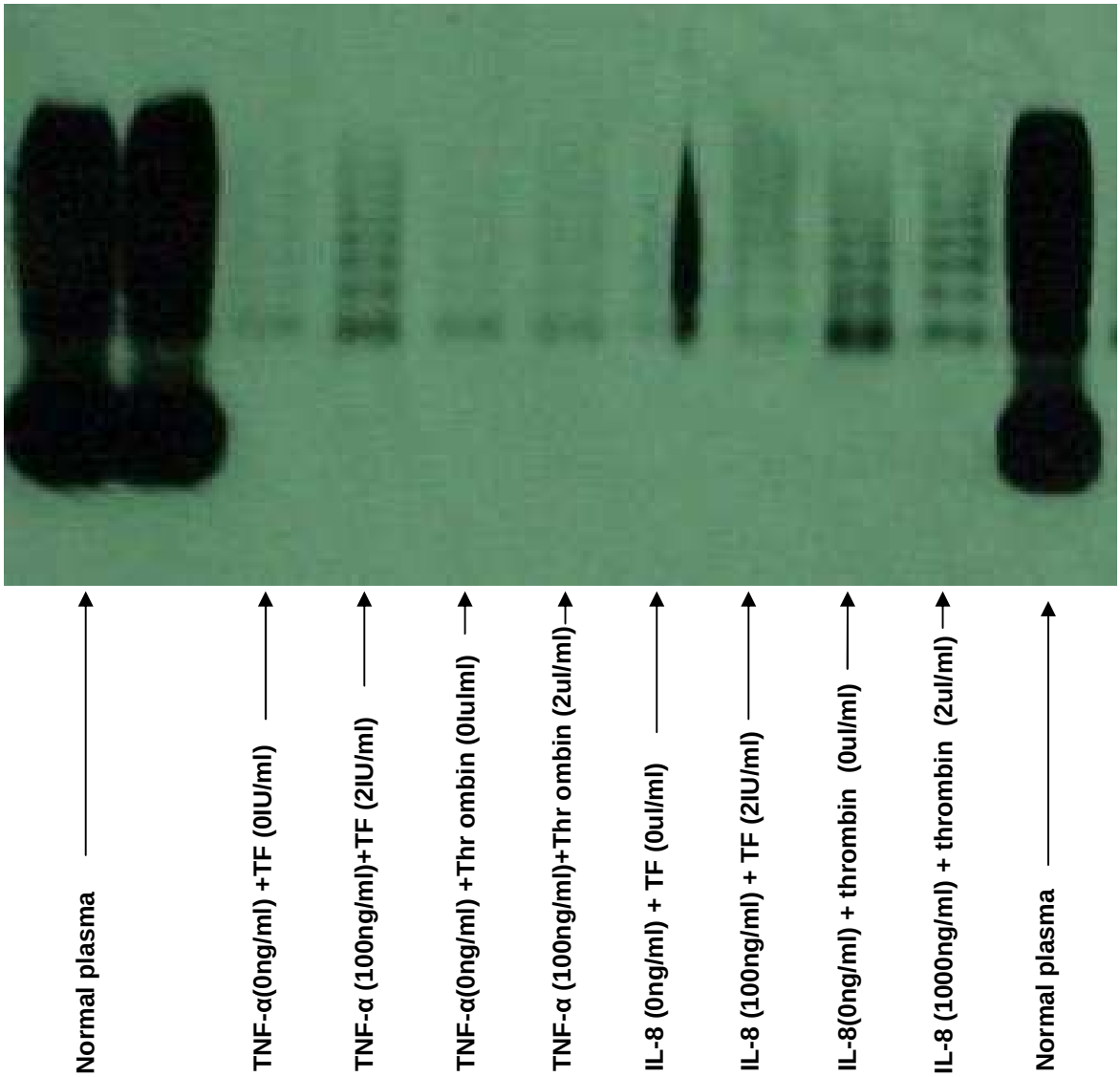

Figure 9b Endothelial microparticle VWF multimeric analysis after different combined treatments

6 DISCUSSION

The role of microparticles in inflammatory and/or thrombotic disorders is not fully understood yet. Endothelial dysfunction is proposed as one of the main triggers of endothelial microparticle formation (Yong *et al.,* 2013). In order to gain a better understanding of the role of endothelial microparticles in inflammation and thrombosis, a study was planned to investigate the effect of the cytokines IL-6, IL-8 and TNF-α and coagulation stimuli, TF and thrombin as well as the combinations thereof on endothelial microparticle formation and on the secretion of VWF and its regulating protease, ADAMTS-13, by endothelial microparticles. The results are discussed under the relevant headings.

6.1 Human umbilical vein endothelial cells

HUVEC are probably the most commonly used cell type to study endothelial function. One of the reasons for this is availability. Another reason is the fact that the majority of available data is on HUVEC, which makes it more easily comparable (Miracle, 2012). This cell line would also be less likely to produce and/or secrete high levels of TF and VWF unless stimulated (McGuigan & Sefton, 2007). HUVEC are capable of expressing VWF and also carry ADAMTS-13, which makes it a viable option for VWF research (Combes et al., 1999, Bernardo et al., 2004, Turner et al., 2006, Liu et al., 2011).

Different endothelial cell lines are similar in many ways. For example, the inflammatory pathway and secretions that are stimulated by IL-6 and its soluble receptor are similar among microvascular endothelial cells and HUVEC (Chatterjee *et al.,* 2009). HUVEC, pulmonary microvascular endothelium and human aortic endothelium also induced similar apoptotic responses to TNF stimulation (Stefanescu *et al*., 2008, Allen *et al.,* 2010). Many endothelial cell types also express similar markers like PECAM-1 and endoglin (Norgall *et al.*, 2007). These markers may predict the severity of disease in patients. HUVEC have also been described as a viable possibility as a blood vessel mimic for physiologically large vessels (Miracle, 2012).

In other ways, endothelial cells are totally different from each other. For example, lung, heart, intestine and skin microvascular endothelium can secrete FVIII upon phorbol myristate acetate treatment, while HUVEC seem to lack the potential to secrete FVIII upon the same stimuli (Shahani *et al.*, 2010). In human aortic endothelial cells, cyclic strain had the

opposite effect of what it had in HUVEC. Human aortic endothelial cells in static conditions induced increased secretion of inflammatory agents like ICAM-1 and VCAM-1 (Sung *et al.*, 2007). Even though endothelial cell phenotypes differ, endothelial cells from different origins produced microparticles that were able to carry VWF that could induce platelet aggregation.

HUVEC were selected for this experiment. It is known that microparticles derived from HUVEC that are able to carry VWF can also restore ristocetin-induced aggregation of platelets and induce very stable aggregates in TTP plasma. Involvement of HUVEC-derived microparticles is important in haemostasis and may be associated with increased aggregation in disorders like TTP and other disorders in which VWF and ADAMTS-13 plays an essential role (Jy *et al.,* 2005, [a]Jimenez *et al.,* 2003). Activated HUVEC are also capable of forming TF-associated endothelial microparticles that are able to induce thrombin generation (Hussein *et al.,* 2008). This study is possibly the first to show how VWF:Ag levels (Figure 6), ADAMTS-13:Ag levels (Figure 7) and thrombin (Figures 8a-d) are influenced *in vitro* by HUVEC-derived microparticles upon stimulation by different cytokine, thrombotic and cytokine-thrombotic combination treatments.

In this study the confluency of the HUVEC in the flasks was estimated to be approximately 80%. Confluency of cells also plays a part in the ADAMTS-13 levels and is shown to be 3 to 4 times higher in subconfluent cells than the constant 2-fold increase observed in confluent cells (Kling *et al.,* 2009). The fourth passage was used for the experiment, since this provided optimum amounts of subconfluent cells and the state of activation of HUVEC as required for the study (Butler *et al.,* 2005). Furthermore, prolonged culturing with trypsin can damage endothelial vessel walls and induce changes in the phenotype of the cells (Luu *et al.,* 2010, Djassemi, 2012).

6.2 Microparticle isolation

The endothelial microparticles were isolated using the Ceveron MFU microparticle filtration unit. This instrument is unique in the fact that it can isolate similarly sized microparticles in the vicinity of 2 µm, reducing the amount of smaller cell debris or larger cells in the sample. It has been proposed in literature that this may be useful for standardisation of microparticle isolation (Lawry *et al.,* 2009). Not only the microparticle numbers, but also the size can influence, for example, thrombin generation (Lawry *et al.,* 2008, Van der Pol *et al.,* 2010). The impact of inflammatory and thrombotic stimuli on the microparticle numbers and the haemostatic characteristics of the microparticles will be discussed.

6.3 Inflammatory stimuli

6.3a Interleukin-6

In this study it is evident from figure 5 that IL-6 had no effect on the relative microparticle numbers. A small decrease in the median endothelial microparticle VWF:Ag levels was found (Figure 5, Table 2). HUVEC do not contain receptors for IL-6; so no marked changes in microparticle numbers or endothelial microparticle VWF:Ag levels were expected (Romano *et al.,* 1997).

Another reason why IL-6 did not influence microparticle numbers is because it may be associated more with apoptotic conditions than inflammation (Borges *et al.,* 2009). In healthy patients, PECAM-1 expression is more increased in endothelial microparticles than the inflammatory marker, E-selectin ([b]Chirinos *et al.,* 2005). In an *in vivo* study of patients with multiple organ dysfunction and sepsis, the number of endothelial microparticles was the same as the number in healthy individuals, while the IL-6 levels were increased (Joop *et al.,* 2001). The results of the *in vivo* study correlate with my results (Figure 5, Table 2). In this study, IL-6 did not induce measurable thrombin generation (Table 5) in endothelial microparticles. This may be due to the lack of microparticles that formed upon IL-6 stimulation of HUVEC.

The literature indicates that IL-6 may inhibit ADAMTS-13 activity (Bernardo *et al.,* 2004). However, in a study of malaria patients, an inverse correlation was found between microparticle ADAMTS-13:Ag and VWF:Ag levels and IL-6 (De Mast *et al.,* 2009). IL-6 also did not show increased ULVWF multimer secretion as in IL-8 and TNF-α-stimulated HUVEC (Bernardo *et al.,* 2004). In this study, IL-6 treatment of HUVEC had no significant effect on microparticle ADAMTS-13:Ag levels (Figure 7, Table 4).

6.3b Tumour necrosis factor alpha and interleukin-8

The number of microparticles as well as the microparticle VWF:Ag levels increased upon TNF-α activation of HUVEC (Figure 5, Table 2). This correlates with other studies (Combes *et al.,* 1999, Bernardo *et al.,* 2004*,* Jy *et al.,* 2005, Faille *et al.,* 2009, Macey *et al.,* 2010). The precise mechanism of how this TNF-α induces such an increase in microparticles is still unkown; however, P38 MAPK phosphorylation that leads to increased ICAM-1 is a proposed mechanism. By increasing ICAM-1, TNF-α may amplify the inflammatory response to induce even more microparticle formation (Curtis *et al.,* 2009). TNF-α treatment of HUVEC is also a known inducer of thrombin generation (Kirchhofer *et al.,* 1994). Microparticles formed from this stimulation contributed to 78% of the thrombin generation (Figure 8a, Table 5).

In a study by Bernardo *et al.* (2004)*,* a dose-dependent increase was observed in the ULVWF multimers upon TNF-α treatment of HUVEC (Bernardo *et al.,* 2004). Overall, IL-8 treatment also induced an increase in ULVWF multimers in endothelial cells. Higher increase in VWF levels at 10 μM concentrations was observed. The highest increase in VWF levels with the IL-8 stimulation was found with the 100 μM treatment. These studies were unfortunately not done on microparticles. It was also demonstrated that microvascular renal and brain endothelial cells stimulated with TNF-α induce endothelial microparticles with functional VWF (Jy *et al.,* 2005). In this study (Figure 6, Table 3) as in others, IL-8 and TNF-α both induced median increases in VWF:Ag levels in microparticles after treatment of HUVEC with 100 ng/ml concentrations of these cytokines (Combes *et al.,* 1999 Bernardo *et al.,* 2004). The first evidence for endothelial microparticle-dependent VWF expression in HUVEC endothelial cells after stimulation thereof with TTP plasma was provided by [a]Jimenez *et al.* (2003). Renal and brain microvascular endothelial cells treated with TNF-α were used as positive control for VWF levels in microvascular endothelial derived microparticles in this study. In my study, TNF-α induced a statistically significant increase in microparticle VWF:Ag levels in HUVEC (Figure 5, Table 2).

In accordance with [b]Cao *et al.* (2008)*,* who found lower ADAMTS-13 mRNA levels after 24 hour treatment with 10 ng/ml, the TNF-α in this study induced decreased ADAMTS-13:Ag levels in microparticles after a higher 100 ng/ml concentration of TNF-α treatment of HUVEC (Figure 7, Table 4). Again, the study of [b]Cao *et al.* (2008) was done under static conditions and on cells and not microparticles. The level of detection of the ELISA used in this study may not be as sensitive as real-time PCR used by [b]Cao *et al.* (2008), but the same effect could be observed.

From figure 7 and table 4 it is evident that IL-8 induced a slight and insignificant increase in HUVEC microparticle ADAMTS-13 levels. To date, no studies have been done on the effect of IL-8 treatment on ADAMTS-13 levels of microparticles.

6.4 Coagulation stimuli:

6.4a Tissue factor

In figure 5 and table 2 an increase in the number of endothelial microparticles after TF treatment is evident. TF is involved in activation of FX by its binding to FVIIa. It is interesting to note that in ovarian cancer and fibroblast cell lines, it took only one minute to reach maximum FX activation, while it took approximately 30 minutes for the TF-FVIIa complex to achieve a state of saturated binding *in vitro.* The faster effect may be due to the presence of microparticles in these disorders (Le *et al.,* 1992). The assembly of the tissue TF-FVIIa complex may also be responsible for formation of these microparticles. Unfortunately, this has not yet been proven. Increased TF levels as well as endothelial microparticle levels, were observed in numerous disorders in which endothelial damage occur, like myocardial infarction, lung cancer and leukaemias (Nakasaki *et al.,* 2000, Morange *et al.,* 2007, Regina *et al.,* 2009, Breen *et al.,* 2011, Fleitas *et al.,* 2011). Therefore, the increased numbers of endothelial microparticles may be due to the activation by TF. Nonetheless, no correlation thereof was found in these studies.

In figure 5 (Table 2) and 6 (Table 3) one can observe an increase in the number of microparticles as well as a statistically significant increase in microparticle VWF:Ag levels upon stimulation with TF. It was suggested that increased VWF levels are involved in patients at risk of thrombosis in atrial fibrillation. TF levels were also increased in these and other patients. Whether TF can be linked to increased VWF:Ag levels *in vivo* is still unknown (Lip, 2003, Sato *et al.,* 2006, Yamashita *et al.,* 2006). If a link could be established between these factors, the findings support the possibility that TF play a part in increased VWF:Ag levels to subsequently promote the prothrombotic state. In this study, (Figure 8c, Table 5) microparticles derived from HUVEC treated with TF contribute to about 30% of the thrombin formation. This correlates with an *in vivo* study in which haematopoietically derived microparticles carrying TF were also involved in thrombus formation (Chou *et al.,* 2004). It is, therefore not surprising that both TF and VWF levels are increased in inflammatory conditions.

In reference to figure 7 (Table 4) no apparent change in the HUVEC microparticle ADAMTS-13 levels upon stimulation with TF was found. This correlates with studies where shiga toxin that stimulates TF expression was used. Cells treated with shiga toxin also did not increase or decrease the ADAMTS-13 levels (Nestoridi *et al.,* 2005, Nolasco *et al.,* 2005, Nguyen & Carcillo, 2006, Liu *et al.,* 2011).

6.4b Thrombin

In various studies, thrombin induces endothelial microparticle formation (Combes *et al.,* 1999, Sapet *et al.,* 2006, Simoncini *et al.,* 2009). In these studies the dosages of thrombin that were used to treat cells range between 0,1 IU/ml and 1 IU/ml. In a study by Šimák *et al.* (2002), stimulation of HUVEC for 30 minutes with 1-50 µmol/l of thrombin did not result in significant microparticle formation. Furthermore, treatment of pulmonary endothelial cells with 100 ng/ml of thrombin did not induce substantial phosphorylation of membrane cytoskeletal proteins (Schaphorst *et al.,* 1997). Similarly, an insignificant decrease was found in the amount of microparticles when HUVEC were stimulated with 2 U/ml thrombin.

Thrombin is a protein known for its pleiotropic effects in vascular cells (Guðmundsdóttir *et al.,* 2008). No recognizable effect of thrombin on microparticle VWF:Ag levels were observed in this study (Figure 6). Gumundsdóttir *et al.* (2008) found no noticeable effect of thrombin receptor PAR-1 stimulation on VWF:Ag levels. Other studies found increases in VWF:Ag levels. A 15-minute stimulation of HUVEC with 0,001-10 U/ml thrombin resulted in increased expression of VWF (Birch *et al.,* 1992). VWF was also shown to be increased by stimulation of 1 U/ml, 2 IU/ml and 5 IU/ml thrombin for 5 to 60 minutes. Then again, after treating cells for 5 minutes with thrombin, washing and immediately treating again with 1 U/ml of thrombin a 0,08% decrease in VWF levels were observed. In this study, the impact of temperature on the thrombin-induced VWF response was investigated. After 5 minutes of stimulation with thrombin, washing of cells and incubating them at 4 °C or 37 °C in culture medium, these cells had a reduced response or no response and strong increased VWF responses subsequently (Storck & Zimmermann, 1996). A slight, insignificant decrease in the amount of endothelial microparticles (Figure 5, Table 2) as well as a decrease in endothelial microparticle-derived VWF levels (Figure 6, Table 3) derived from microparticles was observed in this study.

One of the explanations for these decreased VWF levels in this study may be the possibility of the role that the integral membrane receptor, TM, played in decreasing VWF levels. An

inverse correlation between the levels of TM and VWF levels was found in cancer patients undergoing radiation therapy (Zhou *et al.,* 1992). Furthermore, endothelial cells infected with *Rekettsia conorri* resulted in a decrease in TM receptor levels and an increase in the VWF levels (Teysseire *et al.* 1992). It is possible that TM is expressed on endothelial microparticles and therefore may contribute to the decreased levels of VWF observed in this study (Leroyer *et al.,* 2010). In the intact endothelium, thrombin is expected to contain a more antithrombotic role than in damaged endothelium due to the expression of TM in order to protect the endothelium from excessive thrombosis (Lane *et al.,* 2005).

Endothelial microparticles may also contain the endothelial protein C receptor (EPCR, Pérez-Casal *et al.* 2009, Leroyer *et al.,* 2010). The binding of thrombin to TM receptors on cells or microparticles may induce activation of protein C (provided that protein C is produced by endothelial cells). APC is implicated in anti-inflammatory reactions and is dose and time dependent for inhibiting apoptosis. The EPCR is also recquired for increased activation of protein C (Mosnier & Griffen, 2003, Thiyagarajan *et al.,* 2007).

Another explanation for the observations made in this study could be that the nature of TRAIL induced by thrombin stimulation, may have elicited more non-apoptotic pathways in the HUVEC. TRAIL is involved in inducing apoptosis in cancerous cells. Anti-apoptotic effects of TRAIL in normal cells are under investigation (Collison *et al.,* 2009). In a study by Simoncini *et al.* (2009), TRAIL was shown to be involved in thrombin-induced microparticle formation in HMEC-1 cells. Blocking of TRAIL resulted in a reduction of the amount as well as the procoagulability of the microparticles. However, this study was done on HMECs and my study was done in HUVEC. Secchiero *et al.* (2003) believe that the TRAIL receptor is also involved in protection of HUVEC against apoptosis. In fact, TRAIL receptors in HUVEC cells stimulated proliferation. Activation of TRAIL receptors by TRAIL in HUVEC also did not activate P38, (C-Jun NH2 terminal kinase) JNK or NF-κβ that may lead to increased expression of adhesion molecules, like E-selectin, ICAM-1 and VCAM-1, that advance inflammation but rather have a protective role on HUVEC (Mosnier & Griffin*.,* 2003, Secchiero *et al.,* 2003).

In this study, thrombin induced a slight increase in the microparticle ADAMTS-13 levels (Figure 7, Table 4) but had no effect on thrombin generation in HUVEC derived microparticles (Table 5).

6.5 Combination stimuli:

In a normal *in vivo* environment, many factors are combined and these may induce different effects on cells than only one factor. Thrombin, for example, can exert different functions in combination with different factors. Thrombin and FVIIa may induce an extremely prothrombotic environment, while thrombin and TM may cause exactly the opposite effect (Le *et al.,* 1992, Binette *et al.,* 2007). No studies have been done on the combination effect of inflammatory and coagulation stimuli on microparticles and its specific effect on VWF, ADAMTS-13 and thrombin generation. In the next few paragraphs the effect of the different combinations of cytokines with coagulation stimuli are discussed.

6.5.1 Combinations with thrombin

Stimulation of HUVEC with a combination of thrombin and TNF-α resulted in a decrease in the number of microparticles (Figure 5, Table 2) as well as of microparticle VWF levels (Figure 6, Table 3). It seemed that the effect of thrombin overruled that of TNF-α. It was shown that the expression of PAR-1 and PAR-2 receptors after thrombin stimulation remained unchanged after TNF-α stimuli. Consequently, signaling by APC was still possible (Riewald & Ruf, 2005). Hence PAR-1 activation by thrombin in combination with protein C can still induce anti-apoptotic responses.

PAR-2 also plays an important role in the anti-inflammatory mechanisms of thrombin (Lindington *et al.,* 2005). PAR-2 was shown to be increased by inflammatory cytokines TNF-α and IL-1 (Nystedt *et al.,* 1996). The stimulation of PAR-1 and subsequently PAR-2 is upregulated on HUVEC by inflammatory cytokines in combination with thrombin. PAR-1 and PAR-2 may induce signaling via protein kinase C, which is known for its anti-inflammatory and cytoprotective effects and is involved in the protection of cells against injury mediated by the complement cascades (Lindington *et al.,* 2005). This mechanism may also explain the reduced number of microparticles and VWF levels that were observed in the endothelial microparticles after stimulation of HUVEC with thrombin in combination with the cytokine TNF-α.

In this study, the HUVEC microparticle ADAMTS-13 levels were slightly raised after a combination of TNF-α and thrombin (Figure 7, Table 4). Again, the thrombin negated the effect of the TNF-α in this case. One must keep in mind that these results obtained for HUVEC microparticle ADAMTS-13 levels were not significant and very close to the detection limit.

IL-8, like TNF-α, is principally known for its inflammatory properties and induction of increased vascular permeability (Petreaca *et al.,* 2007). When IL-8 was used in combination with thrombin, small but insignificant increases in VWF- and ADAMTS-13 levels were observed (Figure 7, Table 4). The separate effects of these agents were also very small and insignificant (Figure 7, Table 4). A possible mechanism by which IL-8 and thrombin stimulate endothelial cells may be through endothelial cell permeability. IL-8 may induce increased permeability when eNOS is inhibited (Van Nieuw Amerongen *et al.,* 1998, Wang *et al.,* 2004). Thrombin regulates eNOS in endothelial cells (Eto *et al.,* 2001, Andreeva *et al.,* 2006). Both IL-8 and thrombin can induce increased permeability if endothelial microparticles can express eNOS. However, this has not yet been shown. Thrombin-induced endothelial cell permeability is proposed to be mediated/amplified by more than one pathway (van Nieuw Amerongen *et al.,* 1998).

6.5.2 Combinations with tissue factor

The combination of TF with TNF-α induced an increase in the HUVEC-derived microparticle numbers (Figure 5, Table 2). This increase was not as extensive as that of the TF stimuli alone and was statistically insignificant. This combination also induced an increase of HUVEC microparticles in the VWF. TF, like thrombin, has also recently been implicated in the activation of PAR-1. This was also shown to be able to elicit anti-inflammatory reactions upon certain stimuli (Cenac *et al.,* 2005). This is very novel and knowledge regarding the precise role of TF in this activation is still evolving (Delacroix & Simari, 2012). Activated TF can also activate PAR-2 (Busch *et al.,* 2005). PARs can induce increased or decreased VWF expression (Langer *et al.,* 1999, Klarenbach *et al.,* 2003). The reason for the lower increase of HUVEC microparticles and microparticle VWF with the IL-8 and TF combination stimuli in comparison with TF alone is not known but may be attributed to the pleiotropic effects of PAR-2 (Langer *et al.,* 1999, Klarenbach *et al.,* 2003, Hjortoe *et al.,* 2004, Suen *et al.,* 2010). PAR-2 may have elicited anti-inflammatory effects by downregulating complement gene expression and upregulation of decay-accelerating factor (DAF) in the HUVEC (Suen *et al.,* 2010). This may also suggest a protective role of the PAR-2 receptor in inflammation and thrombosis. Similarly, the combination of TF and IL-8 induced no statistically significant increase in micorparticle VWF levels (Figure 6). Yet, this effect was also less than that of TF alone (Figure 6, Table 3).

The ADAMTS-13 levels after inflammatory cytokine and coagulation stimuli are also still a developing field of research. In this study, slight decreases in endothelial microparticle

ADAMTS-13 was observed after stimulation of HUVEC with TNF-α and TF alone (Figure 7, Table 4). This decrease is probably due to the small effect of TNF-α. Interestingly, in TTP, which is usually associated with decreased ADAMTS-13 activity, TNF-α was increased (Wada *et al.,* 1992). The effect of TF on ADAMTS-13 levels is not yet known. It can be suggested that this increased VWF and decreased ADAMTS-13 levels may be involved in prothombotic processes.

It was shown that TF expression in TNF-α-stimulated endothelial colony-forming cells was increased and that this combination subsequently led to increased thrombin generation potential (Cuccuini *et al.,* 2010). In this study microparticles from TNF-α and TF combination treated HUVEC are involved in 12% of the thrombin generation (Figure 8d, Table 5).

This study suggest that with more research endothelial microparticles in injured or dysfunctional endothelium may prove to be a cause of thrombotic and inflammatory disorders.

7 CONCLUSION

This study focused on the effect of inflammatory and thrombotic stimuli as well as the combination thereof on HUVEC microparticle formation and its haemostatic potential. It is possible that endothelial microparticles may be a cause of several inflammatory and thrombotic disorders but this needs more research. HUVEC can be used in such a study, since this cell line was able to form microparticles upon stimulation and these microparticles contained VWF multimers that were visible by electrophoresis.

The cytokine IL-6 had no effect on HUVEC-derived microparticles due to the lack of the IL-6 receptor in these cells. IL-8 also had only a small effect on microparticle VWF and ADAMTS-13 levels.

TNF-α increased the microparticle numbers significantly but had only a small effect on VWF and the ADAMTS-13 content of the microparticles. These microparticles contributed to almost 80% of the thrombin generated by the stimulation of TNF-α. In this way, TNF-α is a stimulus that may be responsible for the onset of thrombotic disorders by increasing TF-containing microparticles. This is possibly the first study to show the effect of TNF-α on HUVEC microparticle VWF and ADAMTS-13 levels.

TF in contrast to TNF-α had significantly increased VWF levels in microparticles and also increased the number of microparticles. It had no effect on ADAMTS-13 levels in microparticles. TF, therefore, stimulates the formation of microparticles rich in VWF. It is hypothesised that TF is responsible for the increased VWF levels in patients with HIV-associated TTP where inflammation and VWF plays a part.

Thrombin had no effect on microparticle formation and so may protect intact HUVEC. This emphasises the role of thrombin in regulating haemostasis in different cellular environments and protecting against thrombosis in extremely inflammatory environments. It is also not surprising from the microparticle numbers and VWF levels that the combination stimuli of thrombin and inflammatory agents also had a protective effect on HUVEC.

This study contributes to the understanding of the role of endothelial-derived microparticles in inflammatory and thrombotic environments. More research on endothelial microparticles in injured or dysfunctional endothelium may prove to be a cause of thrombotic and/or inflammatory disorders.

8 FUTURE STUDIES

The dose-dependent and protective role of thrombin in the stimulation of intact endothelial cells to form endothelial microparticles is still uncertain. In a possible future study, the effect of different concentrations and length of stimulation on microparticle VWF:Ag levels and numbers must be determined. It will be interesting to determine the possible mechanisms whereby TNF-α increases endothelial microparticle numbers and also mechanisms whereby TF increases VWF:Ag levels in endothelial microparticles.

Since the number of microparticles and their sizes influences the results of the thrombin generation assay, it will be interesting to study how different cytokines or cell types influence the size distribution of microparticles and whether these sizes have an impact on haemostatic potential (Lawry *et al.,* 2008).

Microparticles can be a promising field for future research and development of new diagnostic, prognostic or treatment methods (Bakouboula *et al.,* 2007, Wang *et al.,* 2009, Nozaki *et al.,* 2010, Lev-Ari *et al.,* 2012). This will require research on the number of microparticles in different haemostatic and/or vascular disorders where microparticles may be increased, like TTP and coronary artery disorders, such as angina, acute coronary syndrome, heart failure and hypertention (Simak & Gelderman, 2006, Bakouboula *et al.,* 2007, Nozaki *et al.,* 2010,). The number, origin and thrombotic potential thereof can also be determined. Another perspective will be to assess not only the VWF:Ag and ADAMTS-13:Ag levels in microparticles in disorders, but also to determine other immunological markers such as adhesion molecules that may be specific to certain haemostatic disorders. By doing this immunological profiles can be compiled at different stages of the same or different disorders to ascertain the role of microparticles in diagnostic, prognostic or treatment purposes. For example, in patients with increased VWF:Ag levels, like in TTP (Meiring *et al.,* 2011), the number of microparticles can also be assessed in order to obtain a better prognosis for the risk of developing thrombosis.

Other risk determining factors like VWF:Ag in microparticles can also be assessed in patients with high risk of thrombosis, which may result in high TF levels in the blood, which, in turn, may lead to kidney failure, strokes or infarctions. The correlation between TF levels and microparticle VWF:Ag in these patients may be assessed to determine the role of these microparticles in the pathophysiology of these disorders.

The blood and blood products provided to patients can also be assessed and stored or treated differently for more efficient treatment. Microparticle numbers can increase upon storage thereof. This could have a beneficial or negative impact on freezing and storing different blood or blood products for transfusion or testing purposes. Different storage conditions may account for increased numbers and also for different types of microparticles. A future study can determine more effective methods for storing blood and obtaining the optimal number and type of microparticles for specific purposes using optimal storage conditions (Lawry *et al.,* 2009). In future stored blood products can be manipulated according to specific patient requirements, which may account for a lower risk of thrombosis. For example, decreased TFPI levels that have been found in TTP patients and blood transfusions containing microparticles with higher TFPI levels on microparticles may account for better prognosis (Kobayashi *et al.,* 1995).

9 ABSTRACT

Endothelial microparticle reseach is currently a very novel and exciting topic in the field of haemosistasis and thrombosis. The role of microparticles in inflammatory and thrombotic disorders is however not fully understood. Dysfunction of endothelial cells is hypothesized to be a trigger of microparticle formation. In inflammatory disorders like sepsis and thrombotic disorders like atherosclerosis and thrombotic thrombocytopenic purpura, endothelial microparticle formation is altered and the numbers thereof may increase or decrease. It is not known if microparticles are the cause or the consequence of these disorders.

To understand the role of endothelial microparticles in inflammation and thrombosis, the effect of inflammatory cytokines and coagulation stimuli was studied as well as combinations thereof on endothelial microparticle formation and on microparticle VWF and its regulating protease, ADAMTS-13 in HUVEC.

In this study, the formation of microparticles in cultured human umbilical vein endothelial cells (HUVEC) was stimulated by different inflammatory agents: IL-6 (100 ng/ml), IL-8 (100 ng/ml) and TNF-α (100 ng/ml), coagulation stimuli: TF (2 U/ml) and thrombin (2 U/ml) and combinations thereof. The number of endothelial microparticles that formed was determined using flow cytometry. VWF and ADAMTS-13 levels of the microparticles were assessed by ELISAs and microparticle thrombin generation was measured by thrombin generation assays. VWF multimers were visualized by a Western Blot technique.

IL-6 did not have any effect on HUVEC-derived microparticles due to the lack of the receptor for IL-6 on these cells. IL-8 only slightly increased effect on microparticle VWF and ADAMTS-13 levels. TNF-α had a significant effect on microparticle numbers and contributed to almost 80% of thrombin generated by the microparticles. It has however almost no effect on VWF levels. The coagulation stimulus TF, on the other hand, induced the highest increase in microparticle VWF levels and increased microparticle numbers impressively. Yet, it had no effect on the thrombin generation by the microparticles. TF in combination with TNF-α also induced an increase in microparticle VWF and a small decrease in ADAMTS-13 levels. So, TF may contribute to the increased VWF levels that are commonly found in TTP patients where inflammation and thrombosis occur.

Interestingly, thrombin had a protective effect on the intact HUVEC by preventing microparticle formation. The combination stimuli of thrombin and inflammatory agents also

had a protective effect on HUVEC. This highlighted the regulatory role of thrombin in intact endothelial cells and also the protection that it provides against thrombosis in extremely inflammatory environments.

Endothelial microparticles can therefore be detrimental or beneficial, depending on the different stimuli and different environments. Inflammatory and coagulation stimuli may still pose a significant risk of clotting by altering microparticle quantity and content. This study contributes to understand the role that endothelial microparticles play in inflammation and thrombosis.

10 OPSOMMING

Endoteel-mikropartikelnavorsing is tans ’n baie nuwe en belowende onderwerp in hemostase en trombose. Die rol van mikropartikels in inflammatoriese en trombotiese toestande is nog onbekend. Wanfunksie van endoteelselle word as een van die oorsake van mikropartikelvorming bestempel. In inflammatoriese en trombotiese siektetoestande, soos sepsis, arterosklerose en trombotiese trombositopeniese purpura, vind daar verskeie veranderings in die vorming van mikropartikels plaas. Dit is nog onbekend of mikropartikels die oorsaak of die gevolg van hierdie siektetoestande is.

Om ’n beter begrip van die rol van endoteelmikropartikels te verkry, het ons die uitwerking van inflammatoriese sitokine en stollingsfaktore asook kombinasies van die twee groepe op endoteel-mikropartikelvorming in HUVEC ondersoek. Ons het ook die effek daarvan op die uitdrukking van von Willebrand faktor (VWF) en sy regulerende protease, ADAMTS-13, bestudeer.

Ons het in hierdie studie die vorming van mikropartikels gestimuleer deur menslike naelstringendoteelselle (HUVEC) met inflammatoriese agente, IL-6 (100 ng/ml), IL-8 (100 ng/ml) en TNF-α (100 ng/ml), asook stollingsfaktore, TF (2 U/ml) en trombien (2 U/ml) en kombinasies daarvan, te behandel. Die doel van die studie was nie alleen om die hoeveelheid HUVEC-mikropartikels wat vorm te bepaal nie, maar ook om die ADAMTS-13 en VWF-vlakke, VWF-multimeerpatroon en die trombiengenerasie van die mikropartikels te bepaal. Die hoeveelheid mikropartikels is bepaal deur vloeisitometrie te gebruik. VWF- en ADAMTS-13-vlakke is bepaal deur ELISA tegnieke en die trombiengenerasie is gemeet deur kinetiese reaksies. ’n “Western” kladtegniek is gebruik om die VWF-multimere te besigtig.

Interleukien-6 het geen uitwerking op die vorming van mikropartikels in HUVEC gehad nie. Die rede hiervoor is dat hierdie tipe endoteelselle geen reseptor vir IL-6 besit nie. Interleukien-8 het die mikropartikel VWF- en ADAMTS-13-vlakke slegs in ’n geringe mate verhoog. Tumor-nekrose-faktor-alfa het die hoeveelheid van endoteel-mikropartikels betekenisvol verhoog. Dit het sowat 80% bygedra tot die trombiengenerasie van die mikropartikels, maar het geen effek op die VWF-inhoud van die mikropartikels gehad nie. Tumor-nekrose-faktor-alfa is dus ’n kragtige stimulant van endoteelselle en is moontlik verantwoordelik vir die trombotiese neigings in trombotiese en inflammatoriese siektetoestande. Die stollingsfaktor TF het mikropartikelvlakke die meeste verhoog, maar het geen uitwerking op die trombiengenerasie van mikropartikels getoon nie. Stimulasie met

weefselfaktor in kombinasie met TNF-α het ook ‘n verhoging in mikropartikel-VWF- en ’n klein verlaging in ADAMTS-13 vlakke veroorsaak. Dit is dus moontlik dat TF betrokke is by die verhoogde VWF-vlakke wat in TTP-pasiente voorkom. Inflammasie en trombose speel ‘n hoogs waarskynlike rol in hierdie siektetoestand. Trombien het ‘n beskermende uitwerking op ongeskonde HUVEC, aangesien dit die vorming van mikropartikels bekamp. Die kombinasiestimuli van trombien en sitokine het ook ‘n beskermende uitwerking op HUVEC-selle getoon. Dit beklemtoon dus die regulerende rol van trombien in hemostase, veral op ongeskonde endoteelselle, asook die beskermingseffek daarvan teen trombose in oormatige stollings- en inflammatoriese toestande.

Endoteel-mikropartikels kan dus nadelig of voordelig vir die liggaam wees na aanleiding van verskillende behandelings en in verskillende omgewings. Inflammatoriese en stollingsfaktore is wel risikofaktore vir stolling, aangesien dit die aantal en inhoud van mikropartikes beïnvloed. Hierdie studie dra by tot die kennis van mikropartikels afkomstig van endoteelselle asook die rol daarvan in inflammasie en trombose.

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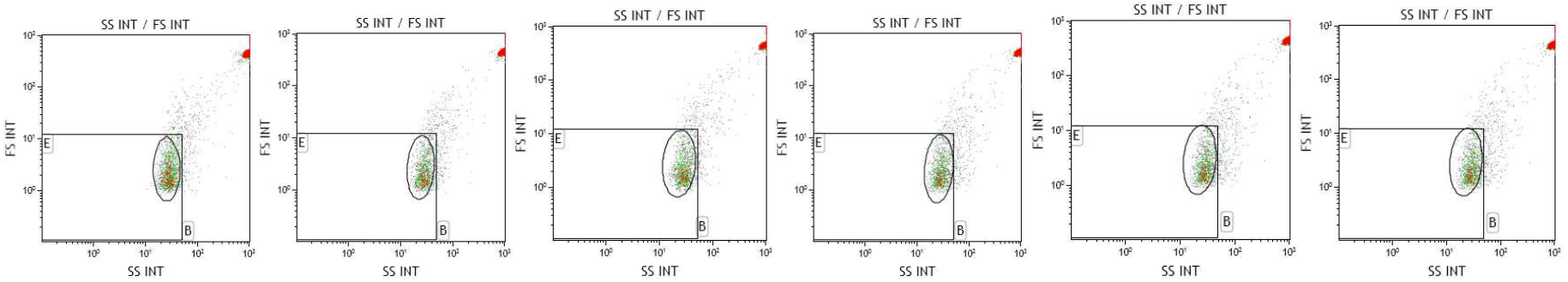
Addendum

Measurement of relative endothelial microparticle numbers after stimulation of HUVEC's with inflammatory/coagulation agents

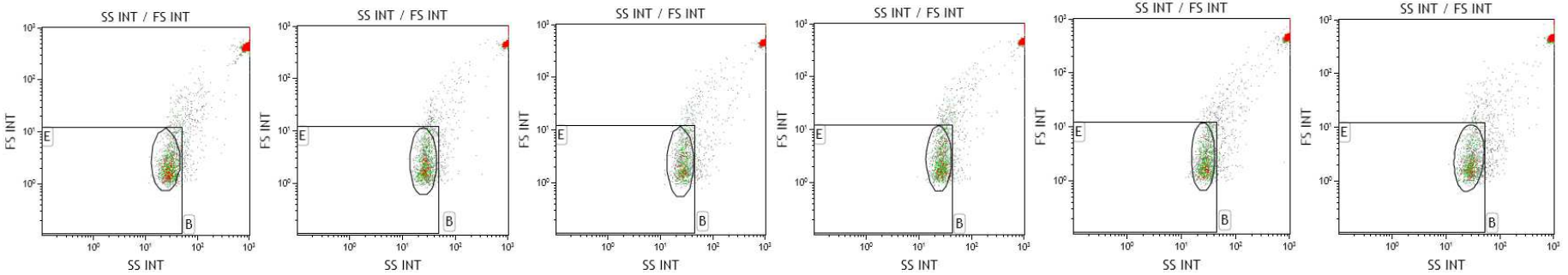
	Repeat 1		Repeat 2		Repeat 3		P-value	Mean	Standard deviation
	untreated	treated	untreated	treated	untreated	treated			
IL-6	540	514	552	653	280	235	0,8475	-0,863	17,521
TNF-α	450	828	540	761	411	876	*0,0382	79,355	36,330
Thrombin	1139	767	547	768	802	591	0,6197	-6,189	40,474
TF	475	618	361	746	367	785	0,068	83,550	46,426
TNF-α & thrombin	773	672	1165	957	573	587	0,2648	-9,492	10,610
TNF-α & TF	534	642	831	1301	342	762	0,099	66,530	52,013

Dot-Plot regions used for the quantification of endothelial microparticles after different stimuli of HUVECs



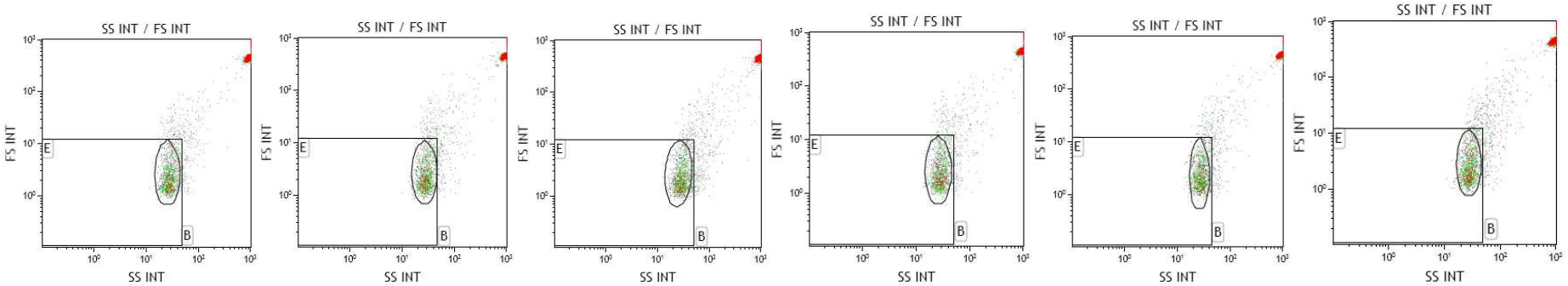
The SS vs. FS dot-plot for Interleukin-6 treated HUVEC

The SS vs. FS dot-plot for Interleukin-6 control HUVEC



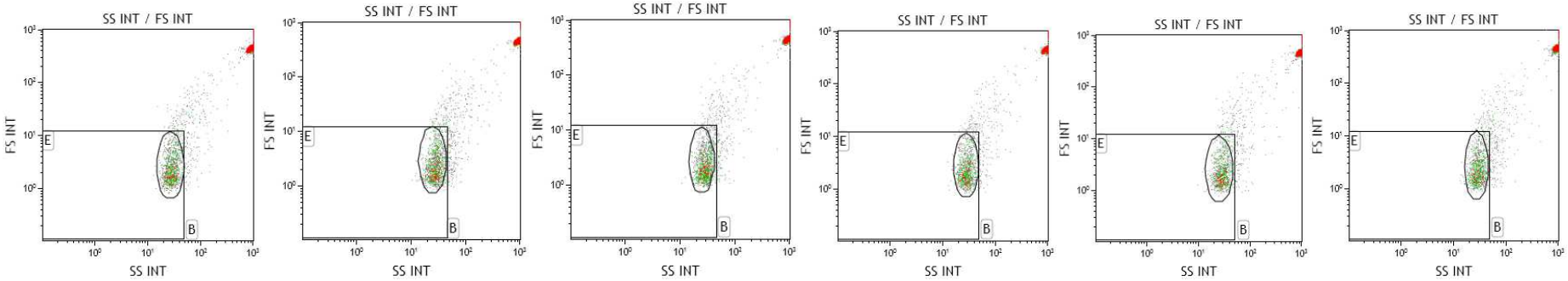
The SS vs. FS dot-plot for TNF-α treated HUVEC

The SS vs. FS dot-plot for TNF-α control HUVEC



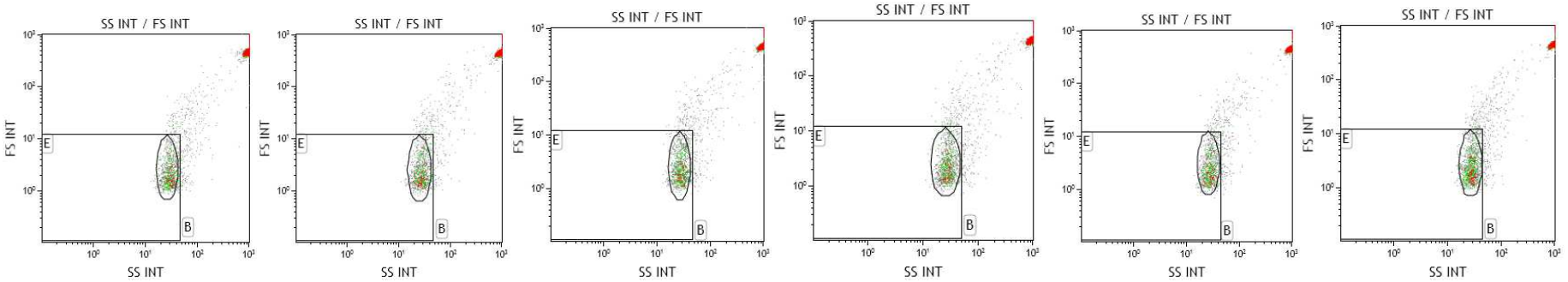
The SS vs. FS dot-plot for Thrombin treated HUVEC

The SS vs. FS dot-plot for Thrombin control HUVEC



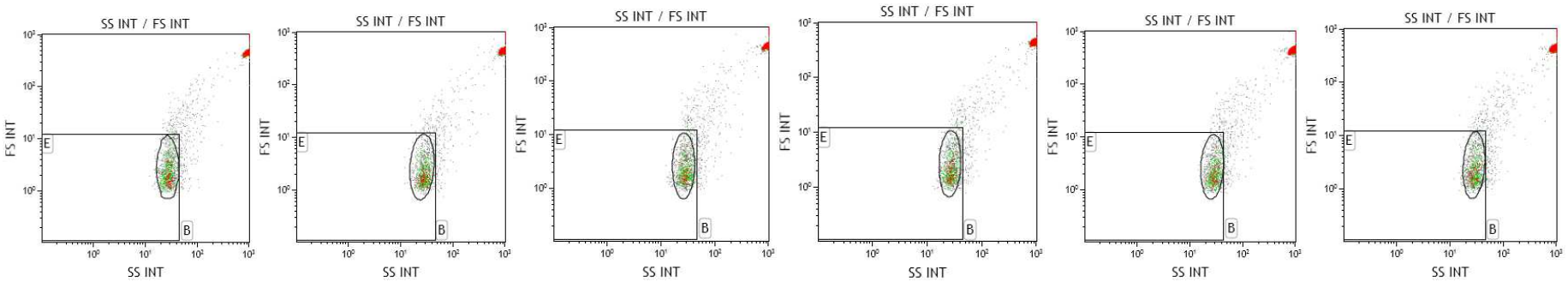
The SS vs. FS dot-plot for Tissue factor treated HUVEC

The SS vs. FS dot-plot for Tissue factor control HUVEC



The SS vs. FS dot-plot for TNF-α & Thrombin treated HUVEC

The SS vs. FS dot-plot for TNF-α & Thrombin control HUVEC



The SS vs. FS dot-plot for TNFα & TF treated HUVEC

The SS vs. FS dot-plot for TNFα & TF control HUVEC

Measurement of VWF levels in microparticles after stimulation of HUVEC's with Inflammatory/ coagulation agents

Treatment	Number of repeats								P-value	Mean	Standard deviation
	Run 1		Run 2		Run 3		Run 4				
	untreated	treated	untreated	treated	untreated	treated	untreated	treated			
IL6	0,139	0,262	0,537	0,231	0,211	0	0,418	0	0,1801	-42,100	89,405
TNF vial A	1,325	2,143	2,219	2,601	4,764	6,049	0,035	7,954	0,0522	51,800	38,132
TNF vial B	0,446	3,903	2,066	4,159							
thrombin	1,099	0,477	0,984	0,867	0,176	0,141	0,141	0,070	0,2232	-34,700	22,093
TF	0,778	1,571	0,925	1,241	0,211	1,132	0,280	1,421	0,0199*	312,900	151,916
TNF vial A & Thrombin	2,041	2,194	3,736	2,96	5,836	3,071	6,401	3,134	0,0775	-27,200	38,537
TNF vial B & Thrombin	2,601	0,598	1,463	1,834							
TNF vial A & TF	0,718	1,597	1,27	3,847	0,682	2,277	0,809	2,216	0,0016*	188,402	57,857
TNF vial B & TF	0,778	1,782	0,658	2,423							
IL-8					0,28	0,552	0,418	0,485	0,3462	56,644	57,357
Thrombin & IL-8					1,727	2,399	1,793	2,358	0,0549	35,211	5,233
TF & IL-8					1,16	2,459	1,372	2,419	0,0681	94,1475	25,223

*Red indicates noticeable outliers

Measurement of ADAMTS-13 levels in microparticles after stimulation of HUVEC's with Inflammatory/coagulation agents (ng/ml)

Treatment	Repeat 1 in ng/ml		Repeat 2 in ng/ml		Repeat 3 in frequency		Repeat 4 in frequency		Repeat 5 in frequency		P-value	Mean	Standard deviation
	untreated	treated	untreated	treated	untreated	treated	untreated	treated	untreated	treated			
IL-6	0,205	0,279	0,242	0,205	0	0,067	0,067	0,586	0,068	0,068	0,7418	6,900	26,386
TNF-α vial A	0,242	0,205	0,427	0,390	0,331	1,102	1,950	1,500	0,068	0,065	0,7233	-1,100	21,148
TNF-α vial B	0,205	0,279	0,427	0,465									
thrombin	0,205	0,242	0,279	0,427	0,396	0,331	1,500	1,950	0,065	0,068	0,2748	17,800	26,165
TF	0,353	0,353	0,242	0,242	0,461	0,396	1,500	1,500	0,065	0,065	0,3739	-2,800	6,306
TNF-α A & Thrombin	0,279	0,353	0,353	0,279	0	0	1,950	1,950	0,068	0,068	0,3939	12,200	25,935
TNF-α B & Thrombin	0,205	0,316	0,279	0,316									
TNF-α A & TF	0,390	0,167	0,427	0,242	0,200	0	1,500	1,500	0,064	0,065	0,046*	-19,400	25,283
TNF-α B & TF	0,205	0,205	0,427	0,353									
IL-8					0,524	0,826	1,720	1,500	0,064	0,066	0,8702	16,000	36,933
IL-8 & thrombin					0,461	0,884	2,880	3,130	0,072	0,073	0,1826	33,900	50,202

*Red indicates data not used due to limit of detection or noticeable outliers