The effect of inflammatory cytokines and coagulation factors on von Willebrand factor synthesis and cleavage

By

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DECLARATION

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Werner Ernst Allers

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- To my Creator for His guidance and peace during the last three years.

'I can do all things through Christ who strengthens me.'

1 Chronicles 4:10

CONTENTS

LIST OF SCIENTIFIC ABBREVIATIONS AND ACRONYMS

α	Alpha
A	Amps
ACE	Angiotensin I-converting enzyme
ADAMTS-13	A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
APS	Ammonium persulphate
BSA	Bovine serum albumin
˚C	Degree Celsius
EC	Endothelial cells
ECL	Enhanced chemiluminescence
ED	Endothelial dysfunction
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-linked immune-adsorbent assay
et al.	*et alii* (and others)
F IX	Factor nine
FBS	Foetal bovine serum
FIXa	Factor nine - activated
FVII	Factor seven

FVIIa	Factor seven - activated
FX	Factor ten
FXa	Factor ten - activated
g	Force of gravity
g	Grams
GP	Glycoprotein
gp130	Glycoprotein subunit 130
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HCl	Hydrogen Chloride
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidise
HUVECs	Human umbilical vein endothelial cells
IFN	Interferons
IgG	Immunoglobin G
IL	Interleukins
IL-1	Interleukin-1
IL-1β	Interleukin-1 Beta
IL-4	Interleukin-4

IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
IL-8	Interleukin-8
JAK	Janus kinase
JAKs	Janus kinases
kb	Kilobase
kDa	kiloDalton
L	Litre
LSGS	Low serum growth supplement
M	Molar
mg	Milligram
$MgCl_2$	Magnesium chloride
µg	Microgram
µl	Microlitre
microL	Microlitre
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
mV	Millivolt

Na2HPO4	Disodium hydrogen phosphate
NaCl	Sodium chloride
$NaH_2PO_4.2H_2O$	Disodium hydrogen phosphate dihydrous
ng	Nanogram
NH_4Cl	Ammonium chloride
NH_4HCO_3	Ammonium bicarbonate
NK	Natural killer cells
nm	Nanometre
NO	Nitric oxide
OPD	Ortho-phenylenediamine
PAF	Platelet activating factor
PARs	Protease activated receptors
PCR	Polymerase chain reaction
%	Percentage
pH	Percentage hydrogen
pmol	Picomole
pp	propeptide
PVDF	Polyvinylidene fluoride
rpm	Revolutions per minute

RNA	Ribonucleic acid
s	Seconds
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STATs	Signal Transducers and Activators of Transcription
T (cells)	T-lymphocyte
TBS	Tris Buffered Saline
TEMED	Tetramethylethylenediamine
TF	Tissue Factor
TF-VIIa	Tissue Factor and Factor seven activated complex
TM	Thrombomodulin
TMA	Thrombotic microangiopathies
TNF-α	Tumour necrosis factor-α
Tris	Hydroxymethyl
TTP	Thrombotic Thrombocytopenic Purpura
U	Unit
ULVWF	Ultra Large Von Willebrand Factor
V	Volts

v/v	Volume to Volume
VWF	Von Willebrand factor
VWF-CP	Von Willebrand factor-cleaving protease
VWF-HRP	Von Willebrand factor-horseradish peroxidase
WHO	World Health Organisation
WPB	Weibel-Palade bodies

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Vascular injury initiates a cascade of events, including inflammation, blood coagulation, new tissue formation, tissue remodelling and ultimately renewal of the injured area (Werner and Grose, 2003). The repair process is immediately initiated after injury by endothelial cells surrounding the wound. The endothelial cells get stimulated to form a site of localized inflammation and at the same time also protect the adjacent healthy tissues (McGill *et al.,* 1998). This leads to the release of various growth factors, cytokines, coagulation factors and the secretion of long strings of multimers known as Ultra Large Von Willebrand Factor (ULVWF) multimers by the endothelial cells (Werner and Grose, 2003).

A disintegrin-like and metalloprotease with thrombospondin type I repeats - nr 13 (ADAMTS-13) is a metalloprotease that is freshly released from the Weibel-Palade bodies in endothelial cells into the plasma. It cleaves these ultra large and hyperactive VWF multimers into smaller and less active forms. These VWF multimers mediate the initial adhesion of activated platelets, the first step in inflammation and thrombosis (Chauhan *et al.,* 2008). Therefore, Weibel-Palade bodies constitute an important link between thrombosis and inflammation. Furthermore, the inflammatory cytokines that are released during injury have stimulatory effects on the synthesis of the ULVWF and inhibitory effects on the ADAMTS-13 cleaving protease (Bernardo *et al.,* 2004; Cao *et al.,* 2008). Today,

inflammatory cytokines are recognized as a possible link between inflammation and coagulation. Coagulation enzymes also play important roles in both inflammation and thrombosis, since thrombin, the key coagulation enzyme responsible for clot formation, has also been shown to induce the release of VWF into plasma (Chauhan *et al.,* 2008).

Ultimately, the increased ULVWF levels and the decreased ADAMTS-13 activity contribute to the development of thrombotic and inflammatory diseases, such as Thrombotic Thrombocytopenic Purpura (TTP). Thrombotic Thrombocytopenic Purpura is a life-threatening disease characterised by micro-vascular platelet deposition and thrombus formation in selected organs resulting in microangiopathic haemolytic anaemia, thrombocytopenia, neurological symptoms, and renal failure. Typically, a very rare disorder, TTP is being seen with increased frequency in patients infected with the human immunodeficiency virus (HIV) (Gunther *et al.,* 2007). However, very little is known about the initial onset of HIV-associated TTP where inflammation and thrombosis play important roles.

In this study, we aim to examine the effects of inflammatory cytokines and coagulation initiators such as tissue factor and thrombin and especially combinations thereof on the release of ULVWF by cultured human umbilical cord endothelial cells (HUVECs) and the cleavage of these ULVWF by ADAMTS-13. This might allow us to evaluate more hypothetical links between inflammation and thrombosis and help us understand the mechanisms that lead to HIV-associated TTP.

CHAPTER 2

LITERATURE REVIEW

2.1 Endothelial Cells

The endothelium is considered an inert barrier to elements contained in the blood. It is a dynamic monolayer of over a trillion cells that cover the inner surface of the entire vascular system. It provides an anticoagulant barrier that separates circulating blood from the tissue also forming a dynamic interface with all other organs in the body (Jaffe, 1987; Shimokawa, 1999; Esper *et al.,* 2006). The human body contains approximately 10^{13} endothelial cells (EC), weighing almost 1 kg and covering a surface area of 4,000 to 7,000 m^2 (Cines *et al.,* 1998). In an adult human, the proliferation rate of EC is very low compared to the other cell types in the body (Fajardo, 1989; Pearson, 1991). Endothelial cells from a large vessel, for instance, an artery or vein will differ in morphology and functionality from those originating from micro vessels like arterioles, capillaries, or venules (García-Cardeña and Gimbrone, 2006; Pober *et al.,* 2009). However, some features of endothelial cells are shared between arteries, veins and capillaries. These are the flat elongated shape of the cells and the content consisting of Weibel-Palade bodies. These are the storage organelles for Von Willebrand factor (VWF) and P-selectin.

The morphology and functionality of EC are largely programmed by the tissue microenvironment, extracellular matrix components, surrounding cells, biological

factors/mediators and shear stress. All of these are responsible for the heterogeneity of the EC (Aird *et al.,* 1997). Furthermore, EC form a unique thrombo-resistant layer between the blood and the potentially thrombogenic sub-endothelial tissue. The vascular endothelium, moreover, functions as a versatile multifunctional organ with many synthetic and metabolic properties (Cines *et al.,* 1998). These properties are responsible for the regulation of vascular tone, vascular growth, thrombosis, atherosclerosis, angiogenesis and inflammation (Schwartz *et al.,* 1983; Larson and Haudenschild, 1988). The function of endothelial cells is discussed in the next section.

2.1.1 Endothelial Cell Functions

All the blood vessels and lymphatic's are lined by EC. These extraordinary cells were once considered for the simple function of keeping cells within the blood from leaking out of the vessels. However, through research on endothelial cells we now know that they have a remarkable array of functional and adaptive qualities. Moreover, EC are the main determinants of health and disease in blood vessels and play a crucial role in arterial disease (Sumpio *et al.,* 2002).

The ability of EC to express procoagulants, anticoagulants, vasoconstrictors, vasodilators, also essential cell adhesion molecules and cytokines, makes it one of the key regulators of haemostasis. Under normal conditions, EC maintain a vasodilatory and local fibrinolytic state where coagulation, platelet adhesion and activation, in addition to inflammation, are suppressed. The non-thrombogenic endothelial surface is sustained through mechanisms which include: the endothelial production of thrombomodulin (TM) and subsequent activation of

protein C and furthermore, endothelial expression of heparansulfate and dermatansulfate which accelerate the activity of anti-thrombin and heparin cofactor (Becker *et al.,* 2000; Wakefield *et al.,* 2008). Endothelial cells are further involved in regulating vascular tone by synthesizing and releasing paracrine agents such as endothelin-1, nitric oxide (NO) and prostacyclin (Barbee *et al.,* 1995; Wu and Thiagarajan, 1996). NO plays a major role in the normal activity of the endothelium (Silva and Saldanha, 2006). Interestingly, endothelial cells can be stimulated to release vasoactive substances in response to different blood flow shear rates (Osanai *et al.,* 2000; Woodman *et al.,* 2005). For instance, the endothelial cells can respond to increased shear stress and decreased shear stress, by releasing NO (a vasodilator) or endothelin-1 (a vasoconstrictor), respectively, in order to normalize flow velocity, and hence stabilize shear stress on the arterial wall (Yoshizumi *et al.,* 1989; Buga *et al.,* 1991). Moreover, prostacyclin, the major vasodilatory prostanoid produced in endothelial cells, can be released in response to shear stress, hypoxia, or to substances that stimulate NO formation (Gryglewski, 1995; Lüscher and Noll, 1995).

The vascular endothelium is also adaptable and multifunctional. Its synthetic and metabolic properties include the regulation of coagulation, thrombosis and thrombolysis, platelet adherence, modulation of vascular tone and blood flow, as well as regulation of immune and inflammatory responses by controlling leukocyte, monocyte and lymphocyte interactions with the vessel wall (McGill *et al.,* 1998; Sumpio *et al.,* 2002). The following sections on inflammation and thrombosis will describe these functions in more detail. However, more importantly relating to the topic of this dissertation; the endothelium functions to and minimize platelet

activation and blood cell adhesion. And, moreover, the aggregation of platelets or leukocytes occurs in response to endothelial cell stress or dysfunction (Stamler *et al.,* 1989; Wu and Thiagarajan, 1996).

2.1.2 Endothelial Cell Dysfunctions

A normal cell with its defined structures and functions, maintain a steady state called homeostasis. Changes in the physical, chemical or biological environment will trigger a cellular response. A cellular response to a mild injurious stimulus consists of adaptations that allow the cell to survive and continue to function. If the stimulus persists or becomes severe, reversible or irreversible injury or even cell death may occur (Growth *et al.,* 2010). Therefore, when the endothelium is exposed to injuring stimuli, the endothelial cells become dysfunctional, a process known as endothelial dysfunction (ED) as presented in Figure 2.1 (Lerman and Zeiher, 2005).

Endothelial dysfunction describes a situation when the equilibrium between vasodilators and vasoconstrictors shifts towards vasoconstrictor and proliferative effects, which leads to the development of hypertension, atherosclerosis, platelet aggregation and ischemia (Cockcroft, 2005; Félétou and Vanhoutte, 2006; Moncada and Higgs, 2006; Yetik-Anacak and Catravas, 2006; Simionescu, 2007). Consequently, during states of endothelial disturbances, whether physical (e.g., vascular injury) or functional (e.g., sepsis), a pro-thrombotic and pro-inflammatory state of vasoconstriction is maintained by the endothelial surface (Becker *et al.,* 2000). Under such conditions, platelets release platelet activating factor (PAF) and

endothelin-1, which promote vasoconstriction. Endothelial cells then also produce VWF, tissue factor (TF), and Factor V that augment thrombosis (Wakefield, 2008).

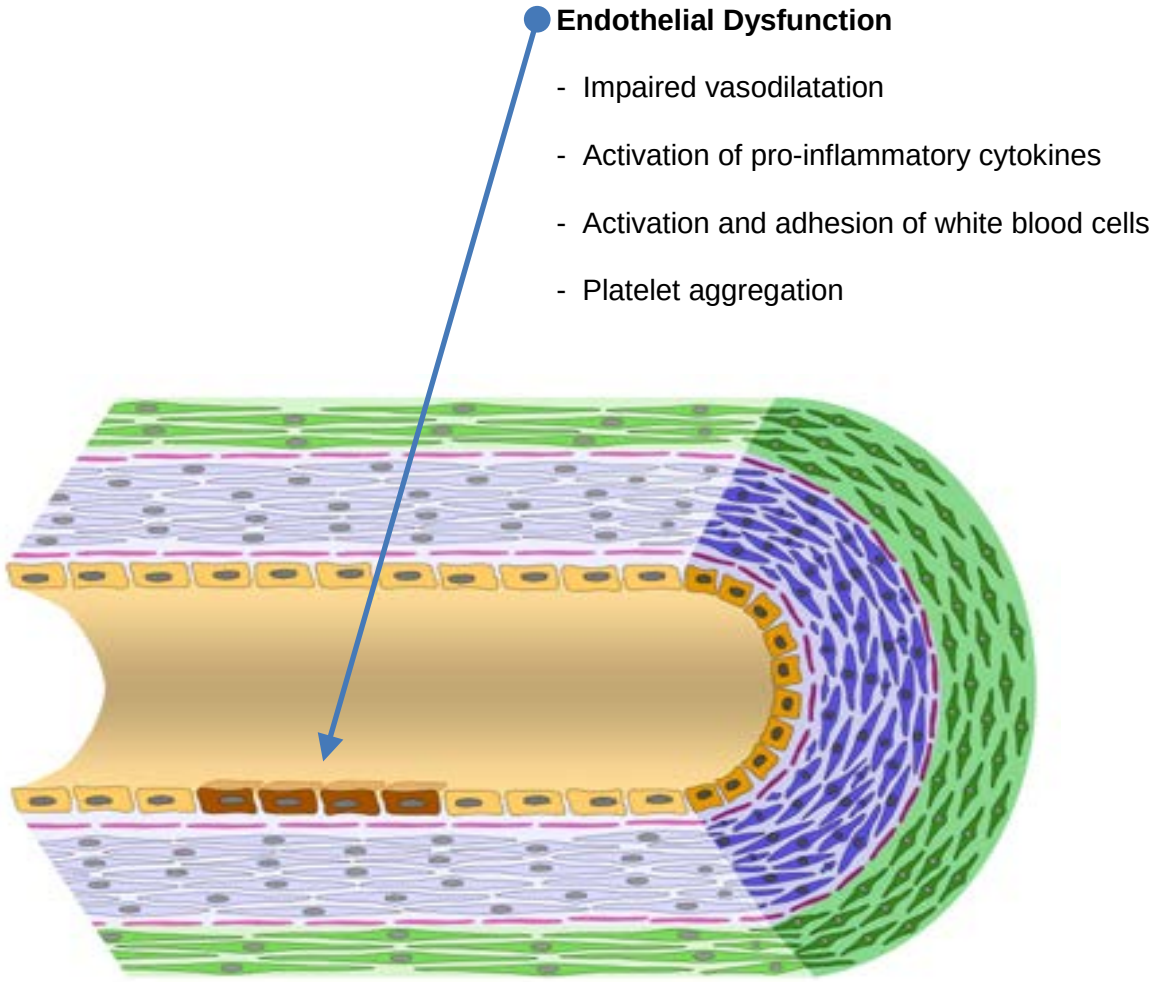


Figure 2.1 Progression of endothelial dysfunction in a blood vessel.

Endothelial cell activation, injury or dysfunction is also a trademark of many pathologic states, which include atherosclerosis, loss of semi-permeable membrane function, altered inflammatory and immune response and thrombosis. Figure 2.2 illustrates examples of situations where the endothelial cell plays a

critical role in initiation and amplification of inflammation and thrombosis by the expression of various products (Sumpio *et al.,* 2002; Wakefield, 2008).

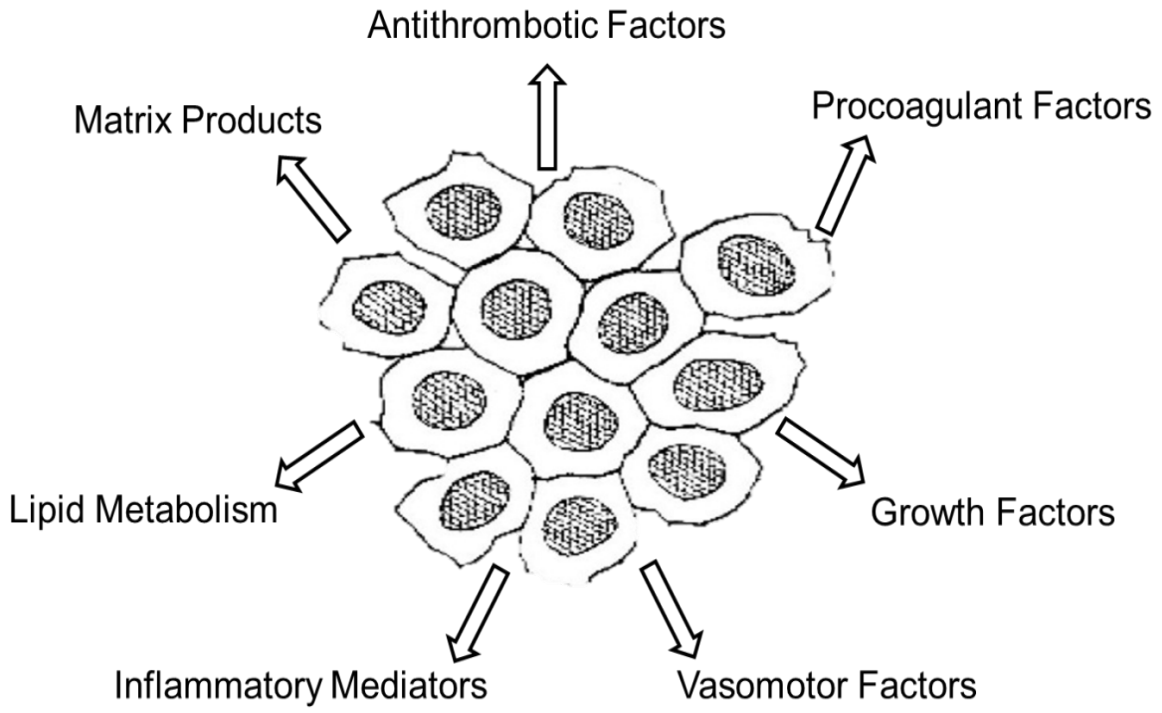


Figure 2.2 Known secretory/expression products of endothelial cells during thrombosis and inflammation. (Sumpio et al., 2002)

2.1.2.1 Inflammation and Thrombosis

It is now known that inflammation and thrombosis are interrelated. Figure 2.3 presents this relationship: inflammation increases tissue factor levels, platelet reactivity and fibrinogen levels, and leads to the release of cytokines during injury, which have stimulatory effects on the synthesis of ULVWF (Bernardo *et al.,* 2004). Furthermore, inflammation has inhibitory effects on the ADAMTS-13 cleaving protease and thus promotes thrombosis (Cao *et al.,* 2008).

The release of ULVWF during inflammation, together with the increased tissue factor levels, leads to thrombus formation. This process is influenced by inflammatory cytokines also released by the endothelium which affects the amount of ULVWF synthesized (Becker *et al.,* 2000; Wakefield, 2008).

Furthermore, inflammation decreases the expression of thrombomodulin (TM). TM is an endothelial cell-surface glycoprotein that interacts with thrombin to activate protein C. Protein C, together with protein S inactivates coagulation factors V and VIII. A deficiency of protein C is associated with an increased risk of thrombosis (Wakefield, 2008). Ultimately, the effect of inflammation interacts with thrombosis via the function of the inflammatory cytokines, coagulation factors, ADAMTS-13 and VWF. As such, inflammation and thrombosis will be separately discussed in more detail, which forms the focus of this dissertation.

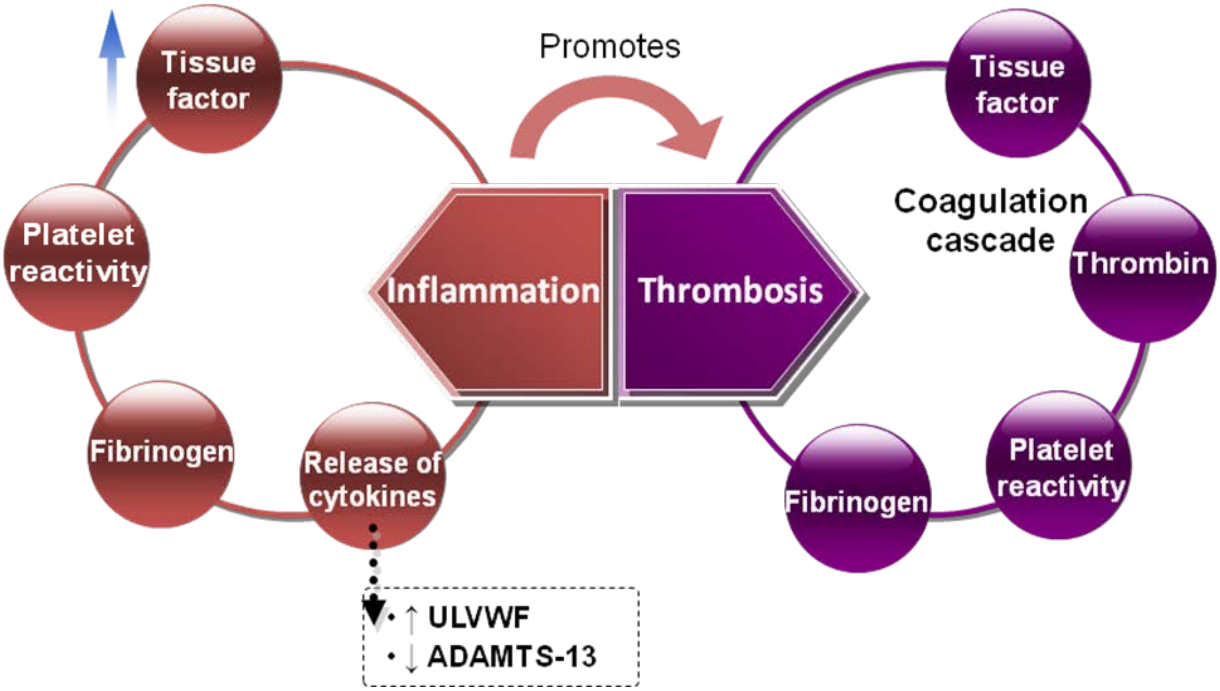


Figure 2.3 Interaction of inflammation and thrombosis.

2.2 Inflammation

The immune system is the body's major defence system and consists of many specialized cell types that cooperatively protect the body from parasitic, bacterial, fungal and viral infections as well as from the growth of tumour cells (Roth, 1994). Inflammation is a response triggered by trauma, toxin exposure, infection, ischemia as well as autoimmune injury and was recognized centuries ago. Today inflammation is known to be the first response of the immune system to infection involving the recruitment of immune cells to the site of injury. Therefore, the purpose of inflammation is to limit damage to the body after injury such as abrasions and lacerations or invasion by foreign organisms, such as bacteria or viruses. It, as a result, serves to create a physical barrier against the spread of infection and promotes healing of damaged tissue resulting in the clearance of pathogens.

Typical signs of inflammation include: rubor (redness), tumour (swelling), calor (heat), dolour (pain), and loss of function (Highlights *et al.,* 2010). There are two forms of inflammation, acute and chronic. Acute inflammation is an immediate and early innate (i.e. intrinsic and not antigen triggered) immune response to tissue injury (Highlights *et al.,* 2010). Granulocytes, monocytes and macrophages as well as mediators such as thromboxane, leukotrienes, PAF, interleukins, tumour necrosis factors and tissue factor are involved in this process. Some mediators are pro-inflammatory (increasing inflammation) while others are anti-inflammatory (decreasing inflammation). Termination of inflammation involves activation of anti-inflammatory mechanisms that prevents further damage to the host. The usual

outcome of acute inflammation is a successful resolution and repair of tissue damage.

In contrast, chronic or persistent inflammatory responses can lead to angiogenesis, fibrosis and further tissue destruction (Growth *et al.,* 2010; Highlights *et al.,* 2010; Stimuli and Injury, 2010). Today chronic inflammation is considered to be a major factor in the pathophysiology of many diseases, including rheumatoid arthritis, asthma, arteriosclerosis, diabetes, neurodegenerative diseases and HIV infection (Winsauer and de Martin, 2007).

To date, extensive progress has been made in the knowledge of inflammation. It is now known that, pro-inflammatory mediators are released or produced from the surrounding tissue and cellular components such as mast cells after injury (Kubes, 1993; Smith, 1993; Granger and Kubes, 1994). Under inflammatory conditions, the endothelium responds by regulating its own permeability and releases pro-inflammatory mediators such as cytokines (Ross, 1999). Cytokines are a group of proteins and peptides used as signalling compounds by organisms. These signalling compounds allow one cell to communicate with another cell. Cytokines have autocrine or paracrine properties that have the ability to affect several target cells through membrane receptors, inducing gene activation and protein synthesis. Cytokines often promote (inflammatory) or inhibit (anti-inflammatory) the synthesis of other cytokines, which in turn forms complex cytokine networks. Monocytes/macrophages are one of the major sources of cytokine production in the body (Boulay *et al.,* 2003; Langer *et al.,* 2004). Monocytes specifically produce a number of cytokines, including IL-1, IL-6, TNF-α,

and transforming growth factor-beta (TGF-β) (Epstein *et al.,* 1994). There are over 50 identified cytokines, which are clustered into several classes, such as interleukins (IL), tumour necrosis factors, interferons (IFN) and chemokines (Boulay *et al.*, 2003; Langer *et al.*, 2004). Among the pro-inflammatory cytokines, tumour necrosis factor-α, IL-6 and IL-8 have been implicated as the primary endogenous mediators of inflammation (Tracey and Cerami, 1994). These cytokines will be described in more detail in the next section.

2.2.1 Inflammatory cytokines

2.2.1.1 Interleukin-6

Interleukin-6 (IL-6), a 26 kDa acute inflammatory cytokine, is produced by activated monocytes, macrophages, and endothelial cells (Aarden *et al.,* 1987; Jirik *et al.,* 1989). Its expression is controlled in response to endotoxins, IL-1, tumour necrosis factor-α (TNF-α) and IL-4 (Kerr *et al.,* 2001).

The biological activities of IL-6 are initiated by binding to the interleukin-6 receptor (IL-6R) on the endothelial surface. This receptor is a 80 kDa protein subunit that binds IL-6, and of a 130 kDa glycoprotein subunit (gp130), that mediates the signal transduction (Rattazzi *et al.,* 2003). Binding of the IL-6/IL−6R complex to gp130 leads to the activation of several transcription factors such as Janus kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs) (Kishimoto *et al.,* 1995). Cells that do not express any IL-6R on their surface can be stimulated only by the IL-6/IL-6R complex and are insensitive towards IL-6 alone. Examples are hematopoietic progenitor cells, endothelial cells in particular

HUVECs, neuronal cells and osteoclasts (Taga and Kishimoto, 1997; Peters *et al.,* 1998).

IL-6 also induces its own release (Von Der Thüsen *et al.,* 2003). It promotes the coagulation cascade through a number of pathways (Kerr *et al.,* 2001). It increases the production of platelets and enhances their activation. Furthermore, IL-6 up-regulates fibrinogen, tissue factor, Von Willebrand factor (VWF), and factor VIII levels (Neumann *et al.,* 1997; Stirling *et al.,* 1998; Kerr *et al.,* 2001). However, this pro-inflammatory cytokine has no stimulatory effects on the endothelial cell release of ULVWF multimers, but showed inhibition on the cleavage of ULVWF by ADAMTS-13, either alone or in complex with IL-6R (Bernardo *et al.,* 2004).

2.2.1.2 Interleukin-8

Interleukin-8 (IL-8), first recognized as a chemotactic protein by Yoshimura and associates in 1987, is translated as a 99-amino acid precursor and is secreted after cleavage of a 20-amino acid leader sequence. Furthermore, to attracting neutrophils along a chemotactic gradient, IL-8 moreover activates these neutrophil cells, in the process triggering degranulation, increasing expression of surface adhesion molecules and producing reactive oxygen metabolites. Interleukin-8 or neutrophil activating protein is a cytokine that is produced by endothelial cells, fibroblasts, keratinocytes and lymphocytes in response to inflammatory stimuli such as TNF-α and lL-1β (Yoshimura *et al.,* 1987). IL-8 induces a shape change of cells, chemotaxis, the release of granule contents, up-regulation of adhesion proteins, formation of bioactive lipids, and the respiratory burst (Gillitzer *et al.,*

1991). It is also present in the synovial fluid of patients with inflammatory joint diseases.

This chemokine has gained considerable attention because of its ability to attract and activate leukocytes and its acknowledged role as a mediator of inflammation. This pro-inflammatory cytokine has distinct stimulatory effects on the endothelial cell release of ULVWF multimers, but not on the cleavage of ULVWF by ADAMTS-13 (Bernardo *et al.,* 2004).

2.2.1.3 Tumour Necrosis Factor-α (alpha)

Tumour necrosis factor (TNF, also known as TNF-α) was first identified as an endotoxin-induced glycoprotein in 1975 (Carswell *et al.,* 1975). TNF-α is primarily produced by activated macrophages and T lymphocytes as a 26 kDa protein. Pro-TNF-α is expressed on the plasma membrane where it undergoes cleavage in the extracellular domain by the matrix metalloproteases resulting in the release of a soluble 17 kDA form. Both membrane-associated and soluble TNF-α's are active in their trimeric forms (Black *et al.,* 1997). TNF-α, one of the most potent pro-inflammatory cytokines, was first discovered as a soluble factor in blood that can cause necrosis of tumours (Hakoshima and Tomita, 1988). Since then, it has been identified as a critical regulator of inflammatory responses through stimulating the expression of adhesion molecules on endothelium and decreasing endothelial NO generation thereby inducing endothelial dysfunction (Bruunsgaard, 2005). TNF-α is mainly produced by activated macrophages and T lymphocytes, but a wide range of cells can produce TNF-α, including endothelial cells, neutrophils, smooth muscle cells, fibroblasts, granulocytes, NK-cells and tumour cells, in response to

certain stimuli (Bradley, 2008). The term “tumour necrosis factor” refers to its ability to suppress certain tumour cells in the defence system of man (Waage *et al.,* 1987). Among other effects, this essential mediator of inflammation also activates leukocytes, enhances adherence of neutrophils and monocytes to endothelium, and triggers local production of other pro-inflammatory cytokines (Tracey and Cerami, 1994). Similarly, TNF-α induces endothelial cells to synthesize and secrete other cytokines such as IL-1 and IL-6 (Cotran and Pober, 1990). This pro-inflammatory cytokine has also shown to have stimulatory effects on the endothelial cell release of ULVWF multimers, but not on the cleavage of ULVWF by ADAMTS-13 (Bernardo *et al.,* 2004).

In this study, endothelial cells were stimulated with IL-6, IL-8 and TNF-α to induce inflammation. The process of thrombosis will be discussed in the next section.

2.3 Thrombosis

Thrombosis is a pathophysiological haemostatic response to vessel trauma in the absence of bleeding. Numerous factors affect the thrombotic process in blood vessels, e.g. the extend of injuries to the vessel wall, the coagulation and fibrinolytic system, circulating blood platelets as well as shear forces (Acland, 1973; Nievelstein and De Groot, 1988; Lassila *et al.,* 1990; Johnson *et al.,* 1993; Maraganore, 1993; Barker *et al.,* 1995; Ruggeri, 1997; Bassiouny *et al.,* 1998). Although it is rarely necessary to interfere with the process of haemostasis, prevention and treatment of thrombosis is therapeutically very important. Thrombosis is a pathological process in which a blood clot termed thrombus is created in a blood vessel. This is due to the most important initial interactions

which occur with circulating blood platelets and the sub-endothelial layers of the injured vessel (Nievelstein and De Groot, 1988; Tangelder *et al.,* 1989). There are three major factors that contribute to formation of the thrombus: endothelial injury, abnormal blood flow and hypercoagulability which are highly interrelated and known as *Virchow's triad* (Figure 2.4) (Pathy *et al.,* 2006).

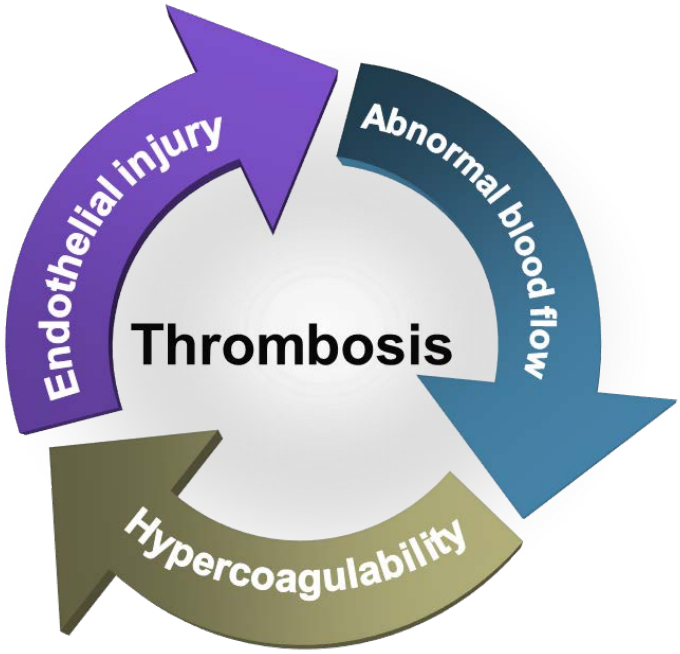


Figure 2.4 Virchow's triad. (Virchow, 1856)

After wessel wall injury tissue factor (TF) is expressed on the endothelial cell surface which binds activated coagulation factor VII. Once formed, the complex TF/FVIIa catalyzes the formation of activated factor VII. Tissue factor/FVIIa complex activates FX. Factor Xa also plays a major role in the process of coagulation. FXa then associates with factor Va to form the prothrombinase complex, which further converts prothrombin (factor II) to thrombin that finally catalyzes the formation of fibrin from fibrinogen (Colman *et al.,* 2006). Thrombin and factor Xa are responsible for their own activation. Thrombin activates factor

XI that, in its turn, activates factor IX. Factor IX, together with factor VIII activates factor X again that activates thrombin. This amplification pathway generates most of the thrombin that is needed to form a fibrin clot (Colman *et al.,* 2006).

Under normal conditions, the intact endothelium presents a non-thrombogenic surface for blood flow. Endothelial cells do not only form a physical barrier; they additionally synthesise, secrete, bind and convert numerous substances such as fibronectin, thrombomodulin, VWF, Factor V, thromboplastin, IL-6, IL-8 and NO. These substances are involved in platelet function, coagulation and fibrinolysis. Two known potent inhibitors of platelet activation secreted by endothelial cells are prostacyclin and nitric oxide (Nievelstein and De Groot, 1988; Makhoul *et al.,* 1999; Sumpio *et al.,* 2002). When inflammation and infection are present due to injury or stimuli, the intact endothelium becomes a site for platelet adherence (Brozović, 1977; Nievelstein and De Groot 1988; Zacharski *et al.,* 1992; Donati, 1995; Ten Cate *et al.,* 1997; Makhoul *et al.,* 1999). Once the endothelium is damaged, and the sub-endothelium or the deeper layers of the endothelium are exposed to the blood, platelets rapidly adhere (Kehrel, 1995). At high rates of shear stress, Von Willebrand factor (VWF) mediates the initial binding of platelets to the sub-endothelium through the platelet membrane glycoprotein (GP) Ib (Nievelstein and De Groot, 1988; Ruggeri 1997). Platelets will also adhere to the exposed collagen directly via glycoprotein Ia/IIa-receptor (Ruggeri, 1997). Subsequently, the platelets are then activated where it covers the exposed surfaces by spreading (Nightingale *et al.,* 1980). VWF and fibrinogen then bind to the activated platelets, in the process linking one to another via the glycoprotein IIb/IIIa-receptors, on platelets and fibrinogen, in a process known as aggregation.

Platelets are strongly activated when they adhere to collagen or other sub-endothelial elements and even stronger when thrombin is formed (Ruggeri, 1997). Most of the activators are released and synthesised at the site of injury.

It is clear that tissue factor and thrombin play an important role in the process of thrombosis and coagulation together with their effect on endothelial cells, and will therefore be discussed separately in more detail.

2.3.1 Coagulation Factors

2.3.1.1 Thrombin

Thrombin, a serine protease of 39 kDa, is generated at sites of vascular damage through the blood clotting cascade. It is best known for its role in haemostasis; however, thrombin is multifunctional, a powerful agonist of cellular responses and also regulates blood coagulation as well as platelet aggregation (Bar-Shavit *et al.,* 1992; Coughlin *et al.,* 1993; Cirino *et al.,* 1996).

Thrombin, generated in large amounts at the site of injury, and the resultant thrombus and exposed extracellular matrix, serve as a reservoir of active thrombin (Marmur *et al.,* 1994; Barry *et al.,* 1996). Thrombin also regulates vessel tone, promotes chemotaxis, smooth muscle cell proliferation, extracellular matrix turnover, release of cytokines, atherogenesis, angiogenesis and, inflammation (see figure 2.5) (Baykal *et al.,* 1995; Fager, 1995; Goldsack *et al.,* 1998; Patterson *et al.,* 2001).

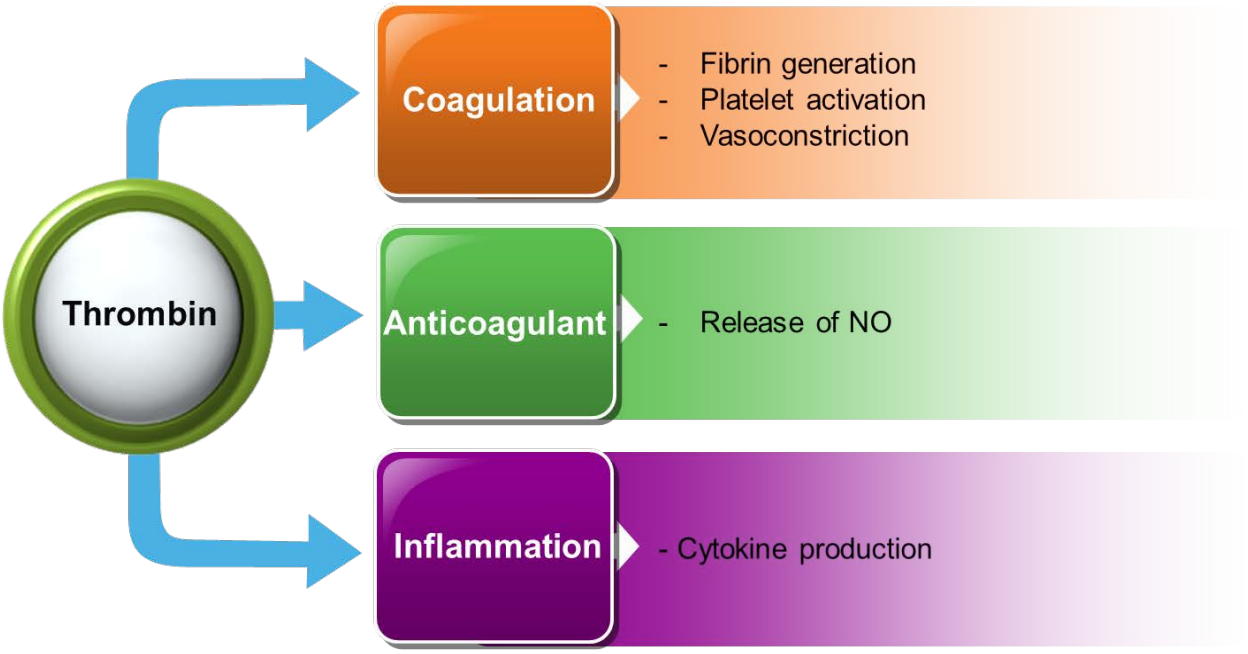


Figure 2.5 Thrombin is a multifunctional serine protease generated at sites of vascular injury. It generates procoagulant, anticoagulant, inflammatory, and proliferative responses by blood cells and blood vessels.

Thrombin is formed from its precursor prothrombin, at sites of vascular injury, by cleavage at two sites by factor Xa (Goldsack *et al.,* 1998). This results in a 39 kDa thrombin molecule that converts fibrinogen to fibrin in the final step of the clotting cascade. Thrombin signalling is mediated in part by a family of protease activated receptors (PARs) (Coughlin, 1999). It functions through the activation of its G-protein-linked receptors PARs (Vu *et al.,* 1991). There are four documented PARs: PAR1, PAR2, PAR3, and PAR4. PAR1, PAR3 and PAR4 are activated by thrombin (Vu *et al.,* 1991; Ishihara *et al.,* 1997; Kahn *et al.,* 1998). PAR2 is activated by trypsin as well as factor VIIa and Xa, but not by thrombin (Nystedt *et al.,* 1994; Camerer *et al.,* 2000). PAR1-3 have been found in human vascular cells and PAR4 in the rats' aorta (Coughlin, 2000; Patterson *et al.,* 2001).

The multiple actions of thrombin are mediated by unique structural features of the thrombin molecule (Figure 2.6, Eisenberg, 1996). The molecule has several distinct receptor (recognition) sites. This includes the catalytic binding site, an anion-binding exosite (exosite-1), an apolar binding site as well as separate sites for binding of heparin (exosite-2) and fibrin (Stubbs and Bode, 1994; Eisenberg, 1996). The catalytic binding site is the active centre, located in a deep narrow slot of the molecule, and is involved in enzymatic activity (see Figure 2.6) (Stubbs and Bode, 1993).

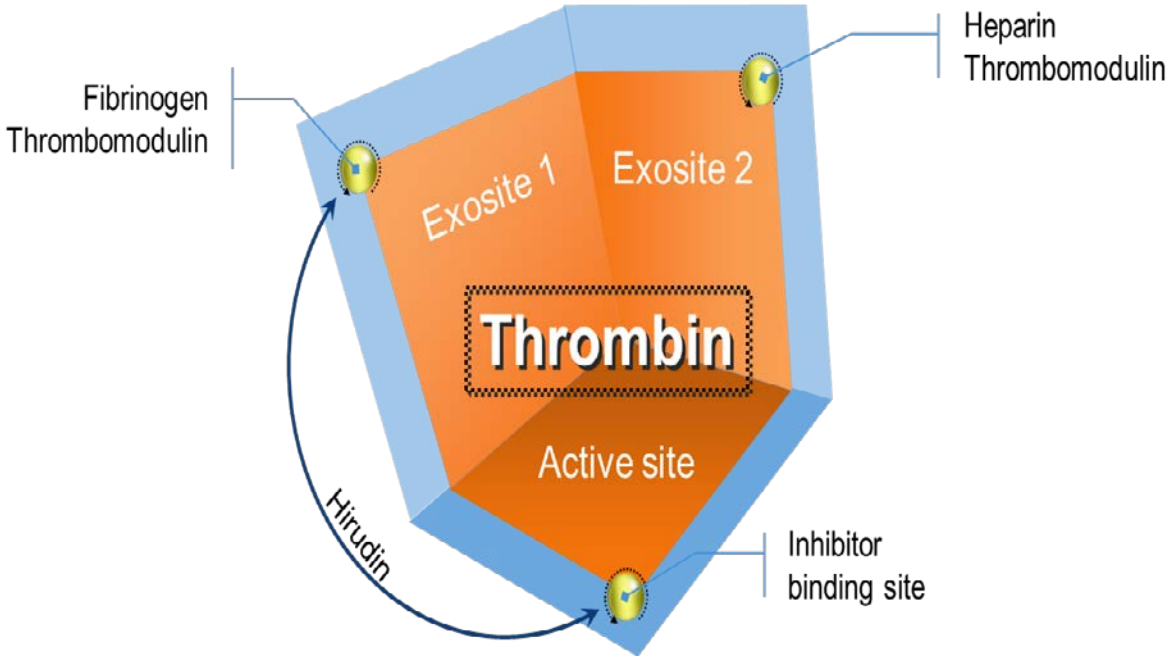


Figure 2.6 Different binding sites on the thrombin molecule. Exosite 1 and 2 are involved in binding substrates, fibrin, heparin, thrombomodulin, and bivalent inhibitors such as hirudin. The active or catalytic site is the binding site for univalent inhibitors and is also involved in enzymatic activity.

Exosite-1 is responsible for the binding of fibrinogen, PAR1, thrombomodulin, heparin cofactor II and the inhibitor hirudin (Fenton *et al.,* 1991; Mathews *et al.,* 1994). Heparin binds to exosite-2 (Sheehan and Sadler, 1994). Heparin together with antithrombin (AT) cannot inactivate clot-bound thrombin, likely because of a conformational change in thrombin's structure once it is bound to fibrin. This conformational change makes the exosite-2 binding site on clot-bound thrombin inaccessible for heparin (Weitz *et al.,* 1990). Some direct thrombin inhibitors bind to the apolar binding site which is adjacent to the catalytic site. These inhibitors are smaller than heparin, need no cofactors and can reach their site on thrombin within the thrombus. The apolar binding site is involved in substrate recognition as well as the interaction of thrombin with platelets, leukocytes and endothelial cells (Moliterno, 2003). Fibrin binds to another part of the thrombin molecule, separated from the other binding sites mentioned.

Thrombin has also stimulatory effects on the endothelial cell release of ULVWF multimers (Wagner, 1990; Chauhan *et al.,* 2008). No other effect of thrombin, regarding the topic of this thesis, has been studied.

2.3.1.2 Tissue Factor

Tissue factor (TF), the protein component of tissue thromboplastin, also known as thromboplastin, coagulation Factor III and CD142, is a 47 kDa transmembrane glycoprotein normally located on the surface of a variety of extravascular cells that initiates the clotting cascade (Nemerson, 1987; Bach, 2006). TF is a high-affinity, cell-surface receptor and is an essential cofactor for the serine protease factor VIIa (Broze, 1982; Bach *et al.*, 1986). The TF-VIIa complex activates factor X (FX)

directly and indirectly via factor IXa (FIXa) generation, which leads to thrombin formation. The ability of TF to serve as a cofactor in the initiation of both the extrinsic and the intrinsic coagulation pathways underscores its critical role in coagulation (Rapaport and Rao, 1995).

Expressed TF has a large extracellular domain (219 residues), a hydrophobic transmembrane domain (23 residues) and a cytoplasmic carboxyterminal domain (22 residues) (Morrissey *et al.*, 1987; Spicer *et al.*, 1987). The extracellular domain of TF is located outside the cell and binds FVIIa. The carboxylated GLA domain of factor VIIa binds in the presence of calcium to negatively charged phospholipids. Binding of FVIIa to negatively charged phospholipids greatly enhances the binding of FVIIa to TF. The transmembrane domain of TF crosses the hydrophobic membrane and the cytoplasmic carboxyterminal domain is involved in the signalling function of TF.

TF is primarily located in the adventitia of blood vessels (the outermost part of arteria, i.e. fibroblast), and comes into contact with blood merely after vascular damage occurred (Drake *et al.*, 1989; Wilcox *et al.*, 1989). TF is a constituent of both the sub-endothelial layer of the vascular wall and the extravascular tissue. It forms a protective lining around the blood vessels and is ready to activate blood coagulation if vascular integrity is compromised (Ryan *et al.*, 1992). Endothelial cells and blood monocytes (in contact with the bloodstream) do not constitutively express functional TF and do not have intracellular stores of TF (Lwaleed *et al.*, 2007). Functional (active) TF is not normally expressed by cells within the bloodstream except in trace amounts in circulating monocytes (Østerud *et al.,*

2008). TF exhibits a non-uniform tissue distribution with high levels in the brain, lungs, and placenta, intermediate levels in the heart, kidneys, intestines, uterus, and testes and low levels in the spleen, thymus, skeletal muscle, and liver (Mackman, 2004). The higher levels of TF in the brain, lungs, placenta, heart, and uterus provide additional haemostatic protection to these vital organs (Drake *et al.,* 1989). An additional source of TF, known as “blood-borne” TF or plasma TF, also contributes to thrombosis. Circulating TF on microparticles is incorporated into arterial thrombi (Giesen *et al.,* 1999; Rauch and Nemerson, 2000). Leukocytes is most likely the main source of circulating blood TF in the form of cell-derived microparticles. Platelets are also a possible source of TF (Müller *et al.,* 2003).

In addition to TF expression in the adventitia of blood vessels, brain (astrocytes), lung (bronchiolar and alveolar cells), heart (cardiac monocytes), kidney (tubular cells) and placenta (trophoblasts), it is also found to be expressed in a number of embryonic cells including epithelial and smooth muscle cells (Eddleston *et al.*, 1993; Lwaleed *et al.*, 1999; Siegbahn, 2000; Luther and Mackman, 2001).

The stimulatory effect of TF on endothelial cells to release ULVWF has not been studied, nor the cleavage of ULVWF by ADAMTS-13.

2.3.2 VWF, ADAMTS-13 and TTP

Upon vascular injury, during the early stage of systemic inflammation endothelial cell stimulation leads to the secretion of a family of monocyte-derived peptides, which include the cytokines IL-6, IL-8 and TNF-α (Paleolog *et al.,* 1990; Bockmeyer *et al.,* 2008; Zhang, 2008). These inflammatory cytokines, including

thrombin, have profound stimulatory effects on the endothelial release of ULVWF multimers and its' synthesis, which is measured by the VWF propeptide (VWFpp) (Bernardo *et al.,* 2004; Suzuki *et al.,* 2004). In addition, inhibitory effects have been observed on the synthesis of the ULVWF cleaving enzyme, ADAMTS-13 (Cao *et al.,* 2008). This ultimately leads to the deficiency of ADAMTS-13 and the over expression of ULVWF multimers, resulting in the initiation of thrombotic thrombocytopenic purpura (TTP) (Veyradier and Meyer, 2005).

Thrombotic thrombocytopenic purpura, first described in 1924 by Dr. Eli Moschowitz, is a rare disease with an estimated incidence of five to ten cases per million per annum in all racial groups (Lämmle *et al.,* 2005; Veyradier and Meyer, 2005; Franchini and Mannucci, 2008; Reininger, 2008). However, the incidence of TTP has increased dramatically in patients infected with the human immunodeficiency virus (HIV). Thrombotic thrombocytopenic purpura forms part of a group of diseases known as thrombotic microangiopathies (TMA) which all share a number of traits (Lämmle *et al.,* 2005; Reininger, 2008). The trait of attention is microvascular occlusion or simply, the blockage of an artery.

Thrombotic thrombocytopenic purpura is a life threatening thrombotic microangiopathy, which is characterised by a pentad of signs and symptoms. These are anaemia, thrombocytopenia, fever, hemiparesis and haematuria (Lämmle *et al.,* 2005; Franchini and Mannucci, 2008). The disease is caused by the massive formation of platelet and Von Willebrand Factor (VWF)-rich thrombi or "clots" in the microcirculation of multiple organs (Veyradier and Meyer, 2005; Reininger, 2008). These clots are caused by a deficiency of the VWF cleaving

protease (VWF-CP), ADAMTS-13, resulting in the excessive presence of ULVWF multimers (Reininger, 2008). In the next two paragraphs, I will describe VWF and its cleaving enzyme ADAMTS-13 before continuing with TTP again.

Von Willebrand factor multimers mediate platelet adhesion to the sub-endothelium that is exposed at the site of vessel injury (Moake JL, 2004; Reininger, 2008). The VWF gene of 178 kb is located on the short arm of chromosome 12 (12p13.3) (Ginsburg *et al.,* 1985; Kuwano *et al.,* 1996; Sadler, 1998). VWF is synthesised by endothelial cells and megakaryocytes. In megakaryocytes, VWF is stored in the alpha (α) granules of megakaryocytes and VWF originating from the endothelial cells is found in plasma, the sub-endothelial connective tissue and is stored in the Weibel Palade bodies of the endothelial cells (Denis *et al*., 2008; López and Dong, 2004; Reininger, 2008). Endothelial cells are the major source of plasma VWF. The primary mRNA product of 52 exons is first translated into a single pre-pro-polypeptide chain of 2,813 amino acids and includes a signal peptide of 22 residues, a large pro-peptide of 741 residues and lastly, a mature subunit of 2,050 residues (Sadler, 1998). The pre-pro-polypeptide undergoes post-translational modifications before entering the circulation as outlined in figure 2.7.

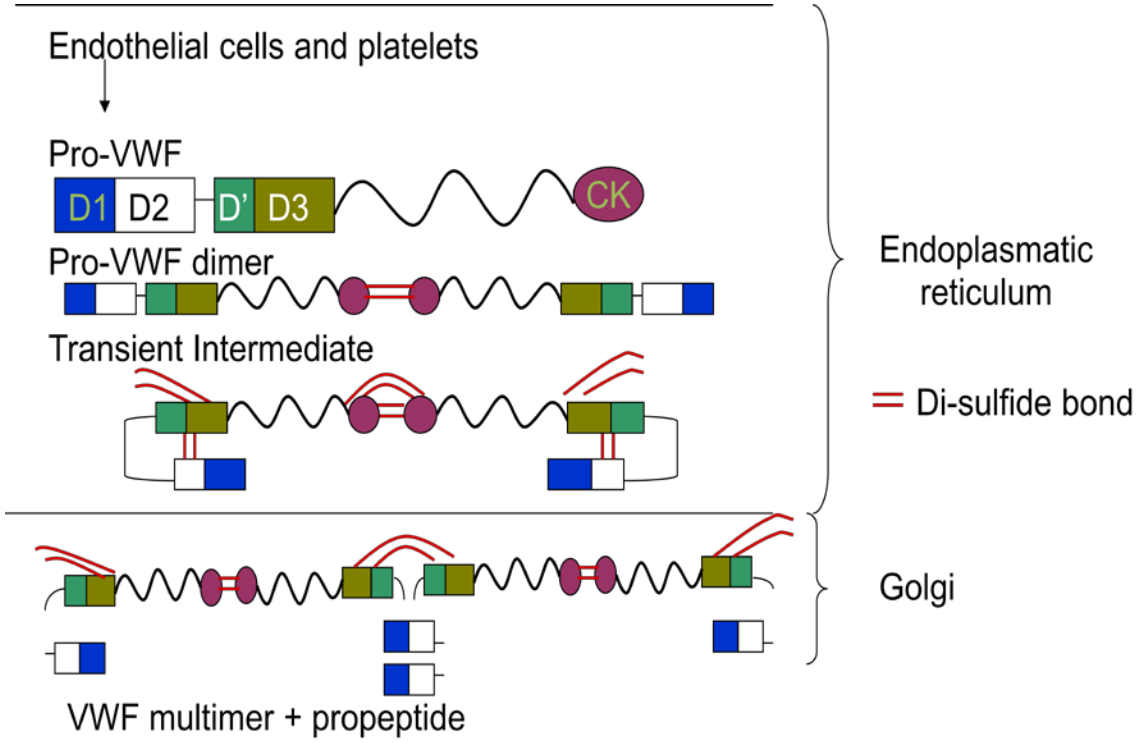


Figure 2.7 Synthesis of VWF: Two pro-VWF subunits are linked tail to tail via disulphide bonding in the ER to form a dimer. The propeptide subunits are cleaved off and the dimers are transported to the trans-Golgi network where they bind to each other to form multimers. Upon secretion, VWF multimers and the propeptide are released simultaneously at a 1:1 ratio.

In the endoplasmic reticulum, the signal peptide is cleaved by signal peptidase. The resulting propeptide undergoes N-linked glycosylation and dimerization. Thus, two pro-VWF subunits undergo tail to tail linkage via disulphide bonding within the cysteine-rich CK-domains to form dimers. In this process, the pro-peptide subunits are cleaved off. The dimers are then transported to the Golgi apparatus and post-Golgi compartments where further processing, sulphation and O-linked glycosylation take place. These dimers are transported to the trans-Golgi network where they bind to each other at the D'-D3 domain to form ultra large VWF multimers (Sadler, 2008). The ultra large VWF multimers are stored together

with the spliced pro-peptide subunits in the α-granules in platelets and Weibel-Palade bodies in endothelial cells and are secreted consecutively or upon stimuli (Denis *et al.*, 2008). The VWF propeptide and the VWF multimers are released simultaneously from the endothelial cells in an equal molar ratio of 1:1 (Wagner *et al.,* 1987; Wagner, 1990; Haberichter *et al.,* 2000). The propeptide circulate independently with a half-life of 2 hours. The amount of VWF secreted is measured by determining the plasma VWF propeptide levels, since the VWF propeptide controls the secretion of VWF and serves as a measurement for it. The VWF propeptide however, does not control the clearance of VWF. The ratio of the VWF propeptide and VWF antigen is used to determine clearance or secretion of VWF (Ragni, 2006). The VWF multimers that are released through stimulation are rich in the ultra-large multimers that are hyperactive (Ruggeri, 2007). These hyperactive ULVWF multimers are normally cleaved by the VWF-cleaving enzyme ADAMTS-13 (Dong, 2005).

ADAMTS-13 is a member of the ADAMTS (a disintegrin and metalloprotease with thrombospondin motif) family (Dong, 2005). The process of ULVWF proteolysis is in constant balance between the amount of ULVWF released from endothelial cells and the proteolytic capacity of ADAMTS-13 present (López and Dong, 2004; Dong, 2005; Doldan-Silvero *et al.,* 2008). Factors that disrupt this balance result in pathologic conditions, which range from bleeding (Von Willebrand disease) to thrombosis (TTP) (Franchini and Mannucci, 2008). For example, a sustained deficiency of ADAMTS-13, either congenital or acquired, results in TTP (Franchini and Mannucci, 2008). The mechanism responsible for the initial onset of TTP remains poorly understood (Cao *et al.,* 2008).

Increased plasma VWF levels have been reported in a wide variety of disease states such as bacterial or viral infections, autoimmune diseases, trauma, coronary and peripheral artery diseases and HIV associated TTP (Dong, 2005; Gunther *et al.,* 2007). Though these diseases cannot be linked by a shared cause, they are by the common pathology of inflammation, suggesting that inflammation may be the shared stimulus for release of EC-derived VWF (Wagner, 2005; Bockmeyer *et al.,* 2008; Chauhan *et al.,* 2008). The next section explains the relationship between inflammation and thrombosis.

2.4 Inflammation and Thrombosis

As mentioned, inflammation and thrombosis are closely interlinked. There is a complex interplay among these two processes. Some diseases as well as endothelial cell injury involve inflammation at every stage, from initiation to progression (Libby, 2002). Thrombosis is also involved in all stages upon endothelial cell injury by the coagulation cascade and platelet activation. Endothelial cells play a key role in vascular oxidative stress, thrombosis and inflammation (Förstermann, 2010). Activated or injured endothelium loses its natural anticoagulant property at the site of the tissue injury. It stop to produce NO and prostacyclin and decreases the expression of thrombomodulin. In addition, activated endothelial cells and monocytes express large amounts of TF, an important trigger of the coagulation cascade that leads to the generation of thrombin. Some coagulation factors have structural similarities to components involved in inflammation. Tissue factor, for instance, has structural homology to the cytokine receptors (Morrissey *et al.,* 1987). Systemic inflammation is thus a potent pro-thrombotic stimulus.

Furthermore, inflammatory mechanisms down regulate natural anticoagulants, upregulate procoagulant factors, increase platelet reactivity, and inhibit fibrinolytic activity (Esmon, 2003). Thrombin generates several inflammatory responses via augmentation of leukocyte adhesion and activation of platelets (Bar-Shavit *et al.,* 1986). In addition, thrombin stimulates production of the pro-inflammatory cytokines IL-6 and IL-8 from monocytes and endothelial cells while thrombomodulin is downregulated by inflammatory cytokines like TNF-α (Conway and Rosenberg, 1988; Fukudome and Esmon, 1994; Johnson *et al.,* 1998).

HIV-associated TTP is one of the disorders where thrombosis and inflammation plays an important role. These patients are characterised by extremely high levels of VWF with very low ADAMTS-13 levels. Although HIV associated TTP is an acquired form of TTP, only 50% of patients present with auto-antibodies to ADAMTS-13. Furthermore, what distinguishes these patients from those with congenital TTP, is the thrombotic potential in these patients. Increased TF levels with increased thrombin generations have been measured in patients with HIV associated TTP (Meiring *et al.,* 2011). The question is whether TF also has a stimulatory effect on endothelial cells to produce increased amounts of VWF and does it suppress the release of ADAMTS-13?

The aim of this study was to examine the separate and combined effects of inflammatory cytokines (IL-6, IL-8 and TNF-α) and the coagulation factors Tissue Factor and Thrombin on the release of ULVWF by cultured endothelial cells and the cleavage of these ULVWF by ADAMTS-13. This will allow us to better

understand the mechanisms that might lead to the increasing onset of the disease HIV-associated TTP where thrombosis and inflammation plays an important role.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study design

This study was an experimental study.

3.2 Experimental design

3.2.1 Procedure rationale

This procedure was based on the induction of inflammation and/or thrombosis on cultured human umbilical vein endothelial cells by cytokines, coagulation factors and combined coagulation-factor/cytokine stimulation, which provokes an increase or decrease of VWF, and the VWF-cleaving enzyme, ADAMTS-13.

We tested the effects of the following compounds on human umbilical vein endothelial cells (HUVECs): Interleukin-6 (IL-6, 100 ng/ml), Interleukin-8 (IL-8, 100 ng/ml), Tumour necrosis factor-α (TNF-α, 100 ng/ml), Thrombin (2 Units/ml) and Tissue factor (TF, 20 µl/ml). We also tested combinations of these compounds, combination of IL-8 and thrombin, IL-8 and TF, TNF-α and thrombin and TNF-α and TF. The control for the model was untreated cells for each treated sample performed. The above cytokine concentrations were based on the experiments done by Bernardo *et al.* and Cao *et al.* where they used the exact cytokine concentrations (Bernardo *et al.,* 2004; Cao *et al.,* 2008). This project was

approved by the Ethics Committee of the University of the Free State (ETOVS number: 31/09).

3.2.2 Endothelial cell culture

The human umbilical vein endothelial cell line (HUVEC cell line) (Cat. no.C-003-5C) was purchased from Invitrogen (Mowbray, South Africa). The endothelial cells were maintained in round tissue culture dishes (Prod. No. 93060, TPP, Separations, South Africa) at a number of 1.25 x 10^4 cells/ml. The dishes were first prepared for cell culturing by coating the surface with 500 µl of Human Fibronectin (Cat. no. PHE0023, Invitrogen, South Africa) at a concentration of 10 ng/ml. The dishes were then placed in a humidified 37°C, 5%CO2/95% air cell culture incubator overnight. Thereafter, the coated surfaces of the dishes were rinsed with Medium 200 (Cat. no. M-200-500, Invitrogen, South Africa). The cells were cultured in Medium 200 supplemented with Low Serum Growth Supplement kit (Cat. no. S-003-10, Invitrogen, South Africa). The Low serum growth supplement kit (LSGS) is an ionically balanced supplement containing foetal bovine serum (FBS), hydrocortisone, human epidermal growth factor, basic fibroblast growth factor and heparin, required for a correct cell growth pattern. After 48 hours, the cells were sub-cultured by incubation at room temperature with 0.025%/0.01% Trypsin/EDTA solution (Cat. no. R-001-100, Invitrogen, South Africa) for 1 to 3 minutes or until the majority of cells had detached from the flask. The action of Trypsin/EDTA was then blocked by the addition of 3 ml of Trypsin Neutralizer solution (Cat. no.R-002-100, Invitrogen, South Africa). Cells were harvested by centrifugation at 180 x *g* for 7 minutes and resuspended in supplemented Medium 200 before plating. The concentration of the cells were determined and the cells

were incubated in a humidified 37°C, 5%CO2/95% air cell culture incubator. Only 4th-passaged HUVECs were used for all experiments, as the phenotype of HUVECs changes with cell passage and HUVEC lose their ability to express certain proteins (Baudin *et al.,* 2007).

3.2.3 Cell culture treatments and experiments under shear stress

The HUVECs were grown until confluent on all tissue culture dishes, the old medium discarded and the dishes prepared for treatment. To induce the release of VWF multimers, ADAMTS-13 and the VWF propeptide, the HUVECs were stimulated with inflammatory cytokines, IL-6 (Cat. no. PHC0064, Invitrogen, South Africa), IL-8 (Cat. no.PHC0084, Invitrogen, South Africa), TNF-α (Cat. no. PHC3015, Invitrogen, South Africa), and also with human tissue factor (TF, HemosIL RecombiPlasTin 2G reagent, Cat. no. 0020003050, Beckman Coulter, South Africa) and bovine thrombin (HemosIL Fibrinogen-C reagent, Cat. no. 0020301100, Beckman Coulter, South Africa). This was done by incubating the cells with the different compounds and combinations of the cytokines with TF or thrombin respectively. The combinations and concentrations were mentioned in section 3.2.1. The cells were incubated with the compounds for 24 hours before applying shear stress. Two flasks were used for treatment: one for the control (untreated) and one for the treatment. The endothelial cells were treated in triplicate. Thus for each stimulant we used six culture flasks, three for the control (untreated) and three for the treatment (treated).

All compounds were reconstituted according to product instructions. The final concentration for each treatment was made up in 5 ml of supplemented Medium 200. In the case of the inflammatory cytokines, the following final concentrations were used: 0 ng/ml as control, and 100 ng/ml as treatment. For the coagulation enzyme, thrombin, we used 0 Units/ml as control, and 2 Units/ml as treatment. Two units of thrombin were calculated according to the final volume of 5 ml. For the coagulation initiator, tissue factor, we used 0 µl/ml as control, and 2 µl/ml as treatment. The exact concentrations were used for the combinations as with the different compounds alone. As mentioned the dishes were treated with the different compounds by incubation for 24 hours in a humidified 37°C, 5%CO2/95% air cell culture incubator (Napco, Thermo Fisher Scientific, South Africa).

After the treatment period, the dishes were carefully removed from the incubator, and placed onto a ROTEM orbital shaker for 1 hour to generate a wall shear stress of 2.5 dyne/cm^2 as previously described by Sargent et al. (Zhang et al., 2005; Carpenedo et al., 2007; Sargent et al., 2009; Sargent et al., 2010). Lastly, the flasks were removed from the ROTEM and the perfusate collected, aliquoted and stored at -80ºC until the measurements were performed. We measured the VWF levels, the ADAMTS-13 content and the VWF propeptide levels in the perfusates.

3.2.3.1 VWF levels

The VWF concentration in the perfusates was measured with an enzyme-linked immune-adsorbent assay (ELISA) as previously described (Favaloro et al., 2007).

In short, an ELISA plate was coated at 4ºC overnight with a rabbit anti-human von Willebrand factor (VWF) antibody (DAKO, South Africa, 1:6000 dilution in PBS: 5.84 $g.L^{-1}$NaCl, 4.76 $g.L^{-1}$ Na_2HPO_4, and 2.64 $g.L^{-1}$ $NaH_2PO_4.2H_2O$, pH 7.2). This antibody captures the VWF to be measured. The plate was then blocked with 4% bovine serum albumin (BSA, Sigma,South Africa) in PBS for 2 hours at room temperature. One hundred µl of each perfusate (stimulated and controls) was added in duplicate to the wells and incubated for 2 hours at 37ºC. After 7 washing steps with PBS/0.1% Tween-20 (Merck, South Africa), a rabbit anti-VWF antibody conjugated to peroxidase (DAKO, South Africa, 1:8,000 dilution in PBS/2% BSA) was added and incubated for one hour at room temperature. This antibody binds to the remaining free antigenic determinants of VWF and forming the “sandwich”. The bound enzyme peroxidase is revealed by its activity in a predetermined time on the substrate ortho-phenylenediamine (OPD) in the presence of hydrogen peroxide (10 ml of 0.2 M Na_2HPO_4, 10 ml of 0.1 M Citric Acid, 200 µl of 50 mg/L OPD, and 8 µl of 30% H_2O_2). The intensity of the colour produced is direct related to the VWF concentration present in the perfusate. The reaction was stopped after 3 minutes by adding 4 M H_2SO_4 (sulphuric acid; 30 µL/well), and the absorbance measured at 490 nm minus 630 nm with a plate reader (Bio Tek SYNERGY HT, Analytical & Diagnostic Products, South Africa). A standard curve of calibrated human plasma (WHO 6thFVIII/VWF standard) was used as the standard against which the perfusates were measured. The data were analysed using the accompanying *Gen5* software.

3.2.3.2 VWF multimeric analysis

The multimeric structure of VWF in the perfusates was determined by a highly sensitive and rapid method that is used routinely in our laboratory (Meiring *et al.,* 2005). This method utilises submerged horizontal agarose gel electrophoresis, followed by transfer of the VWF onto a polyvinylidine fluoride membrane, and immuno-localisation and luminographic visualisation of the VWF multimer pattern. The density of the high, intermediate and low molecular weight multimers of each multimer pattern were determined using a Geldoc XR geldocumentation system (Bio-Rad, CA, USA).

A 0.65% agarose gel was prepared in 100 ml Tris-acetate electrophoresis buffer (40 mMTris, 0.1% SDS, 1 mM EDTA, pH 7.8). The agarose was then poured into a horizontal gel apparatus with a 20 tooth comb in place and after solidification, the gel was placed at 4°C for 30 minutes. Samples were prepared by thawing each sample at 37°C and diluted 1:30 in sample buffer (0.01 M Na_2HPO_4, 37 mM iodoacetamide and 1% SDS, pH 7.0). After incubation at 37°C for 60 minutes, 10 µl bromophenol blue was added in a 1:10 ratio to the diluted sample and centrifuged at 14,000 rpm for 1 minute in an Eppendorf centrifuge.

The prepared gel was then set in place. Pre-cooled electrophoresis buffer was poured onto the gel to overlay it and 10 µl of the diluted sample was loaded into each well. The electrophoresis was performed in electrophoresis buffer for approximately 2 to 3 hours at 50 milli-ampere, followed by the transfer. The gel was first equilibrated for 30 minutes in transfer buffer (2.5 mMTris, 19.2 mM glycine, 20% methanol, 0.01% SDS, pH 8.8) and then placed onto a Semi-Dry

Blotter (Bio-Rad, South Africa) for Western blot analysis. The Western blot "sandwich" was assembled by placing a polyvinylidene fluoride (PVDF) 0.45 µm membrane (Biorad, South Africa), pre-soaked in methanol for 1 to 2 minutes, on top of the gel together with transfer buffer soaked filter papers on the outside of the "sandwich". Electrophoresis transfer conditions were maintained at 15 V for 1 hour at a current limit of 0.300 A.

After blotting, the PVDF membrane was placed in a blocking agent that contains 5% skimmed milk powder in TBS-0.1% Tween-20 for 1 hour at room temperature. After washing 6 times with TBS-0.1% Tween-20, the membrane was then placed into a 1:2,666 dilution of Rabbit anti-human VWF-HRP conjugated antibody (DAKO, South Africa) in TBS-0.1% Tween for 1 hour 15 minutes. It was then washed again for 8 times with TBS-0.1% Tween-20. Equal volumes of ACL Western blot detection reagent 1 and 2 (AEC Amersham, UK) were mixed and poured onto the membrane. After 1 minute, the excess detection reagent was dripped off and the membrane was sealed with plastic film and exposed to an X-ray film for 1 minute in the dark. Finally, the X-ray film was removed and developed in an automated film developer (Kodak, CA, USA). A picture was taken of the multimer patterns using the SYNGENE G-box gel documentation system (Vacutec, South Africa).

3.2.3.3 ADAMTS-13 levels

An ELISA plate was coated overnight at 4ºC with a mouse monoclonal antibody against ADAMTS-13 (R&D Systems, 1:1,000 dilution in PBS, 100µl per well). The next morning the plate was blocked with 4% bovine serum albumin (BSA) in PBS

(200 µl/well) for 2 hours at room temperature. After a wash step (with PBS/0.1%Tween-20, 4 X wash), the perfusates were added in duplicate (100 µl/well) and incubated for 2 hours at 37ºC. After another wash step, a rabbit polyclonal IgG antibody against ADAMTS-13 (Santa Crux Biotechnology, CA, USA) was added (1:100 dilution) and incubated for 1 hour at room temperature. A polyclonal goat anti- rabbit antibody conjugated with horseradish peroxidise (HRP) was used to detect the amount of ADAMTS-13 in the perfusates. This antibody was added in a 1:2,000 dilution after washing and incubated for another 1 hour at room temperature. We used OPD (50 $mg.L^{-1}$) as the substrate for HRP (the same concentration as with the VWF levels). As with the VWF antigen assay, the WHO 6^{th} FVIII/VWF standard was used as the standard against which the perfusates were measured. The results were expressed at percentage ADAMTS-13.

3.2.3.4 Detection of ADAMTS-13 in the perfusates by SDS-PAGE and Western Blot

The ADAMTS-13 protein in the perfusates was detected with a SDS-PAGE, followed by Western blot detection. The SDS-PAGE was prepared and performed using a 12% separating gel and a 4% stacking gel. The separating gel consists of 40% (v/v) polyacrylamide (30%), 11.25 mM Tris pH 8.8, 0.1% (v/v) SDS, 300 µl ammonium persulphate (APS) and 30 µl TEMED and the stacking gel of 13.3% (v/v) polyacrylamide (30%), 3.75 mM Tris pH 6.8, 0.1% SDS, 300 µl APS and 30 µl TEMED. Once the gels were prepared and polymerized on glass plates, the polyacrylamide gels were mounted in a Mini-II apparatus (Bio-Rad, South Africa) and covered with 1 X running buffer (10 X running buffer: 250 mM Tris, 1.92 M glycine and 1% SDS in an aqueous solution). The molecular weight protein

marker, Roti®-Mark-prestained (Carl Roth, Germany) was loaded (5 µl for Coomassie staining) in one of the twelve wells during sample preparation. Concentrated Laemmli buffer (4 X denaturing buffer: 200 mM TrisHCl, pH 6.8, 40% Glycerol, 30% β-mercaptoethanol (disulphide bridge reduction), 10% SDS and 0.2% bromophenol blue) was added to the samples and put on a heating block at 95°C for 5 minutes. Ten micro-litres of each 20 µl sample was loaded. Two gels were run at a constant voltage (200 mV/s) using a Bio-Rad electrophoresis apparatus.

Following SDS-PAGE, one gel was incubated overnight in Coomassie blue staining solution (25% isopropanol, 10% acetic acid and 0.05% Coomassie Brilliant Blue per litre). After careful removal, the gel was bathed in de-staining solution (10% ethanol, 10% acetic acid) until bands appear. For the efficient removal of excess Coomassie staining, absorbing paper were used along with the exchange of de-staining solution.

For the identification of the ADAMTS-13 protein in the perfusates, the unstained gel was blotted onto a PVDF membrane, as previously described in Section 3.2.3.2.

After blotting, the PVDF membrane was placed in a blocking agent that contains 2% skimmed milk powder in TBS-0.1% Tween-20 for 1 hour at room temperature and washed 6 times with TBS-0.1% Tween-20. The membrane was then placed into a 1:100 dilution of a rabbit polyclonal IgG antibody against human ADAMTS-13 (Santa Crux Biotechnology, CA, USA) and incubated for 1 hour at room

temperature. After washing again for 6 times with TBS-0.1% Tween-20, a polyclonal goat anti- rabbit antibody conjugated with horseradish peroxidise (HRP) (1:2,000 dilution) was used to detect the presence of ADAMTS-13. After incubation for another hour at room temperature, the membrane was washed again and equal volumes of ACL Western blot detection reagent 1 and 2 (AEC Amersham, UK) were mixed and poured onto the membrane. After 1 minute, the membrane was sealed with plastic film and exposed to an X-ray film for 1 to 10 minutes in the dark. Finally, the film was removed and developed in an automated film developer (Kodak, CA, USA). A picture was taken of the multimer patterns using the SYNGENE G-box gel documentation system (Vacutec, South Africa).

Next, the picture was scanned into a computer and the strength of the signal of ADAMTS-13 was quantified by densitometric analysis using the ImageJ software. Densitometric analysis of ADAMTS-13 content was expressed as area pixel intensity from which the relative density of the signal (band) was calculated. The relative density was calculated by dividing the percent value for each sample per treatment by the percent value for the control (standard) sample.

3.2.3.5 VWF propeptide levels

VWF propeptide levels in the perfusates were determined using an ELISA. In short, a 96-well ELISA plate was coated overnight at 4ºC with a monoclonal antibody against the VWF propeptide (CLB-Pro 35, Euro-Immune, Germany, 1:100 dilution in PBS, 100 µl per well). The next morning the plate was blocked with 4% bovine serum albumin (BSA) in PBS (200 µl/well) for 2 hours at room temperature and washed with PBS/0.1%Tween-20, 4 X wash. The perfusates

were added in duplicate (100 µl/well) and incubated for 2 hours at 37ºC. An HRP-conjugated monoclonal antibody against VWF propeptide (CLB-Pro 14.3, Euro-immune, Germany) was added in a 1:100 dilution after another wash step, and incubated for 1 hour at room temperature in order to detect the concentration of VWF propeptide in the perfusate. We used OPD (50 mg.L-1) as the substrate for HRP, again the same as with the VWF levels. The WHO (World Health Organisation) 6th FVIII/VWF standard was again used as the standard against which the perfusates were measured. The results were expressed at percentage VWF:propeptide.

It is important to notice that the whole experiment was done three times. Thus for each stimulant we culture 6 flasks, three control and three treated flasks. We thus had 6 perfusates per stimulant, and each perfusate was measured in duplicate for the VWF, ADAMTS-13 and VWF propeptide levels. The percentage increase or decrease in the mean measurements of the treated samples was calculated from the control samples. All the experimental data were presented as mean±SD (standard deviation). The unpaired 2-tailed Student t-test was used for data analysis of all ELISA assays and P values less than $P<0.05$ were considered statistically significant.

CHAPTER 4

RESULTS

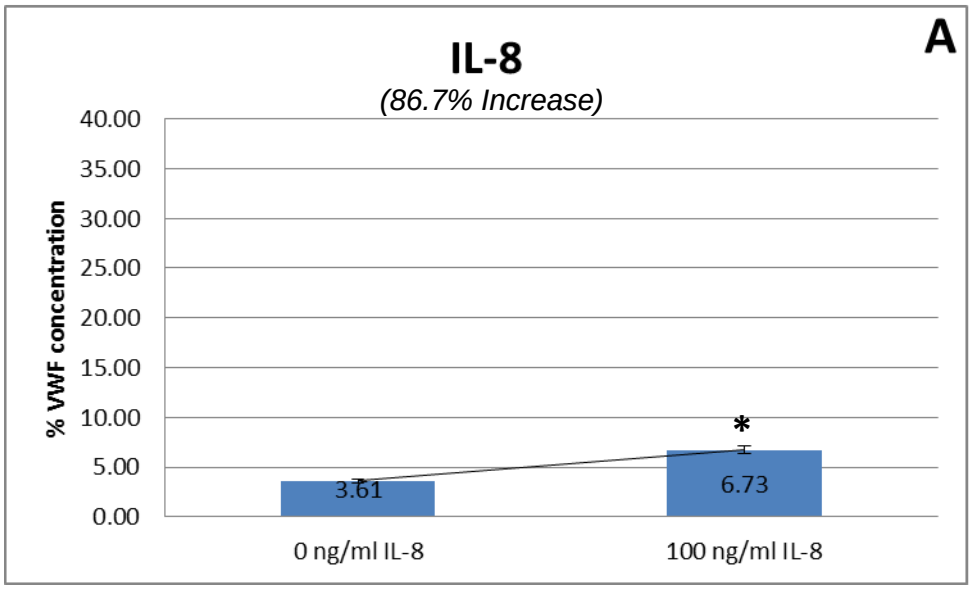
4.1 Endothelial cell culture

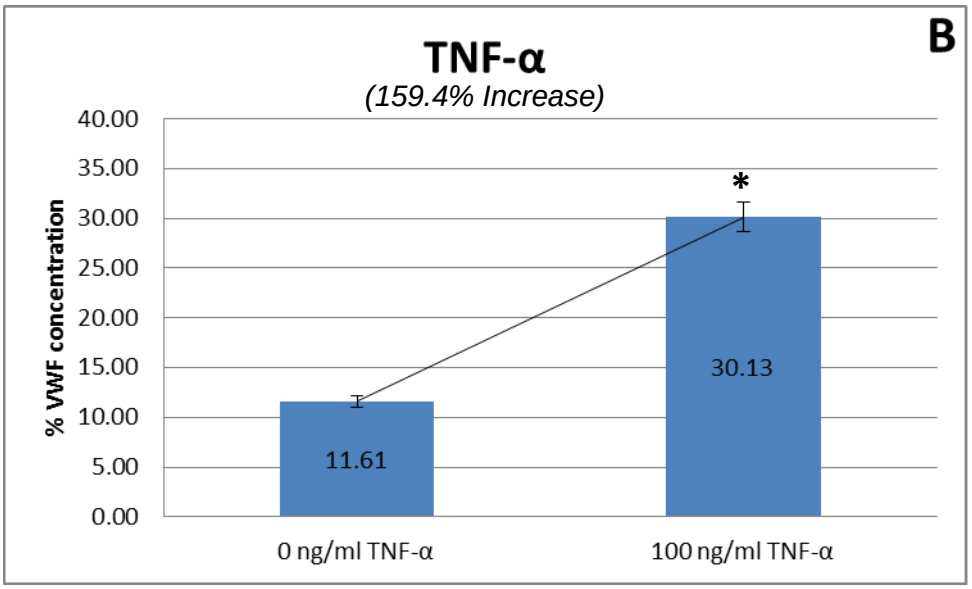
The HUVECs were grown from a concentration of 1.25 x 10^4 cells/ml and a viability of +95% to a confluency of 100% through microscopic examination before each experiment. Each dish was individually inspected for contamination, concentration, viability and confluency after the 4^{th} passage. After treatment (treated) or non-treatment (controls), the dishes were placed in a 37°C, 5%CO_2/95% air, humidified cell culture incubator under the same conditions. The treatment and control flasks were placed under the same conditions on the ROTEM orbital shaker to induce shear stress, and the perfusates of the treatment and control flasks were collected and stored in the same way.

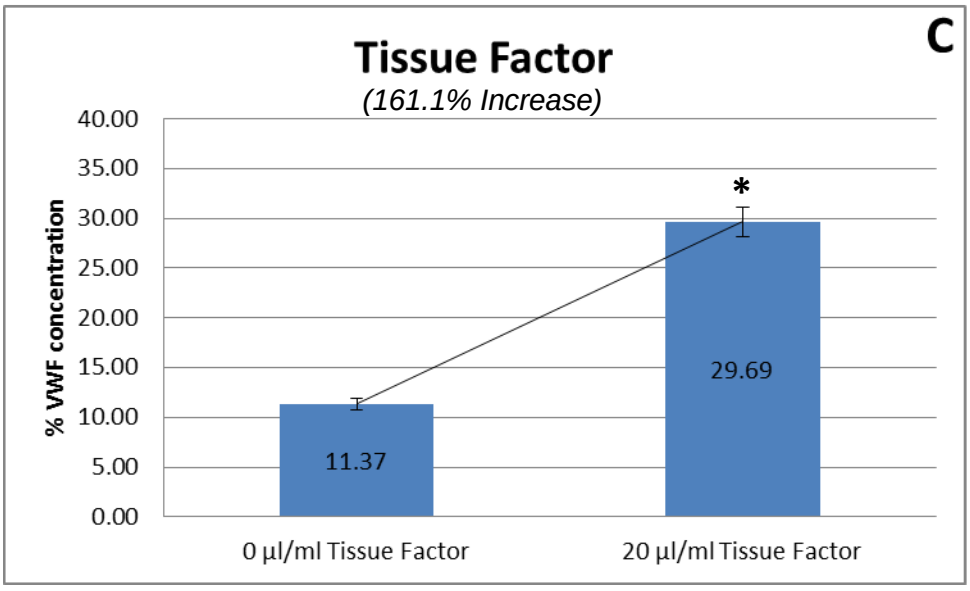
4.2 VWF levels

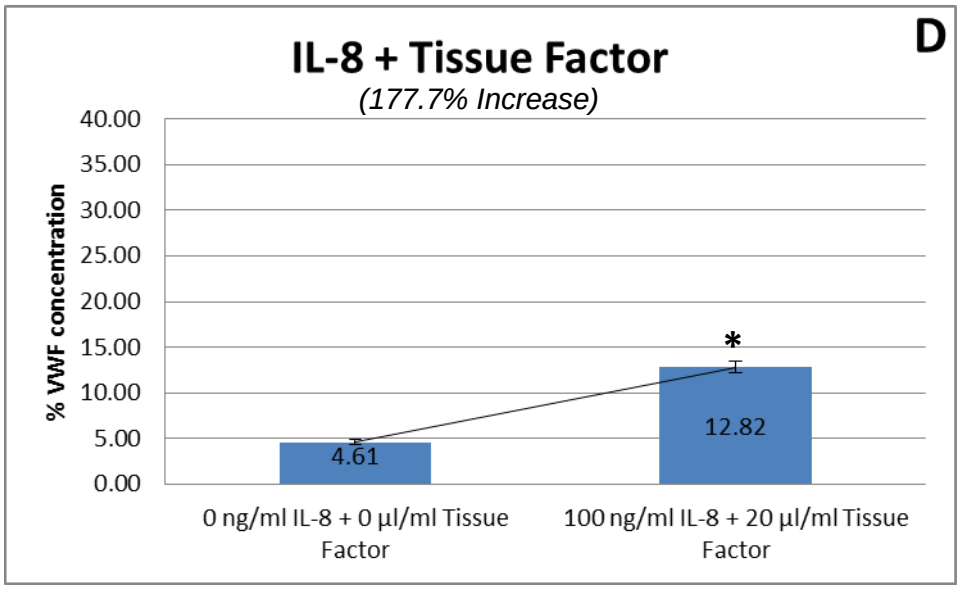
The stimulatory effect of cytokines (IL-6, IL-8 and TNF-α), coagulation factors (thrombin and tissue factor) and combinations of coagulation factors and cytokines (IL-8+thrombin, TNF-α+thrombin, IL-8+tissue factor and TNF-α+tissue factor) on human umbilical vein endothelial cells (HUVECs) were tested by measuring the levels of VWF in the perfusates.

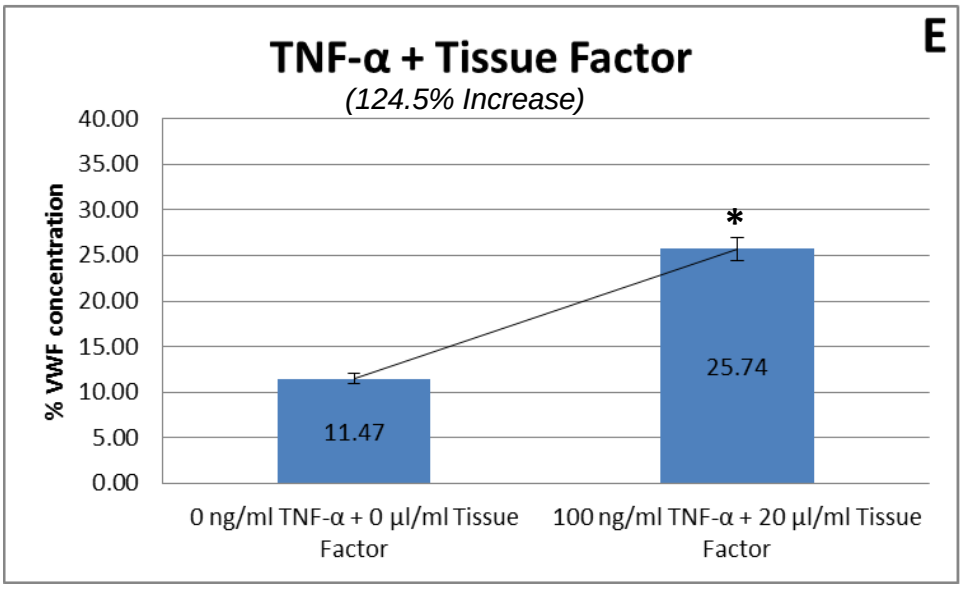
The amounts of VWF secreted by the HUVECs were increased in the presence of all treatments as shown in Figure 4.1. IL-8 alone increased the VWF levels by 86.7% (P = 0.002, Figure 4.1 A). However, in combination with TF, the highest increase of 177.7% (P = 0.00002) increase was observed (Figure 4.1 D). In combination with thrombin, an increase of 72.2% (P = 0.008) was obtained (Figure 4.1 G) thus no difference than that of IL-8 alone. TNF-α alone showed a remarkable increase in VWF levels of 159.4% (P = 0.0001, Figure 4.1 B). This is surely the cytokine with the most potent stimulatory effect on VWF secretion. In combination with thrombin or TF, TNF-α showed the same potent effect (Figure 4.1 E). TF alone showed a remarkable stimulatory effect of 161.1% (P = 0.001) on VWF secretion (Figure 4.1 C). This is the first time where the effect of tissue factor was tested on VWF secretion in HUVEC cells. Thrombin also showed a strong stimulatory effect of 113.6% (P = 0.0275) on VWF secretion (Figure 4.1 F). Both coagulation factors showed the same potent effect in combination with IL-8 and TNF-α than alone. The highest effect on VWF secretion was, however, observed with the combination of IL-8 and tissue factor (P = 0.00002, Figure 4.1 D). IL-6, showed no effect on the release of VWF from HUVECs (P = 0.40949, Figure 4.1 I). The data of individual runs are given in Appendix 1.

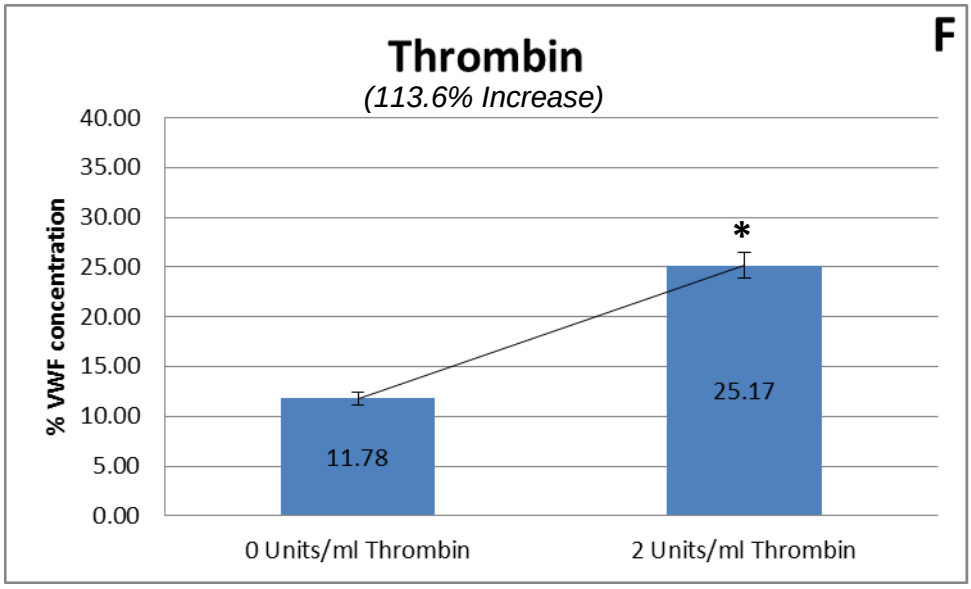

A
IL-8
(86.7% Increase)
% VWF concentration
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
3.61
6.73
*
0 ng/ml IL-8
100 ng/ml IL-8


B
TNF-α
(159.4% Increase)
% VWF concentration
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
11.61
30.13
*
0 ng/ml TNF-α
100 ng/ml TNF-α


C
Tissue Factor
(161.1% Increase)
% VWF concentration
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
11.37
29.69
*
0 µl/ml Tissue Factor
20 µl/ml Tissue Factor


D
IL-8 + Tissue Factor
(177.7% Increase)
% VWF concentration
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
4.61
12.82
*
0 ng/ml IL-8 + 0 µl/ml Tissue Factor
100 ng/ml IL-8 + 20 µl/ml Tissue Factor


E
TNF-α + Tissue Factor
(124.5% Increase)
% VWF concentration
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
11.47
25.74
*
0 ng/ml TNF-α + 0 µl/ml Tissue Factor
100 ng/ml TNF-α + 20 µl/ml Tissue Factor


F
Thrombin
(113.6% Increase)
% VWF concentration
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
11.78
25.17
*
0 Units/ml Thrombin
2 Units/ml Thrombin

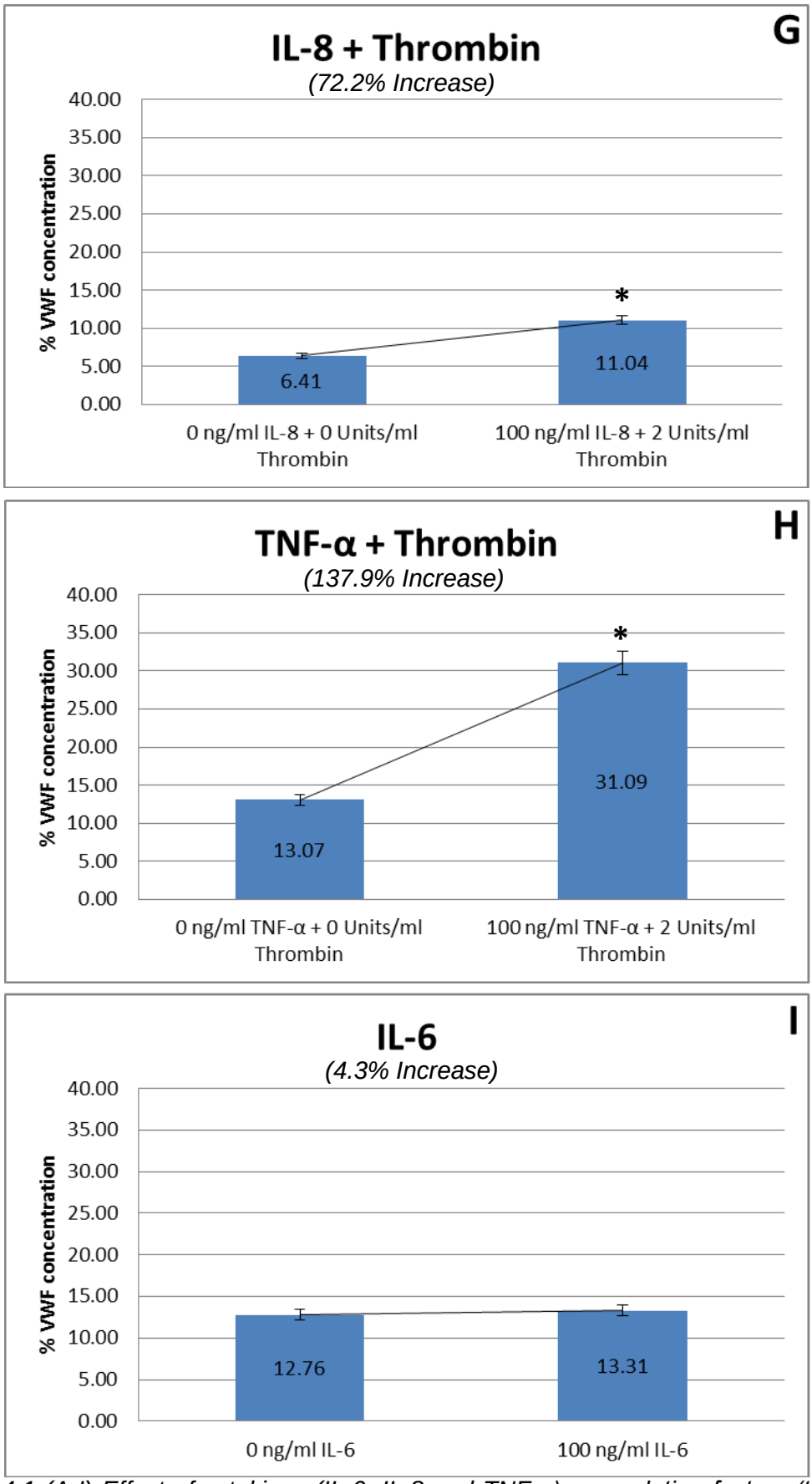


*Figure 4.1 (A-I) Effect of cytokines (IL-6, IL-8 and TNF-α), coagulation factors (thrombin and tissue factor) and combined coagulation factor/cytokine stimulations (IL-8+thrombin, TNF-α+thrombin, IL-8+tissue factor and TNFα+tissue factor) on the release of VWF from HUVECs (n = 6, mean ±SD, *P<0.05).*

4.3 VWF multimeric analysis

The VWF multimeric analysis was performed to determine whether the cytokines (IL-6, IL-8 and TNF-α), coagulation initiators (thrombin and tissue factor) and combined coagulation-initiator/cytokine stimulations (IL-8+thrombin, TNF-α+thrombin, IL-8+tissue factor and TNF-α+tissue factor) affected the multimeric structure of VWF. Via a Western blot technique, we observed the distribution of the VWF multimer patterns from large, intermediate to small multimers. When stimulated with IL-6, IL-8, TNF-α, thrombin, tissue factor, IL-8+thrombin, TNF-α+thrombin, IL-8+tissue factor and TNF-α+tissue factor, normal VWF multimer patterns were observed in all treated and untreated samples, distributing from large to small multimers (Figure 4.2). It is, however, important to notice the absence of the VWF dimer peak as seen in normal plasma.

Due to the normal distribution of VWF multimers, we assume that the VWF cleaving protease, ADAMTS-13 was present. Therefore, the next logical step was to measure the levels of ADAMTS-13.

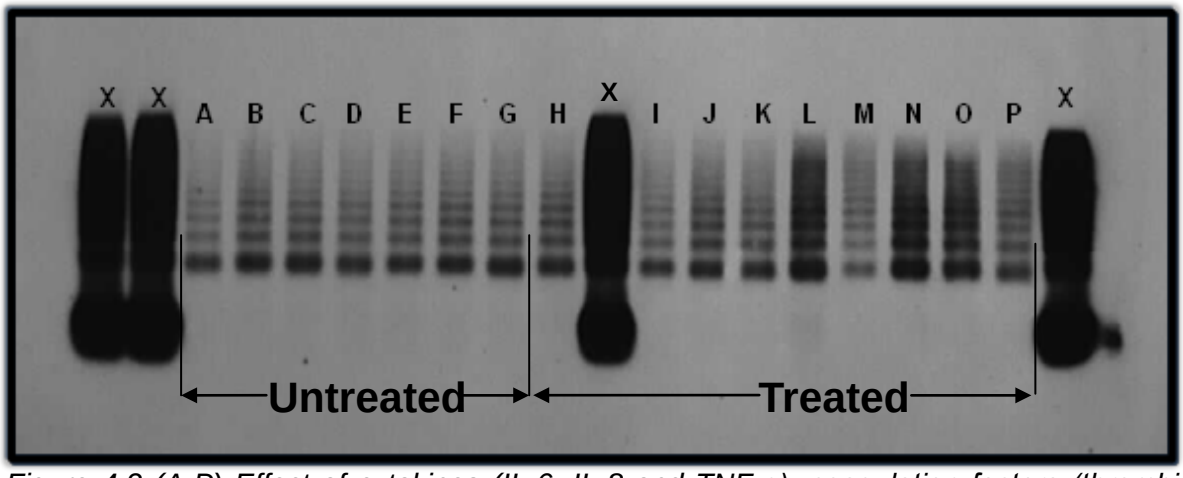
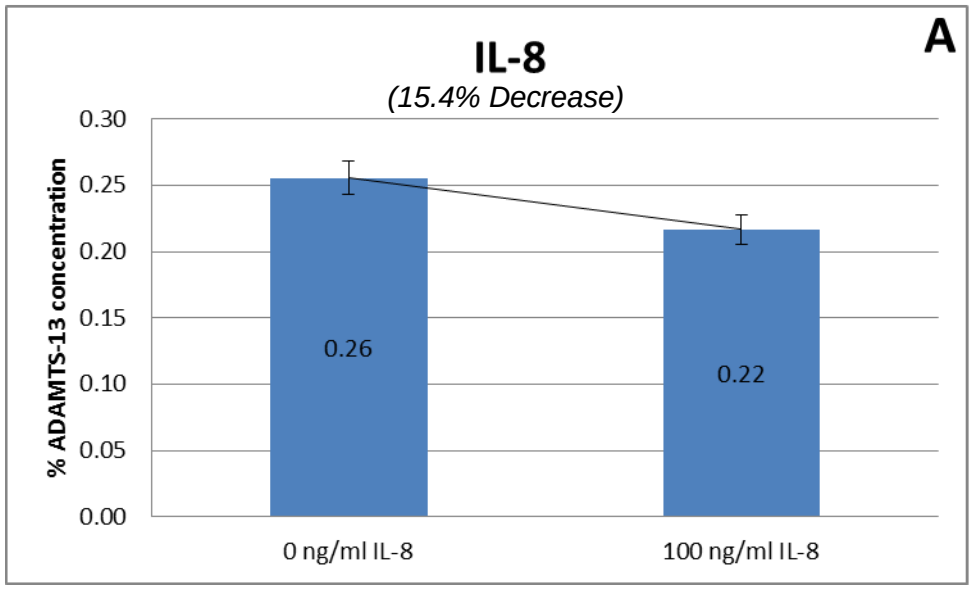


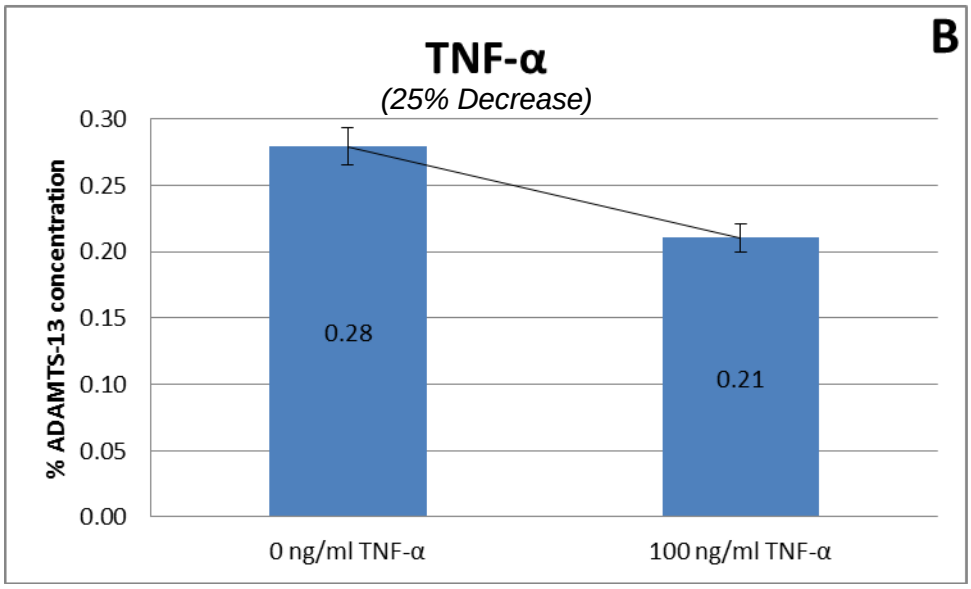
Figure 4.2 (A-P) Effect of cytokines (IL-6, IL-8 and TNF-α), coagulation factors (thrombin and tissue factor) and combined coagulation-initiator/cytokine stimulations (IL-8+thrombin, TNF-α+thrombin, IL-8+tissue factor and TNF-α+tissue factor) on the multimer structure of VWF from human umbilical vein endothelial cells (HUVECs).IL-6 (A & I), IL-8 (B & J), TNF-α (C & K), thrombin (D & L), tissue factor (E & M), IL-8+thrombin(F & N), TNF-α+thrombin (G & O), IL-8+tissue factor (H)and TNF-α+tissue factor (P). X was the VWF multimer pattern of normal plasma.

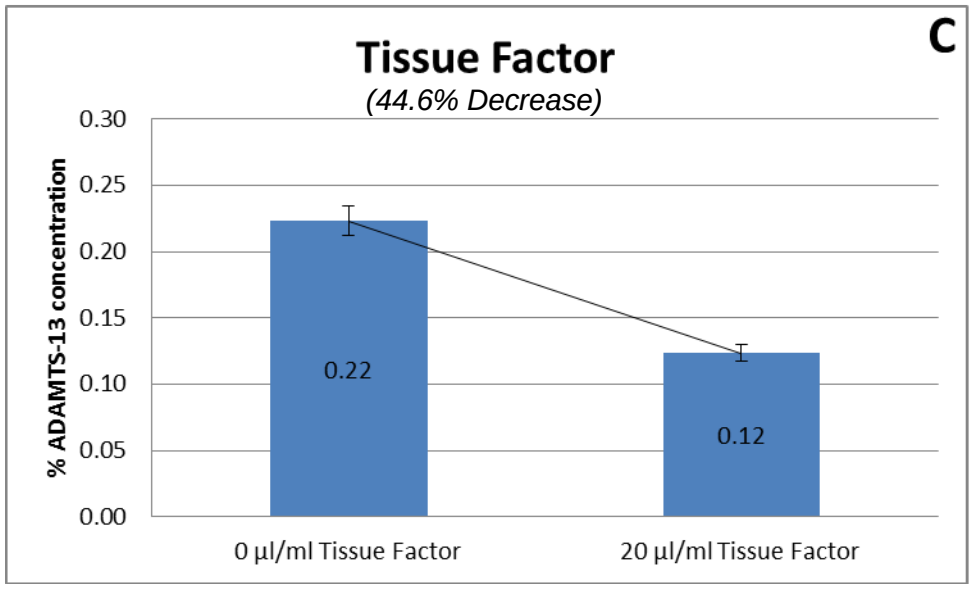
4.4 ADAMTS-13 levels

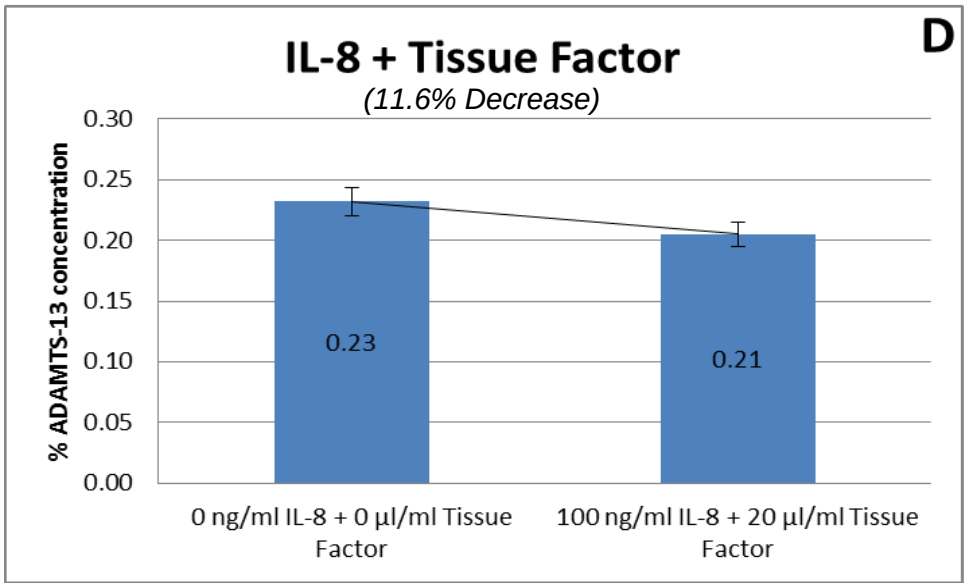
In addition to the effects on the synthesis and multimer patterns of VWF from HUVECs, we also determined whether these cytokines (IL-6, IL-8 and TNF-α), coagulation factors (thrombin and tissue factor) and combinations thereof (IL-8+thrombin, TNF-α+thrombin, IL-8+tissue factor and TNF-α+tissue factor) did affect the secretion of the VWF cleaving protease, ADAMTS-13. We have previously demonstrated (section 4.3) that VWF strings are cleaved, just by looking at the VWF multimer patterns. To determine the effects on ADAMTS-13 levels, we stimulated HUVECs with IL-6, IL-8, TNF-α, thrombin, tissue factor, IL-8+thrombin, TNF-α+thrombin, IL-8+tissue factor and TNF-α+tissue factor. The same perfusates were used as those that we used for the VWF level measurements. ADAMTS-13 levels were detected in both treated and untreated samples and revealed the complete opposite results to VWF levels in figure 4.1.

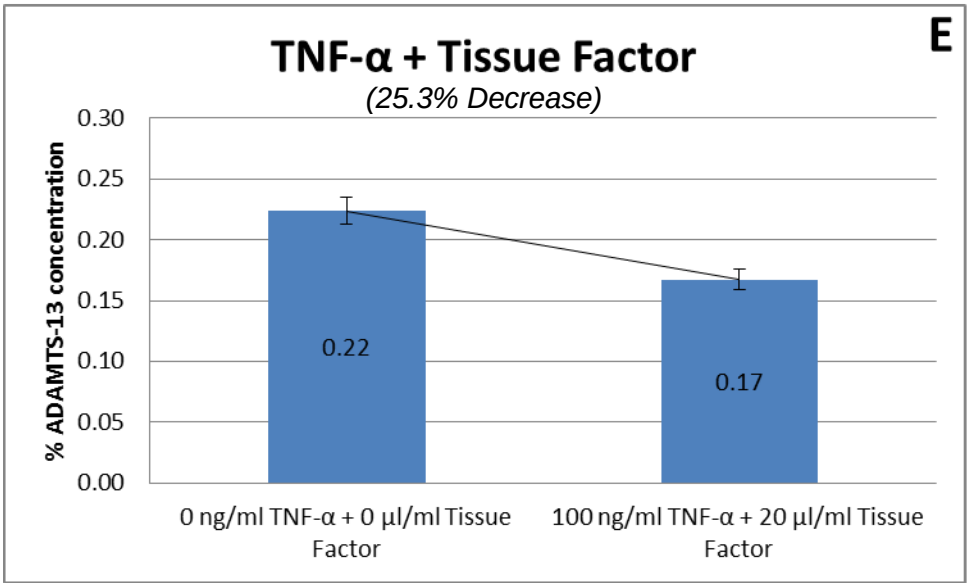
The levels of ADAMTS-13 detected in the presence of IL-8, TNF-α, tissue factor, and combinations of the IL-8 and tissue factor or TNF-α and tissue factor, decreased with the dose administered (Figure 4.3). In contrast, thrombin, and combinations of IL-8 and thrombin or TNF-α and thrombin, increased the secretion of ADAMTS-13 levels a little in the ELISA (Figure 4.3 F-H). However, IL-6 had no stimulatory or inhibitory effects on the release of ADAMTS-13 from HUVECs (Figure 4.3 I). As a result of the exceedingly low ADAMTS-13 levels in the perfusates, the results of the ELISA are not convincing and moreover, presented no statistical significance ($P>0.05$). An SDS-PAGE and Western blot were performed to support the position of ADAMTS-13 levels detected. The data of individual runs are given in Appendix 2.

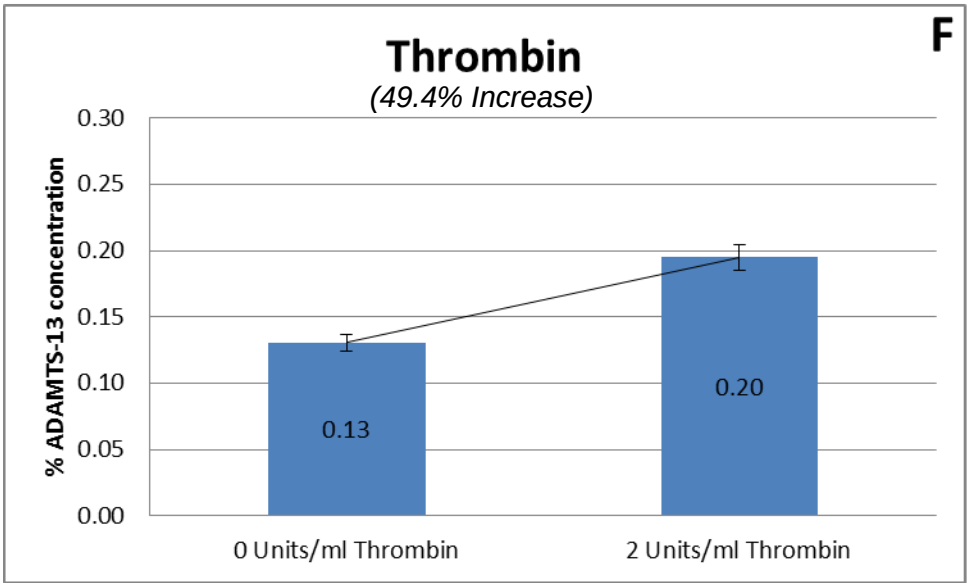

A
IL-8
(15.4% Decrease)
% ADAMTS-13 concentration
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.26
0.22
0 ng/ml IL-8
100 ng/ml IL-8


B
TNF-α
(25% Decrease)
% ADAMTS-13 concentration
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.28
0.21
0 ng/ml TNF-α
100 ng/ml TNF-α


C
Tissue Factor
(44.6% Decrease)
% ADAMTS-13 concentration
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.22
0.12
0 µl/ml Tissue Factor
20 µl/ml Tissue Factor


D
IL-8 + Tissue Factor
(11.6% Decrease)
% ADAMTS-13 concentration
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.23
0.21
0 ng/ml IL-8 + 0 µl/ml Tissue Factor
100 ng/ml IL-8 + 20 µl/ml Tissue Factor


E
TNF-α + Tissue Factor
(25.3% Decrease)
% ADAMTS-13 concentration
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.22
0.17
0 ng/ml TNF-α + 0 µl/ml Tissue Factor
100 ng/ml TNF-α + 20 µl/ml Tissue Factor


F
Thrombin
(49.4% Increase)
% ADAMTS-13 concentration
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.13
0.20
0 Units/ml Thrombin
2 Units/ml Thrombin

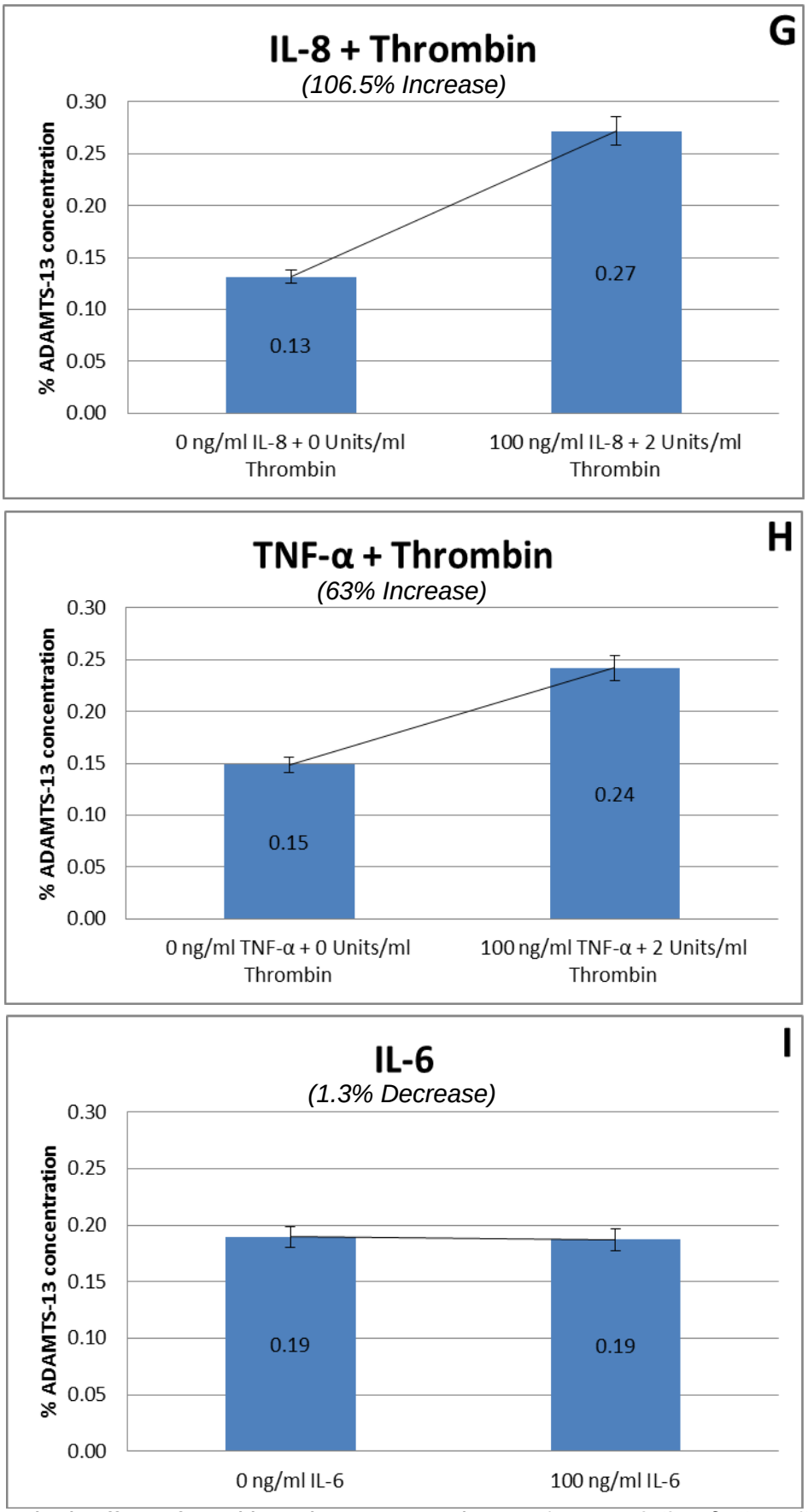


Figure 4.3 (A-I) Effect of cytokines (IL-6, IL-8 and TNF-α), coagulation factors (thrombin and tissue factor) and combined coagulation factor/cytokine stimulations (IL-8+thrombin, TNF-α+thrombin, IL-8+tissue factor and TNF-α+tissue factor) on the secretion of the VWF cleaving protease, ADAMTS-13, from HUVECs (n = 6, mean ±SD).

4.5 Presence of ADAMTS-13

After detecting extremely low levels of ADAMTS-13 in the perfusate, supporting evidence had to be gathered. Two approaches were followed to identify the presence of ADAMTS-13 in the perfusates after the stimulation of HUVECs. First, an SDS-PAGE was done to separate the proteins in the perfusate. The results are presented in Figure 4.4 A and B. The ADAMTS-13 protein were present in both the SDS PAGE and the Western blot. This confirmed that ADAMTS-13 protein was present in both treated and untreated samples.

Secondly, Western blotting densitometric analysis showed a decrease in the relative density of the treated samples compared to that of the controls (untreated samples) (Figure 4.5 A-I). The density of all stimulated samples ranges between a ratio of 0.3 and 0.5 of that of the control samples. This is, however, not a quantitative measurement, but it shows that less ADAMTS-13 is secreted when endothelial cells were stimulated with the IL-6, IL-8, TNF-α, thrombin and TF and combinations of TF and thrombin with the cytokines. The data of densitometric analysis are given in Appendix 3.

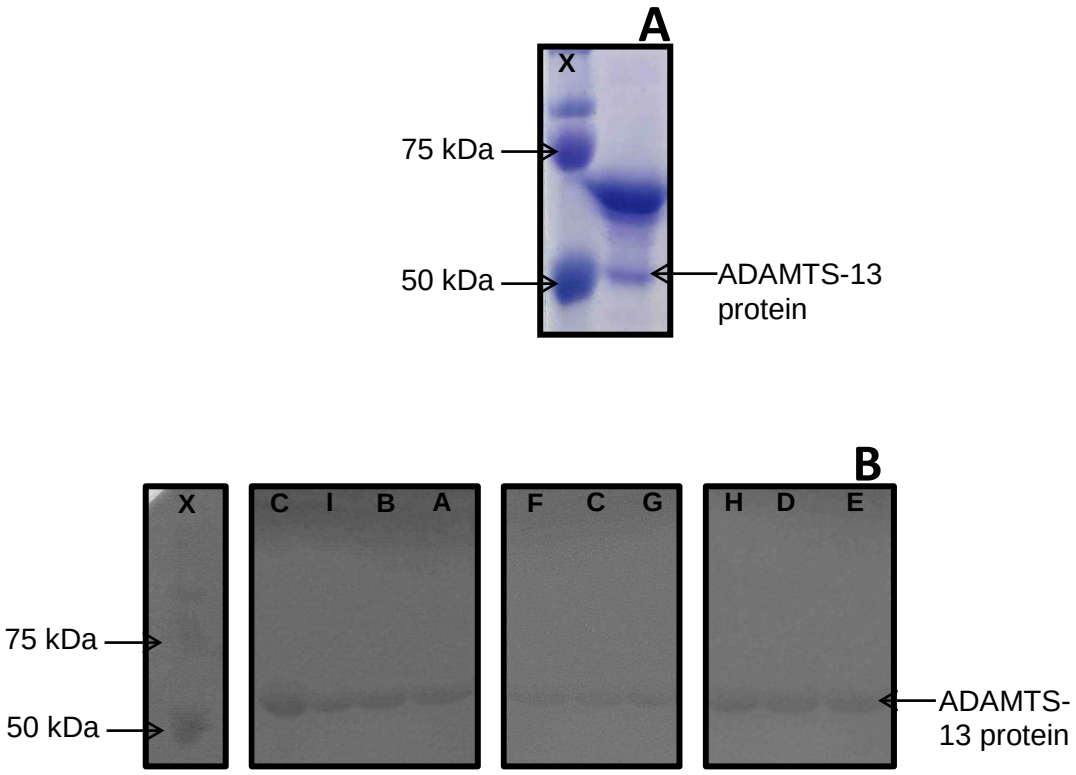
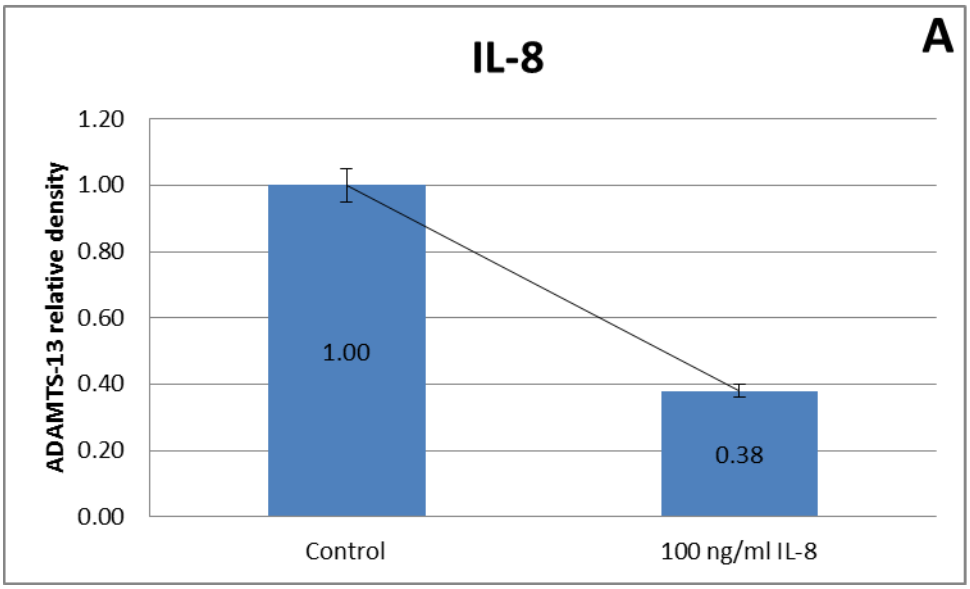
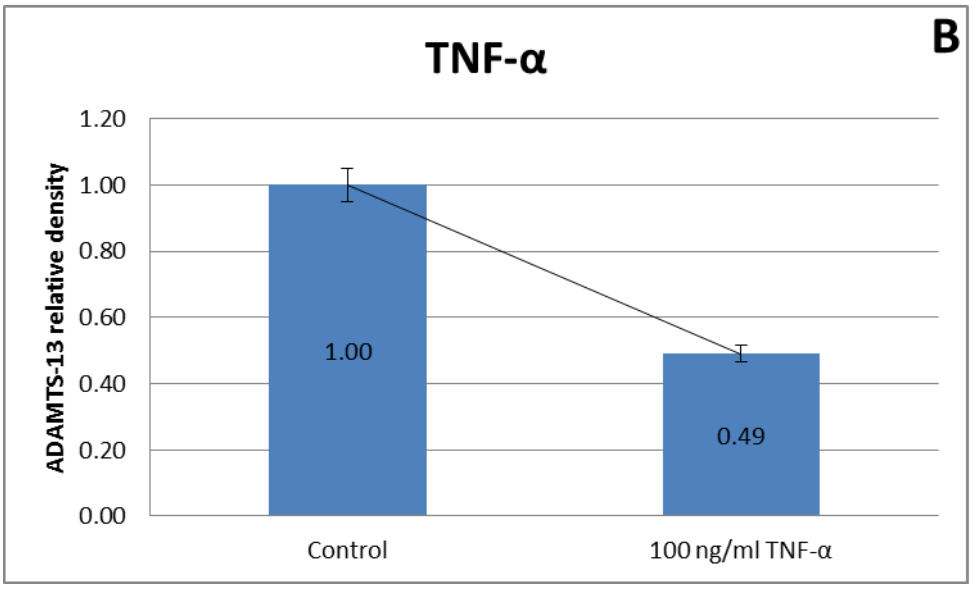
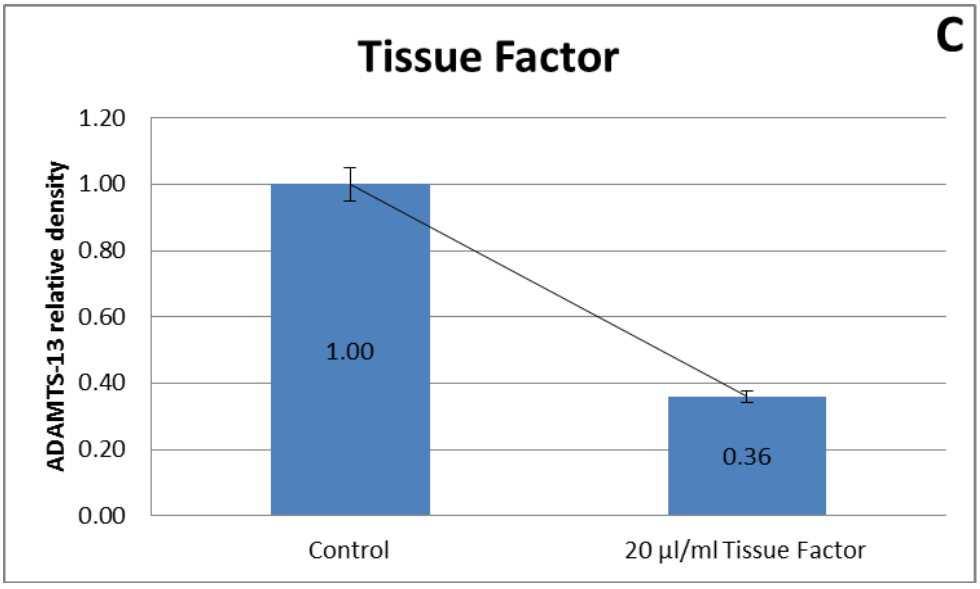
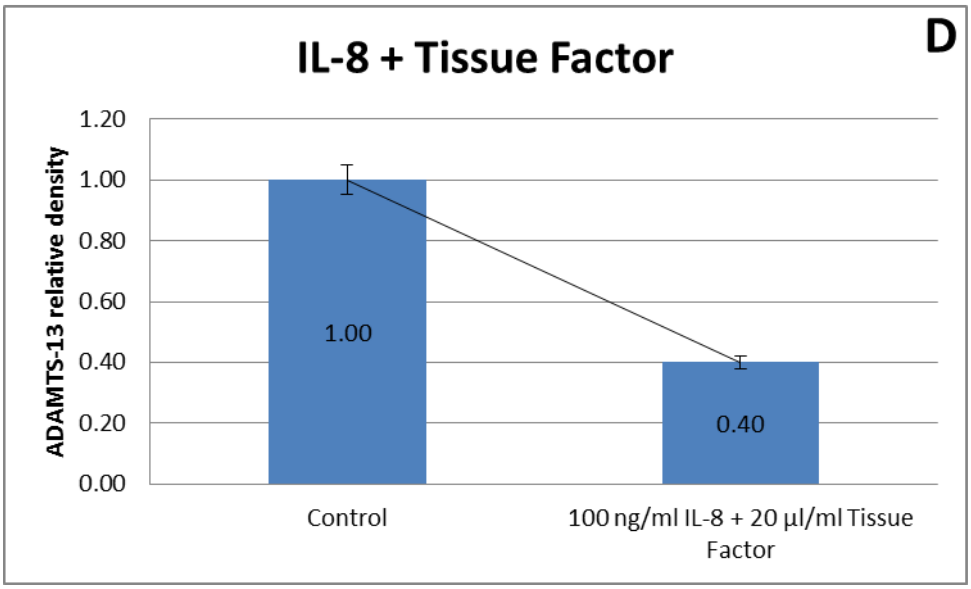


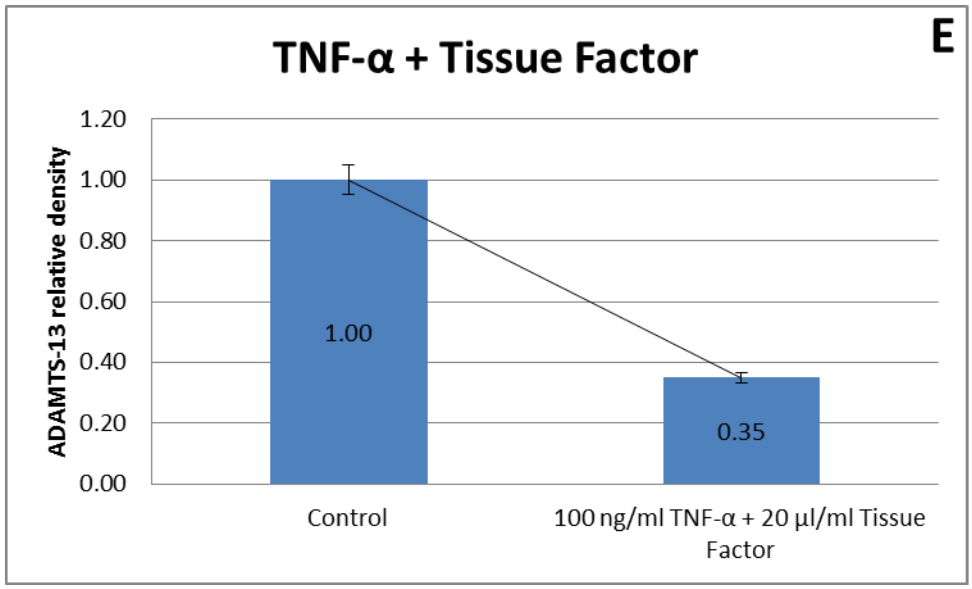
Figure 4.4 (A-B) SDS-PAGE (A) and Western blot (B) indicating the presence of the ADAMTS-13 protein in all samples. For illustration purposes, only some samples are shown. IL-6 (I), IL-8 (A), TNF-α (B), thrombin (F), tissue factor (C), IL-8+thrombin(G), TNF-α+thrombin (H), IL-8+tissue factor (D)and TNF-α+tissue factor (E). X represented the ladder and C the 0 ng/ml control.

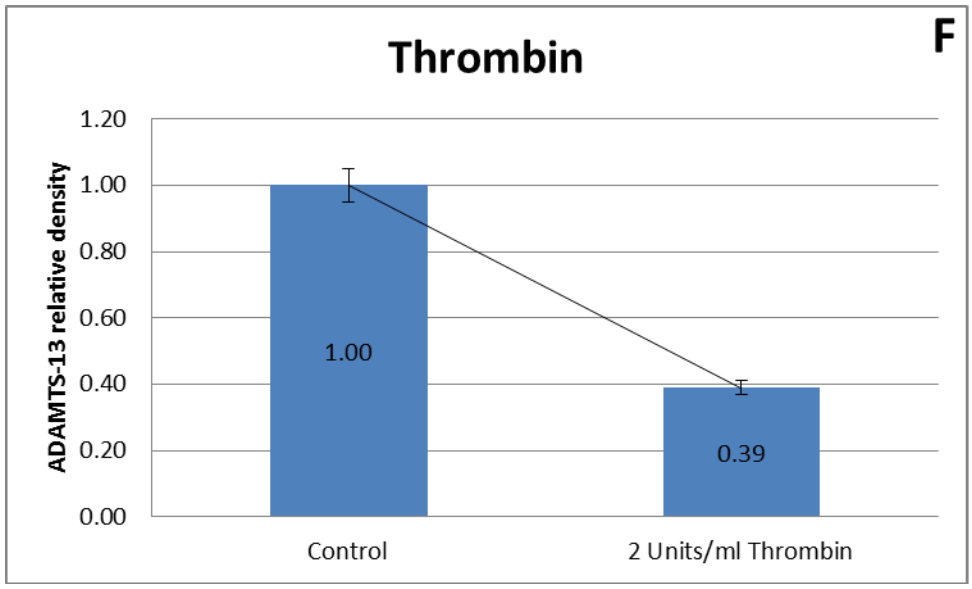

A
IL-8
ADAMTS-13 relative density
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1.00
0.38
Control
100 ng/ml IL-8


B
TNF-α
ADAMTS-13 relative density
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1.00
0.49
Control
100 ng/ml TNF-α


C
Tissue Factor
ADAMTS-13 relative density
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1.00
0.36
Control
20 µl/ml Tissue Factor


D
IL-8 + Tissue Factor
ADAMTS-13 relative density
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1.00
0.40
Control
100 ng/ml IL-8 + 20 µl/ml Tissue Factor


E
TNF-α + Tissue Factor
ADAMTS-13 relative density
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1.00
0.35
Control
100 ng/ml TNF-α + 20 µl/ml Tissue Factor


F
Thrombin
ADAMTS-13 relative density
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1.00
0.39
Control
2 Units/ml Thrombin

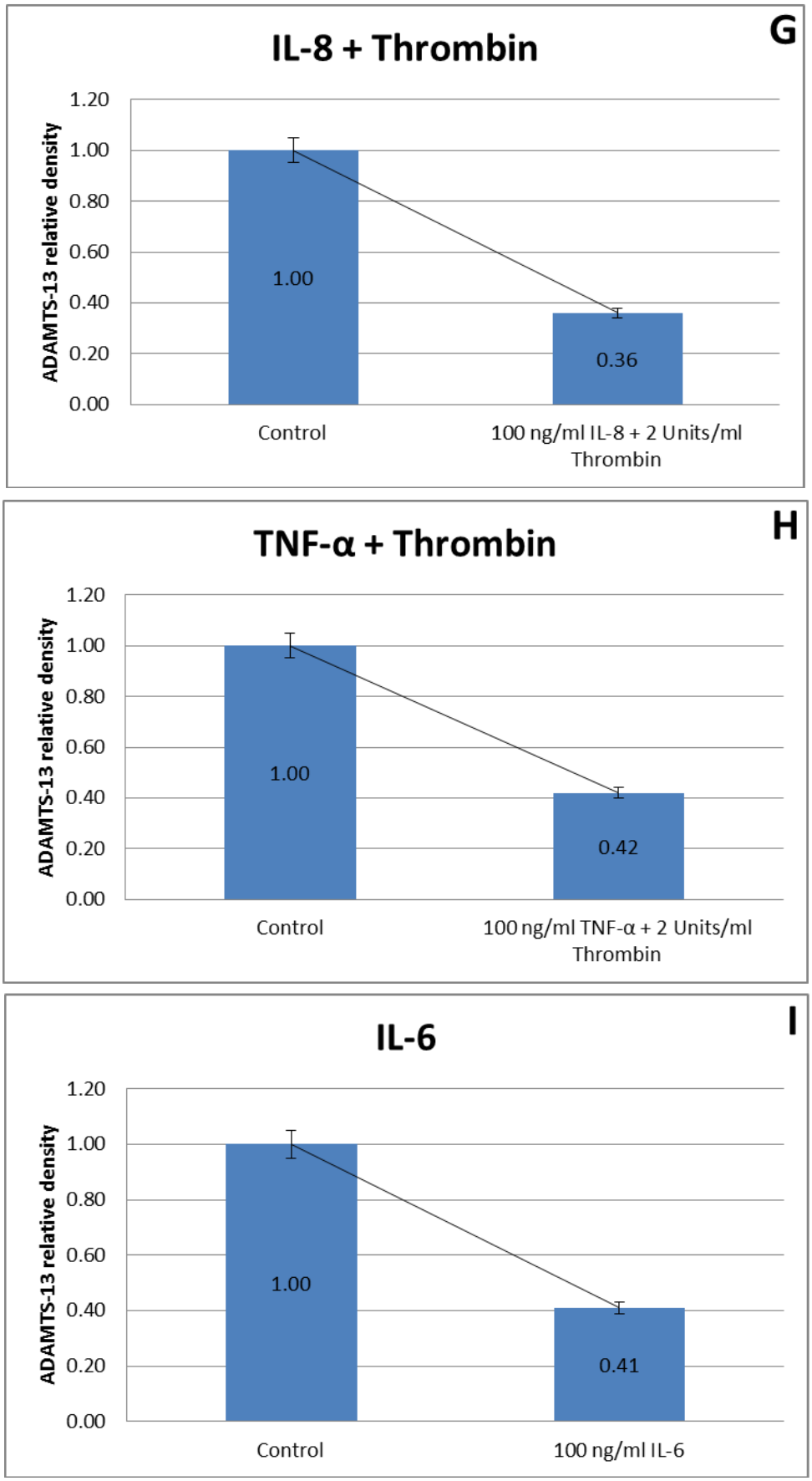


Figure 4.5 (A-I) Densitometric ratios of the effect of cytokines (IL-6, IL-8 and TNF-α), coagulation initiators (thrombin and tissue factor) and combined coagulation-initiator/cytokine stimulations (IL-8+thrombin, TNF-α+thrombin, IL-8+tissue factor and TNF-α+tissue factor) on the synthesis of the VWF cleaving protease, ADAMTS-13, from HUVECs (n = 3, mean ±SD).

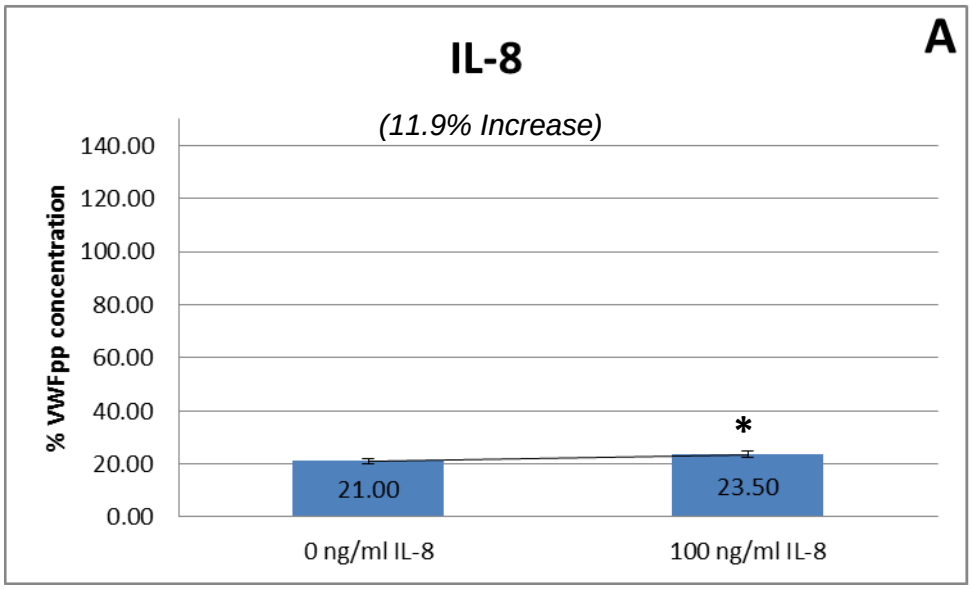
4.6 VWF propeptide levels

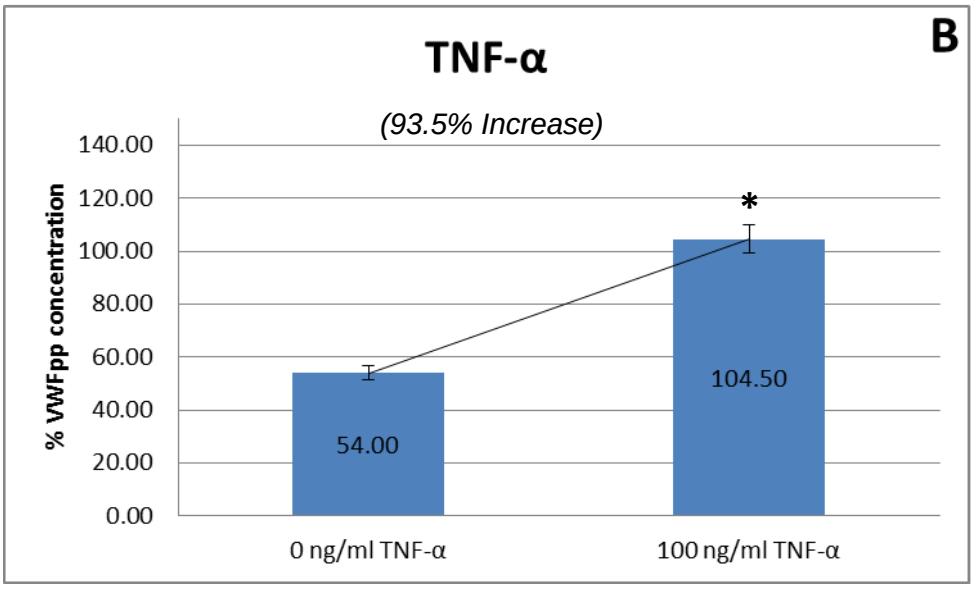
The VWF propeptide (VWF:pp) levels were also measured in the perfusate, since it provides a more accurate measurement of VWF secretion (Ragni, 2006). The effect of cytokines (IL-6, IL-8 and TNF-α), coagulation factors (thrombin and tissue factor) and combined coagulation factor/cytokine stimulations (IL-8+thrombin, TNF-α+thrombin, IL-8+tissue factor and TNF-α+tissue factor) on the release of the VWF:pp were determined by measuring the levels of VWF:pp in the perfusate. The VWF:pp levels were detected in both treated and untreated samples (Figure 4.6).

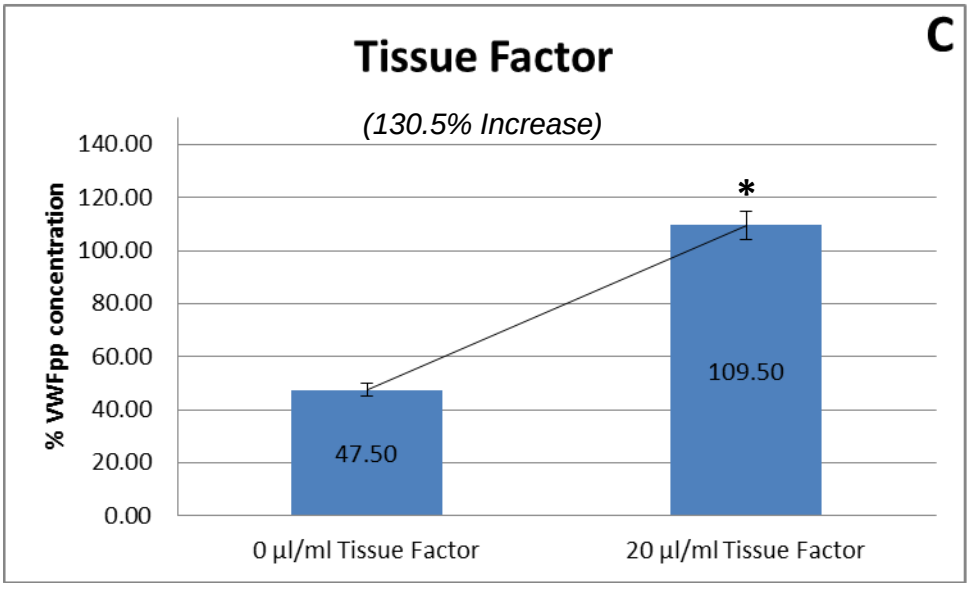
The levels of VWF:pp secreted in the presence of all treatments, except for IL-6, increased with the dose administered. The levels of VWF:pp secreted by endothelial cells increased in the following order from low to high: IL-8, IL-8+thrombin, thrombin, TNF-α, TNF-α+thrombin, tissue factor, IL-8+tissue factor, and with the highest increase seen at TNF-α+tissue factor treatment (Figure 4.6 A-H). These results resemble that of the VWF antigen levels in the perfusate.

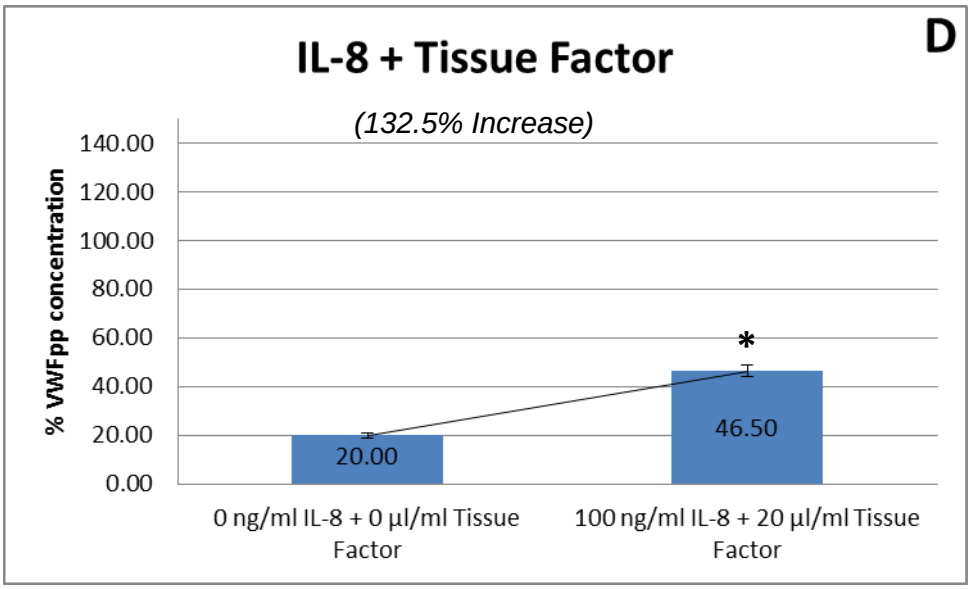
Stimulation with IL-8 alone increased the VWF:pp levels by 11.9% ($P = 0.00995$, Figure 4.6 A). In combination with TF, a high increase of 132.5% ($P = 0.00089$) was observed (Figure 4.6 D). In combination with thrombin, an increase of 64.8% ($P = 0.01823$) was obtained (Figure 4.6 G). This is not as much as with the TF combination. TNF-α increased VWF:pp levels markedly by 93.5% ($P = 0.00022$, Figure 4.6 B). This is surely the cytokine with the most potent stimulatory effect on VWF:pp secretion. In combination with thrombin or TF, TNF-α was even more potent and increase the VWF:pp by 132.5% ($P = 0.00089$) with TF and 115.5% (P

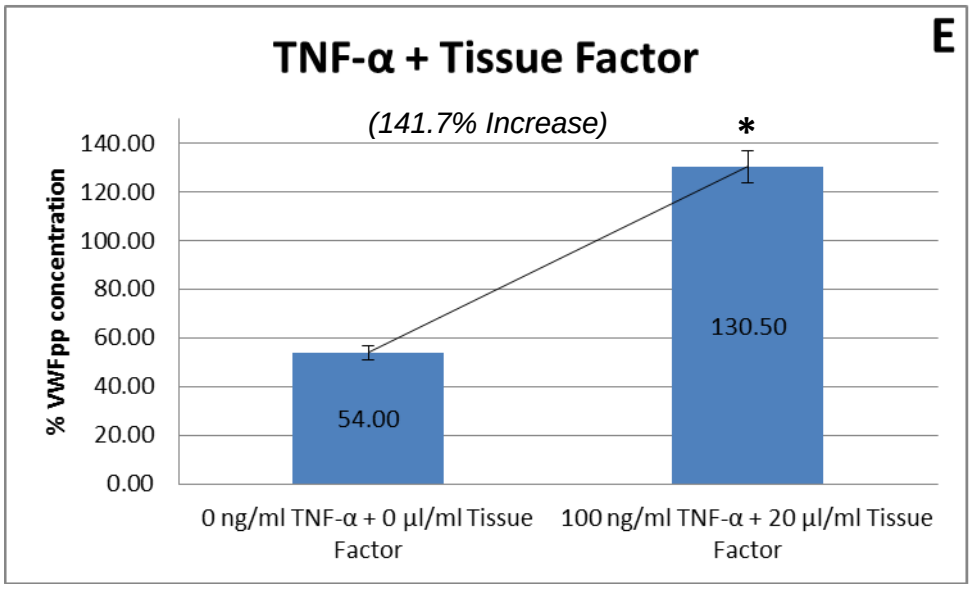
= 0.00009) with thrombin (Figure 4.6 E and H). TF had a remarkable stimulatory effect of 130.5% (P = 0.00016) on VWF:pp secretion (Figure 4.6 C). This is the first time where the effect of tissue factor was tested on VWF:pp secretion in HUVEC cells. Thrombin also stimulated VWF:pp secretion by 90.5% (P = 0.00037) on VWF secretion (Figure 4.6 F). Both coagulation factors had the same potent effect in combination with IL-8 and TNF-α than alone. The highest effect on VWF:pp secretion was, however, observed with the combination of TNF-α and tissue factor (Figure 4.6 E). IL-6 had almost no effect on the release of VWF from HUVECs (Figure 4.1 I). The data of individual runs are given in Appendix 4.

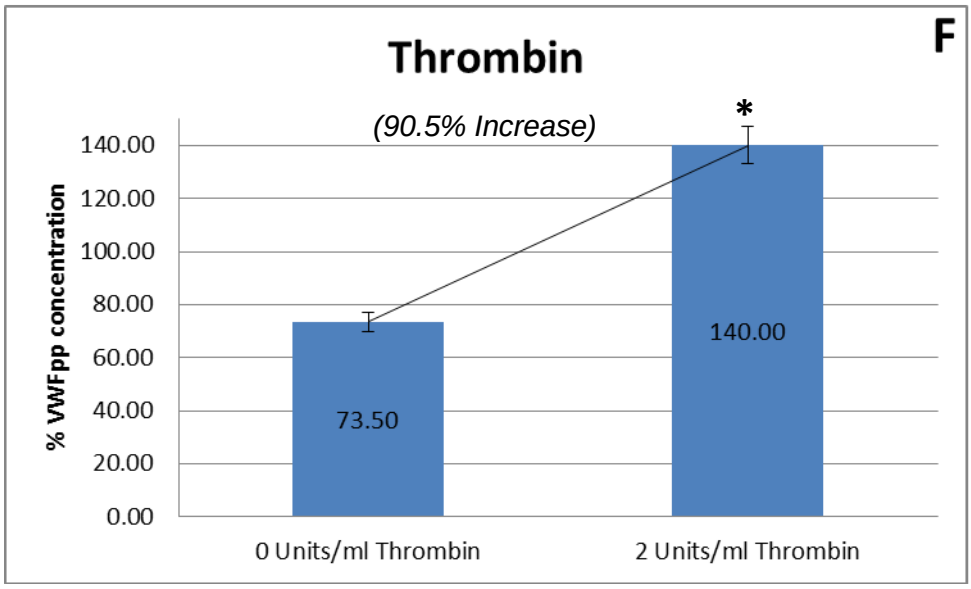

A
IL-8
(11.9% Increase)
% VWFpp concentration
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
21.00
23.50
*
0 ng/ml IL-8
100 ng/ml IL-8


B
TNF-α
(93.5% Increase)
% VWFpp concentration
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
54.00
104.50
*
0 ng/ml TNF-α
100 ng/ml TNF-α


C
Tissue Factor
(130.5% Increase)
% VWFpp concentration
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
47.50
109.50
*
0 µl/ml Tissue Factor
20 µl/ml Tissue Factor


D
IL-8 + Tissue Factor
(132.5% Increase)
% VWFpp concentration
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
*
20.00
46.50
0 ng/ml IL-8 + 0 µl/ml Tissue Factor
100 ng/ml IL-8 + 20 µl/ml Tissue Factor


E
TNF-α + Tissue Factor
(141.7% Increase)
% VWFpp concentration
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
*
54.00
130.50
0 ng/ml TNF-α + 0 µl/ml Tissue Factor
100 ng/ml TNF-α + 20 µl/ml Tissue Factor


F
Thrombin
(90.5% Increase)
% VWFpp concentration
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
*
73.50
140.00
0 Units/ml Thrombin
2 Units/ml Thrombin

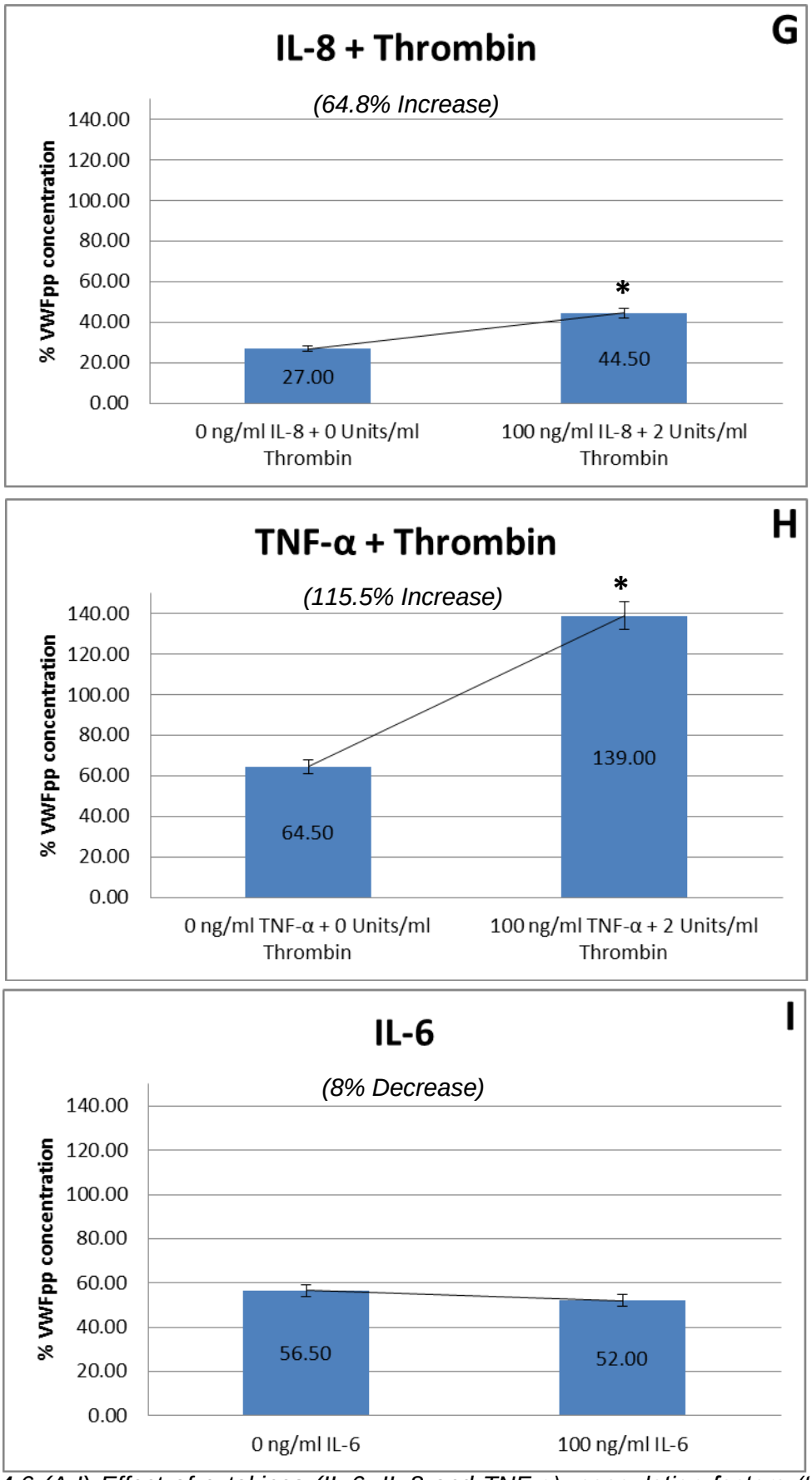


*Figure 4.6 (A-I) Effect of cytokines (IL-6, IL-8 and TNF-α), coagulation factors (thrombin and tissue factor) and combined coagulation factor/cytokine stimulations (IL-8+thrombin, TNF-α+thrombin, IL-8+tissue factor and TNF-α+tissue factor) on the synthesis of the VWF propeptide from HUVECs (n = 6, mean ±SD, *P<0.05).*

To summarise, the results showed that when endothelial cells (HUVECs) are stimulated with cytokines and coagulation factors, more VWF and VWF:pp are secreted and less ADAMTS-13. This is a possible mechanism for the development of the fatal disease HIV-associated thrombotic thrombocytopenic purpura where very low levels of ADAMTS-13 and extremely high levels of VWF, and the VWF:pp are present.

CHAPTER 5

DISCUSSION

Little is known about the initial onset of HIV-associated TTP. The mechanisms underlying the initial onset and/or burst of TTP episode also remains poorly understood. Interrelated components, such as coagulation factors and inflammatory cytokines, all contribute to the development of TTP. Therefore, we hypothesised that the combination of certain inflammatory cytokines and coagulation factors that are released during inflammation may stimulate the release of VWF and simultaneously inhibit the synthesis of ADAMTS-13. This result in an acquired deficiency of plasma ADAMTS-13 that present as TTP. To test this hypothesis, we determined the effects of several inflammatory cytokines in particular interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor-α (TNF-α), which are released during the early stages of systemic inflammation and evidently elevated in patients with an acute episode of TTP, on the synthesis and cleavage of VWF in human primary umbilical vein endothelial cells (HUVECs), a major cell type that produces ADAMTS-13 in humans (Wada *et al.,* 1992; Esmon, 2000; Furlan and Lämmle, 2001; Hillyer *et al.,* 2003; Tousoulis *et al.,* 2003; Bernardo *et al.,* 2004; Uemura *et al.,* 2005; Shang *et al.,* 2006; Stefanescu *et al.,* 2008). We also studied the effect of coagulation factors and their combined effect with the cytokines on VWF synthesis and cleavage. This allowed us to evaluate hypothetical links between inflammation and thrombosis and help us to understand the mechanisms that lead to HIV-associated TTP where inflammation and

thrombosis occur. This study presents the first *in vitro* evidence on the effects of thrombin and tissue factor, combined with inflammatory cytokines, on VWF synthesis and cleavage in HUVECs.

In this study, HUVECs were cultured and used for all experiments. For each cell line, there is a specific doubling time where the population of cells doubles. Thereafter, the cells must be sub-cultured for further use. This will then count as one passage. In 1999, Kalogeris *et al.* observed that endothelial cell responses to TNF-α were similar from the first passage through to the sixth passage. Moreover, each passage leads to the decrease in expression of many proteins due to a mechanism of accelerated senescence (aging), with spontaneous apoptosis (organized cell death). For example, both angiotensin I-converting enzyme (ACE) and prostacyclin synthesis decrease as a function of the number of passages. Therefore, in all our experiments, HUVECs in passage four were used (Noveral *et al.,* 1987; Kalogeris *et al.,* 1999; Dimmeler and Zeiher, 2000).

Shear stress, a mechanical force induced by the pulsatile flow of blood, plays an important role in cardiovascular regeneration and remodelling. In particular, the endothelium is uninterruptedly exposed to different fluid shear stresses generated by flowing blood (Sumpio, 1991; Hsieh *et al.,* 1993; Malek and Izumo, 1995). Moreover, shear stress is responsible for protein balance synthesis, morphology, migration, proliferation and survival of EC (Gimbrone *et al.,* 2000; Walshe *et al.,* 2005; Kadohama *et al.,* 2006). As previously discussed, VWF plays a key role in the primary haemostatic processes by inducing platelet adhesion and aggregation at the sites of vascular injury under conditions of high shear stress (Zimmerman

and Ruggeri, 1973). Under these shear stress conditions, VWF becomes more susceptible to proteolysis by ADAMTS-13. In our study, we used a fluid shear stress of 2.5 dyne/cm^2, a shear stress normally found in venules. This shear stress proved to be ideal for HUVECs to be exposed to result in the formation and proteolysis of VWF (Dong *et al.,* 2002).

We found that inflammatory cytokines, coagulation factors, and their combinations, have contrasting effects on the release of VWF and the release of the VWF-cleaving metalloprotease, ADAMTS-13, from HUVECs. IL-6 alone did not stimulate the release or inhibition of either VWF or ADAMTS-13 from HUVECs respectively (Figure 4.1), all due to the lack of the IL-6 receptor (IL-6R) on the surface of HUVECs (Peters *et al.,* 1997; Romano *et al.,* 1997). This result was confirmed by the findings of Bernardo *et al.,* who reported that IL-6 alone does not stimulate the VWF release from HUVECs; however, that IL-6 in complex with its receptor induced a small but significant release of VWF from HUVECs. They also reported that IL-6 significantly inhibited the rate of the cleavage of ULVWF-platelet strings by ADAMTS-13, but how IL-6 inhibits ADAMTS-13 activity still remains unclear. One potential mechanism for this inhibitory effect of IL-6 on ADAMTS-13 can be that IL-6 might impair the docking of ADAMTS-13 to the VWF strings under flow conditions (Dong *et al.,* 2003). We, however, did not do experiments with IL-6 combined to its receptor, IL-6R, and recommend it for future studies.

All other treatments increased the release of VWF from endothelial cells (Figure 4.1). This is also the first time where the effect of tissue factor was tested on VWF secretion in HUVEC cells. Tissue factor had a potent effect on VWF secretion.

The highest increase in VWF levels was found with stimulation by the combination of tissue factor and IL-8 (Figure 4.1). In HIV where tissue factor levels are increased, it is possible that the constant stimulation of VWF release by tissue factor might contribute to the extremely high levels of VWF in these patients. Our results with IL-8 and TNF-α-stimulation were consistent with a study done by Bernardo *et al.,(2004)* indicating the same effect of IL-8 and TNF-α on VWF release from endothelial cells.

We found total reverse results with ADAMTS-13 compared to VWF. IL-8, TNF-α, tissue factor, IL-8+tissue factor, and TNF-α+tissue factor decreased the levels of ADAMTS-13 (Figure 4.3). However, the results were not significantly different and are therefore not credible. Due to the exceedingly low levels of ADAMTS-13 we did SDS-PAGE and Western blot analysis to support the findings of ADAMTS-13. Western Blot, a technique to analyse proteins based on their molecular weight, was done to examine VWF multimer patterns and ADAMTS-13 levels. The Western blot analysis showed that inflammatory cytokines, coagulation initiators, nor their combinations had any effect on the VWF multimeric pattern. This could be observed by the distribution of large, intermediate to small VWF multimers (Figure 4.2). Moreover, these results suggest that VWF was cleaved, confirming the presence of ADAMTS-13. Although the levels of ADAMTS-13 were very low (Figure 4.3), Western blot analysis was performed. It indicated that ADAMTS-13 was present (Figure 4.4). Our results clearly indicated a lower relative density of ADAMTS-13 on the Western blot in all the treated samples compared to that of the control sample. These results propose an alternative method for quantitative detection of ADAMTS-13 levels in the future. The affected inhibition of the

synthesis of ADAMTS-13 in human umbilical endothelial cells by IL-8, TNF-α, thrombin and tissue factor and their combined effects, may offer a logical explanation of how systemic inflammation and/or infection might trigger the onset and/or burst of TTP in patients by increasing VWF secretion and decreasing ADAMTS-13 secretion.

The Weibel-Palade bodies (WPB) in endothelial cells are specialised cellular compartments for the storage of VWF multimers (Van Mourik *et al.,* 2002; Michaux and Cutler, 2004; Rondaij *et al.,* 2006). The stoichiometry of the storage of the Von Willebrand factor propeptide (VWF:pp) and mature VWF in WPBs are 1:1 (Wagner *et al.,* 1987; Wagner, 1990; Haberichter *et al.,* 2000). The VWF multimers are internalized, and VWF secretion from WPBs are interconnected to secretagogues stimuli. Upon stimulation, we observed an increase of the secretion of VWF:pp in all treatments except IL-6 (Figure 4.6). VWF:pp levels were also measured in the perfusate, since it provides a more accurate measurement of VWF secretion (Ragni, 2006). This was also the first time where the effect of tissue factor was tested on VWF:pp secretion in HUVEC cells. We have also measured the levels of VWF:pp in an attempt to assess if the elevated VWF levels represent acute endothelial damage as found, for example, in patients with sepsis and TTP, or, are the increased VWF levels due to chronic endothelial perturbation such as found in patients with diabetes (Van Mourik *et al.,* 1999). As the half-life of VWF:pp is much shorter than that of VWF, only elevated levels of VWF and normal to slightly elevated levels of VWF:pp are found in patients with chronic endothelial damage (Van Mourik *et al.,* 1999). However, the highly elevated propeptide levels found in our study suggest that the increased propeptide compared to the VWF levels may

be explained by a reduced clearance of this molecule, since we operated in a closed system. It may also reflect persistent enhanced VWF synthesis by stimulated HUVECs (Hollestelle *et al.,* 2004). The VWF:pp may have physiological relevance in the plasma environment in processes related to inflammation (Van Mourik and Romani De Wit, 2001). Whether the highly elevated VWF:pp levels in patients with TTP interfere with inflammatory processes is unknown.

Our results suggest that certain inflammatory cytokines and coagulation factors that are released during pathological conditions may affect the balance between the quantity of VWF multimers and ADAMTS-13 released from endothelial cells. More VWF and VWF:pp were secreted and less ADAMTS-13. As a result, the over expression of VWF multimers may deplete the quantity of ADAMTS-13 released from HUVECs, potentially leading to thrombosis. This is a possible mechanism for the development of HIV-associated TTP where very low levels of ADAMTS-13 and extremely high levels of VWF are seen. Therefore, these findings may provide a link between inflammation and thrombosis, which may also be of therapeutic importance for future studies.

CHAPTER 6

CONCLUSION

The research outlined in this thesis focused on the effects of inflammatory cytokines and coagulation initiators such as tissue factor and thrombin as well as combinations thereof on the release of ULVWF by HUVECs in addition to the cleavage of these ULVWF by ADAMTS-13. The outcomes lead us to evaluate more hypothetical links between inflammation and thrombosis and helped to understand the mechanisms that lead to HIV-associated TTP. Different aspects of these mechanisms were studied.

This study showed that inflammatory cytokines such as interleukin-8 and tumour necrosis factor-α as well as coagulation factors thrombin and tissue factor and combinations of the cytokines with the coagulation factors increased VWF and comparatively VWF propeptide secretion. It also decreased ADAMTS-13 secretion by human umbilical vein endothelial cells. This can indicate an over expression of VWF multimers that may deplete the quantity of ADAMTS-13 released from HUVECs. This may potentially lead to thrombosis and ultimately to TTP. The comparison between VWF levels and VWF propeptide levels, suggest that VWF propeptide may be a better measure of VWF secreted from endothelial cells.

We present the first report on the individual effects of TF and thrombin and their combined effect with inflammatory cytokines on the release of VWF by HUVECs

and the cleavage of VWF by ADAMTS-13. All treatments increased VWF and VWF propeptide levels and decreased ADAMTS-13 levels.

In HIV patients where repeated inflammatory responses takes place and where the levels of tissue factor are increased, we postulate that the increase in VWF secretion and the decrease in ADAMTS-13 secretion might lead to the onset of HIV-associated TTP. The results of this study may offer a logical explanation of how systemic inflammation and thrombosis trigger the onset and/or burst of TTP in patients with HIV-associated TTP.

CHAPTER 7

FUTURE STUDIES

In the last few years, a great amount of work generated valuable data that provided insight into the link between inflammation and thrombosis. Our study showed that inflammatory cytokines and coagulation factors are equally involved in the early stages of vascular injury and in the early steps of the inflammatory process ultimately promotes thrombosis. However, it remains difficult to separate the effects of the inflammatory cytokines and coagulation factors and the combinations thereof and to survey the complete process. We believe that future research should aim at a better understanding of these effects at cellular level from where the mechanisms involved can be studied further.

In this dissertation, two possible approaches were used to quantitatively measure ADAMTS-13 levels. We were unable to present credible results using ELISA analysis. We then and thus did Western blot analysis with densitometry. The last approach is not quantitative. Therefore, the quantification of the messenger RNA (mRNA) of ADAMTS-13 using real-time polymerase chain reaction (PCR) should be considered. However, given the number of choices available for every aspect of real-time PCR, it may be difficult to determine what detection chemistry, quantitation method and normalization gene to use. Finally, the current research can be improved by using more concentration points of the stimulants and to do different time exposures in order to gain a better understanding of the effect on

responses to a stimulating agent. This might provide more insight into the onset of HIV-associated TTP where thrombosis and inflammation plays an important role.

ABSTRACT

When injured, endothelial cells secrete inflammatory cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), and tumour necrosis factor-α (TNF-α). These inflammatory cytokines stimulate the endothelial release of ultra large Von Willebrand factor (ULVWF) multimers that bind platelets to form thrombi in small vessels. The interaction between thrombosis and inflammation is not fully elucidated. A disintegrin-like metalloprotease with thrombospondin type I repeats-13 (ADAMTS-13) is freshly released from Weibel-Palade bodies of endothelial cells into the plasma and it cleaves the ultra large and hyperactive VWF multimers into smaller and less active forms. These VWF multimers mediate the initial adhesion of activated platelets, the first step in both inflammation and thrombosis. This process may be affected by the amount of ULVWF released and the processing capacity of ADAMTS-13. Little is known about the initial onset of HIV-associated TTP, a fatal thrombotic disease that is characterised by the absence of ADAMTS-13. The mechanisms underlying the initial onset and/or burst of TTP episodes still remain poorly understood. Interrelated components, such as coagulation factors and inflammatory cytokines, all contribute to the development of TTP, since increased levels of cytokines interleukin-6 and tumour necrosis factor and the coagulation factor, tissue factor is measured in these patients. Therefore, we hypothesised that certain inflammatory cytokines and coagulation factors released during inflammation may stimulate the release of VWF simultaneously while inhibiting the synthesis of ADAMTS-13, which results in an acquired deficiency of plasma ADAMTS-13 and ultimately in a TTP episode. Our aim was to examine the effects of inflammatory cytokines and coagulation factors

such as tissue factor and thrombin as well as combinations thereof on the release and cleavage of ULVWF by cultured human umbilical vein endothelial cells (HUVECs). HUVECs were treated with cytokines, IL-6, IL-8, and TNF-α and coagulation factors, tissue factor and thrombin, and their combinations, for 24 hours under static conditions. The cells were then exposed to a shear stress of 2.5 dyne/cm^2 to expose the VWF cleaving sites. The VWF, VWF propeptide and ADAMTS-13 secretion were measured by an ELISA technique. ADAMTS-13 content was measured using Western blot technology with densitometry. All treatments and their combinations, excluding IL-6, significantly stimulated the release of VWF and VWF propeptide from HUVECs. The VWF propeptide levels were constantly higher than the major VWF protein levels suggesting that the measurement of the VWF propeptide levels may be a better representation of the amount of VWF secreted from endothelial cells. Tissue factor alone and in combination with inflammatory cytokines increase the amount of VWF release from endothelial cells substantially. This correlates with the situation in thrombotic patients with inflammation where extremely high VWF levels are measured. Densitometric analysis of the Western blots indicated that lower levels of ADAMTS-13 secretion were found with all treatments. These results suggest that inflammatory cytokines such as IL-8 and TNF-α, coagulation factors such as thrombin and tissue factor, as well as combinations thereof, stimulate the release of ULVWF and inhibit the release of ADAMTS-13 in HUVECs, resulting in the accumulation of hyperreactive ULVWF in plasma and on the surface of endothelial cells to induce platelet aggregation and adhesion on the vascular endothelium. Our study may offer a logical explanation of how systemic inflammation and

thrombosis might trigger the onset and/or burst of TTP in patients with HIV-associated TTP.

ABSTRAK

Endoteelselle stel inflammatoriese sitokienes soos interleukin-6 (IL-6), interleukin-8 (IL-8) en tumor nekrosefaktor-α (TNF-α) vry tydens vaskulêre beserings. Hierdie inflammatoriese sitokienes stimuleer die endoteelselle om ekstra groot von Willebrand faktor (VWF) multimere vry te stel. Die von Willebrandfaktor bind aan plaatjies en vorm dan trombusse in die kleiner bloedvate. Die interaksie tussen trombose en inflammasie is nog steeds nie volledig bekend nie. 'n Disintegrienagtige metalloprotease met trombospondien-tipe herhalings nommer 13 (ADAMTS-13) word deur endoteel selle uit Weibel-Palade liggame storingsgranules in die plasma vrygestel. ADAMTS-13 sny hierdie ekstra groot en hiperaktiewe VWF multimere in kleiner en minder aktiewe vorms. Hierdie VWF multimere bemiddel die aanvanklike klewing van geaktiveerde bloedplaatjies; die eerste stap in inflammasie en trombose. Hierdie proses kan beïnvloed word deur die hoeveelheid ULVWF wat vrygestel word en die vlakke en aktiwiteit van die snydingsprotease ADAMTS-13. Daar is min bekend oor die aanvang van MIV-verwante trombotiese trombositopeniese purpura (TTP), 'n noodlottige trombotiese siekte wat gekenmerk word deur die afwesigheid van ADAMTS-13. Die meganismes onderliggend aan die aanvang en/of uitbarsting van ‘n TTP episode word nog steeds swak verstaan. Opeenvolgende komponente soos stollingsfaktore en inflammatoriese sitokienes dra almal by tot die ontwikkeling van TTP want daar is verhoogde vlakke van sitokienes, asook die stollingsfaktor, weefsel faktor, in hierdie pasiënte gevind. Ons het daarom gehipotiseer dat sekere inflammatoriese sitokienes en stollingsfaktore wat tydens inflammasie vrygestel word, die vrystelling van VWF stimuleer en so ook gelyktydig die vrystelling van

ADAMTS-13 inhibeer. Dit lei tot 'n verworwe tekort aan plasma ADAMTS-13 die gevolg daarvan is die ontstaan van ‘n TTP episode. Die doel van hierdie studie was om die effek van sitokienes en stollingsfaktore weefsel faktor en trombien, sowel as die kombinasies daarvan op die vrylating en snyding van ULVWF deur gekultuurde mens naelstringaar-endoteelselle (HUVECs) te ondersoek. Die HUVECs is met sitokiene IL-6, IL-8, en TNF-α en stollingsfaktore, weefsel faktor en trombien, en hul kombinasies behandel vir 24 uur onder statiese omstandighede. Die selle is toe blootgestel aan 'n skuifkrag van 2,5 dyne/cm^2 om die VWF kliewingspunte bloot te stel. Die VWF, VWF propeptied (VWF:pp) en ADAMTS-13 vrystelling is toe gemeet deur middel van 'n ELISA tegniek. Die ADAMTS-13 inhoud was verder gemeet deur “Western”-klad tegnologie wat digtheidstoetsing insluit. Alle behandelings en hul kombinasies, met die uitsondering van IL-6, het die vrystelling van ULVWF en VWF:pp deur HUVECs aansienlik verhoog. Die effek van IL-6 op VWF vrystelling was verwag as gevolg van die afwesigheid van die IL-6 reseptor op HUVECs. Die VWF:pp vlakke was voortdurend hoër as die volle VWF proteïen vlakke wat daarop dui dat die VWF:pp vlakke ‘n beter voorstelling is van VWF vrystelling deur endoteel selle en is dus 'n meer betroubare meting van VWF vrystelling. Weefsel faktor alleen en in kombinasie met die sitokienes, het die VWF vrystelling deur endoteel selle aansienlik verhoog. Dit is soortgelyk aan die situasie in trombotiese pasiënte met inflammasie. Die digtheidsmetings het gewys dat laer vlakke van ADAMTS-13 vrygestel is tydens al die behandelings. Hierdie resultate dui dus daarop dat sitokienes soos IL-8 en TNF-α, stollingsfaktore soos trombien en weefsel faktor, sowel as die kombinasies daarvan, die vrystelling van ULVWF stimuleer en die vrystelling van ADAMTS-13 in HUVECs inhibeer. Dit lei moontlik tot die

opeenhoping van hiperaktiewe ULVWF in plasma op die oppervlak van die endoteel selle om sodoende plaatjie klewing en aggregasie op die vaskulêre endoteel selle te bewerkstellig. Ons studie kan dus ‘n logiese verduideliking bied oor hoe sistemiese inflammasie en trombose die aanvang en/of uitbarsting van TTP in pasiënte met MIV-verwante TTP aktiveer.

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APPENDIXES

Appendix 1: VWF levels

No (n=6)	Treatments																	
	IL-6		TNF-α		IL-8		Thrombin		Tissue Factor		IL-8 + Thrombin		TNF-α + Thrombin		IL-8 + Tissue Factor		TNF-α + Tissue Factor	
	0 ng/ml IL-6	100 ng/ml IL-6	0 ng/ml TNF-α	100 ng/ml TNF-α	0 ng/ml IL-8	100 ng/ml IL-8	0 Units/ml Thrombin	2 Units/ml Thrombin	0 µl/ml Tissue Factor	20 µl/ml Tissue Factor	0 ng/ml IL-8 + 0 Units/ml Thrombin	100 ng/ml IL-8 + 2 Units/ml Thrombin	0 ng/ml TNF-α + 0 Units/ml Thrombin	100 ng/ml TNF-α + 2 Units/ml Thrombin	0 ng/ml IL-8 + 0 µl/ml Tissue Factor	100 ng/ml IL-8 + 20 µl/ml Tissue Factor	0 ng/ml TNF-α + 0 µl/ml Tissue Factor	100 ng/ml TNF-α + 20 µl/ml Tissue Factor
1	12.06	13.12	12.96	32.77	3.18	4.59	5.03	12.96	12.65	38.32	7.23	13.16	13.92	33.42	4.74	14.26	13.78	34.39
2	13.46	13.49	10.27	27.49	4.04	8.88	18.53	37.37	10.09	21.06	5.59	8.92	12.22	28.76	4.49	11.38	9.15	17.08
3	12.75	12.01	11.87	34.53	3.65	7.86	13.00	15.77	11.42	23.71	7.37	8.21	10.72	17.21	8.10	10.64	10.21	30.31
4	13.83	13.98	15.71	30.66	3.62	6.45	14.21	17.87	12.45	35.55	8.22	15.39	16.44	37.53	2.33	12.19	12.54	23.53
5	14.01	14.23	10.84	20.57	2.11	5.61	8.31	34.21	10.87	27.64	6.91	11.20	10.66	34.63	3.42	14.29	8.32	20.43
6	10.46	13.05	8.03	34.77	5.05	6.99	11.58	32.83	10.72	31.88	3.11	9.33	14.48	34.98	4.61	14.16	14.81	28.72
Mean	**12.76**	**13.31**	**11.61**	**30.13**	**3.61**	**6.73**	**11.78**	**25.17**	**11.37**	**29.69**	**6.41**	**11.04**	**13.07**	**31.09**	**4.61**	**12.82**	**11.47**	**25.74**
SD	**1.34**	**0.79**	**2.60**	**5.41**	**0.97**	**1.54**	**4.70**	**10.77**	**1.01**	**6.75**	**1.83**	**2.79**	**2.29**	**7.38**	**1.94**	**1.63**	**2.63**	**6.52**
P-Value	**0.40949**		**0.00011**		**0.00267**		**0.02750**		**0.00103**		**0.00835**		**0.00129**		**0.00002**		**0.00193**	

Appendix 2: ADAMTS-13 levels

No (n=6)	Treatments																	
	IL-6		TNF-α		IL-8		Thrombin		Tissue Factor		IL-8 + Thrombin		TNF-α + Thrombin		IL-8 + Tissue Factor		TNF-α + Tissue Factor	
	0 ng/ml IL-6	100 ng/ml IL-6	0 ng/ml TNF-α	100 ng/ml TNF-α	0 ng/ml IL-8	100 ng/ml IL-8	0 Units/ml Thrombin	2 Units/ml Thrombin	0 µl/ml Tissue Factor	20 µl/ml Tissue Factor	0 ng/ml IL-8 + 0 Units/ml Thrombin	100 ng/ml IL-8 + 2 Units/ml Thrombin	0 ng/ml TNF-α + 0 Units/ml Thrombin	100 ng/ml TNF-α + 2 Units/ml Thrombin	0 ng/ml IL-8 + 0 µl/ml Tissue Factor	100 ng/ml IL-8 + 20 µl/ml Tissue Factor	0 ng/ml TNF-α + 0 µl/ml Tissue Factor	100 ng/ml TNF-α + 20 µl/ml Tissue Factor
1	0.129	0.127	0.279	0.242	0.205	0.142	0.130	0.179	0.279	0.130	0.131	0.322	0.130	0.279	0.252	0.231	0.205	0.167
2	0.250	0.247	0.232	0.191	0.353	0.279	0.131	0.211	0.167	0.117	0.132	0.221	0.167	0.205	0.212	0.179	0.242	0.167
3	0.324	0.332	0.356	0.331	0.371	0.357	0.132	0.355	0.389	0.131	0.113	0.397	0.131	0.132	0.212	0.179	0.112	0.171
4	0.142	0.151	0.284	0.221	0.231	0.231	0.133	0.139	0.254	0.112	0.142	0.117	0.112	0.182	0.179	0.172	0.205	0.171
5	0.133	0.141	0.241	0.152	0.194	0.153	0.134	0.172	0.117	0.130	0.154	0.112	0.212	0.393	0.233	0.254	0.221	0.172
6	0.137	0.139	0.288	0.121	0.182	0.154	0.135	0.134	0.132	0.117	0.117	0.442	0.171	0.221	0.321	0.238	0.323	0.167
Mean	**0.19**	**0.19**	**0.28**	**0.21**	**0.26**	**0.22**	**0.13**	**0.20**	**0.22**	**0.12**	**0.13**	**0.27**	**0.15**	**0.24**	**0.23**	**0.21**	**0.22**	**0.17**
SD	**0.08**	**0.08**	**0.04**	**0.07**	**0.08**	**0.09**	**0.00**	**0.08**	**0.10**	**0.01**	**0.02**	**0.14**	**0.04**	**0.09**	**0.05**	**0.04**	**0.07**	**0.00**
P-Value	**0.93988**		**0.07995**		**0.47286**		**0.10565**		**0.06517**		**0.06287**		**0.08389**		**0.32056**		**0.13938**	

Appendix 3: Densitometric analysis

No (n=3)	Treatments									
	Control	IL-6	TNF-α	IL-8	Thrombin	Tissue Factor	IL-8 + Thrombin	TNF-α + Thrombin	IL-8 + Tissue Factor	TNF-α + Tissue Factor
	0 ng/ml Control	100 ng/ml IL-6	100 ng/ml TNF-α	100 ng/ml IL-8	2 Units/ml Thrombin	20 µl/ml Tissue Factor	100 ng/ml IL-8 + 2 Units/ml Thrombin	100 ng/ml TNF-α + 2 Units/ml Thrombin	100 ng/ml IL-8 + 20 µl/ml Tissue Factor	100 ng/ml TNF-α + 20 µl/ml Tissue Factor
1	1.00	0.42	0.51	0.37	0.44	0.32	0.41	0.45	0.38	0.38
2	1.00	0.37	0.45	0.35	0.37	0.34	0.29	0.41	0.43	0.35
3	1.00	0.45	0.50	0.41	0.35	0.42	0.37	0.39	0.39	0.32
Mean	**1.00**	**0.41**	**0.49**	**0.38**	**0.39**	**0.36**	**0.36**	**0.42**	**0.40**	**0.35**
SD	**0.00**	**0.04**	**0.03**	**0.03**	**0.05**	**0.05**	**0.06**	**0.03**	**0.03**	**0.03**

Appendix 4: VWF propeptide levels

No (n=6)	Treatments																	
	IL-6		TNF-α		IL-8		Thrombin		Tissue Factor		IL-8 + Thrombin		TNF-α + Thrombin		IL-8 + Tissue Factor		TNF-α + Tissue Factor	
	0 ng/ml IL-6	100 ng/ml IL-6	0 ng/ml TNF-α	100 ng/ml TNF-α	0 ng/ml IL-8	100 ng/ml IL-8	0 Units/ml Thrombin	2 Units/ml Thrombin	0 µl/ml Tissue Factor	20 µl/ml Tissue Factor	0 ng/ml IL-8 + 0 Units/ml Thrombin	100 ng/ml IL-8 + 2 Units/ml Thrombin	0 ng/ml TNF-α + 0 Units/ml Thrombin	100 ng/ml TNF-α + 2 Units/ml Thrombin	0 ng/ml IL-8 + 0 µl/ml Tissue Factor	100 ng/ml IL-8 + 20 µl/ml Tissue Factor	0 ng/ml TNF-α + 0 µl/ml Tissue Factor	100 ng/ml TNF-α + 20 µl/ml Tissue Factor
1	55.00	50.00	54.00	100.00	20.00	23.00	75.00	139.00	47.00	110.00	19.00	29.00	73.00	134.00	19.00	48.00	53.00	139.00
2	58.00	54.00	54.00	109.00	22.00	24.00	72.00	141.00	48.00	109.00	35.00	60.00	56.00	144.00	21.00	45.00	55.00	122.00
3	56.00	55.00	59.00	108.00	22.00	23.00	79.00	155.00	52.00	74.00	20.00	34.00	43.00	106.00	31.00	59.00	50.00	151.00
4	57.40	57.00	60.00	121.00	19.00	25.00	72.00	101.00	34.00	121.00	24.00	38.00	93.00	149.00	27.00	34.00	49.00	111.00
5	58.00	55.00	50.00	112.00	23.00	22.00	67.00	155.00	76.00	132.00	37.00	54.00	40.00	138.00	12.00	35.00	59.00	123.00
6	54.60	41.00	47.00	77.00	20.00	24.00	76.00	149.00	28.00	111.00	27.00	52.00	82.00	163.00	10.00	58.00	58.00	137.00
Mean	**56.50**	**52.00**	**54.00**	**104.50**	**21.00**	**23.50**	**73.50**	**140.00**	**47.50**	**109.50**	**27.00**	**44.50**	**64.50**	**139.00**	**20.00**	**46.50**	**54.00**	**130.50**
SD	**1.51**	**5.87**	**5.02**	**15.08**	**1.55**	**1.05**	**4.14**	**20.27**	**16.71**	**19.50**	**7.56**	**12.49**	**21.57**	**19.06**	**8.20**	**10.78**	**4.10**	**14.45**
P-Value	**0.12158**		**0.00022**		**0.00995**		**0.00037**		**0.00016**		**0.01823**		**0.00009**		**0.00089**		**0.00002**	