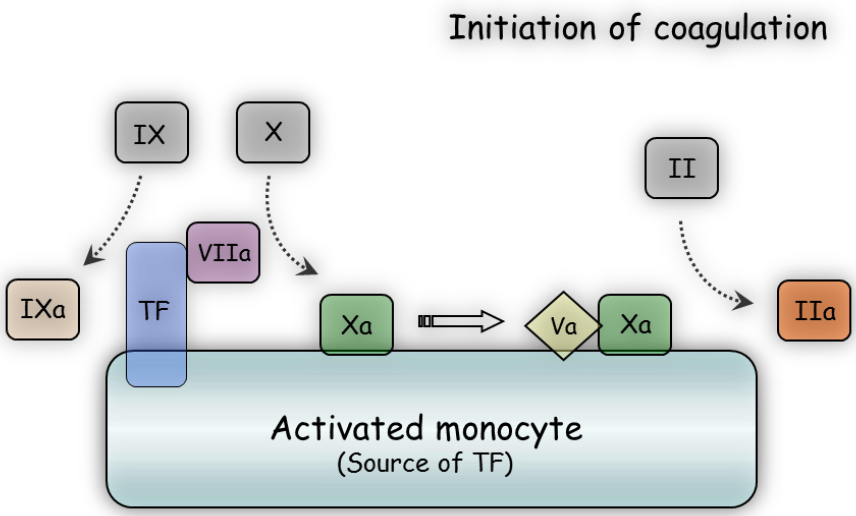
UNIVERSITY OF TROMSØ UIT

FACULTY OF HEALTH SCIENCES
DEPARTMENT OF CLINICAL MEDICINE

Role of monocyte procoagulant functions in the pathophysiology of arterial and venous thrombosis



Manjunath Goolyam Basavaraj

A thesis submitted for the degree of Philosophiae Doctor

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Role of monocyte procoagulant functions in the pathophysiology of arterial and venous thrombosis

by

Manjunath Goolyam Basavaraj
Hematological Research Group

A thesis submitted according to the requirements

for the degree of Philosophiae Doctor

Department of Clinical Medicine
Faculty of Health Sciences
University of Tromsø
Tromsø, Norway

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....to my parents
for their love and encouragement

Thrombosis is "haemostasis in the wrong place"

-Macfarlane RG (1977)

Contents

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List of papers

This thesis is based on the following papers:

I. Basavaraj MG, Gruber FX, Sovershaev M, Appelbom HI, Østerud B, Petersen LC, Hansen JB.
The role of TFPI in regulation of TF-induced thrombogenicity on the surface of human monocytes.
Thromb Res. 2010 Nov; 126(5): 418-425.

II. Basavaraj MG, Olsen JO, Østerud B, Hansen JB.
Differential impact of tissue factor antibody clones on tissue factor antigen expression in blood cells and microparticles.
Manuscript.

III. Basavaraj MG, Sovershaev MA, Egorina EM, Gruber FX, Bogdanov VY, Fallon JT, Østerud B, Mathiesen EB, Hansen JB.
Circulating monocytes mirror the imbalance in TF and TFPI expression in carotid atherosclerotic plaques with lipid-rich and calcified morphology.
Submitted to Thrombosis Research

IV. Basavaraj MG, Brækkan SK, Brodin E, Østerud B, Hansen JB.
Monocyte count and procoagulant functions are associated with risk of venous thromboembolism: the Tromsø study.
J Thromb Haemost. 2011 Aug; 9(8): 1673-1676.

Abbreviations

APC	activated protein C
ApoA-1	apolipoprotein A-1
ApoB	apolipoprotein B
AT	antithrombin
BSA	bovine serum albumin
CAT assay	calibrated automated thrombogram assay
CD	cluster of differentiation
cDNA	complementary DNA
Cq	quantification cycle
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DVT	deep vein thrombosis
EDTA	ethylenediaminetetraacetic acid
EPCR	endothelial cell protein C receptor
FVII	coagulation factor VII
FVIIa	activated FVII
GPI	glycosylphosphatidylinositol
GUS	β-glucuronidase
HDL	high density lipoprotein
HR	hazard ratio
IgG	immunoglobulin G
IL-6	interleukin-6
kDa	kilo Dalton

K-domain	kunitz-domain
LDL	low density lipoprotein
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MFI	median fluorescence intensity
MMPs	monocyte derived microparticles
MPs	microparticles
mRNA	messenger RNA
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PSGL-1	p-selectin glycoprotein ligand-1
RNA	ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute-1640 medium
RT-qPCR	reverse transcription quantitative real-time PCR
SEM	standard error of mean
SD	standard deviation
SMC	smooth muscle cell
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TM	thrombomodulin
TNF-α	tumor necrosis factor-α
VTE	venous thromboembolism

1 General introduction

Thrombotic diseases are the leading cause of morbidity and mortality worldwide. Thrombosis is the formation of a thrombus inside a blood vessel, obstructing the blood flow through the circulatory system. Thrombus formation can occur either in the arteries (leads to e.g., myocardial infarction, stroke) or veins (leads to venous thromboembolism, e.g., deep vein thrombosis or pulmonary embolism). Hemostasis is the protective physiological response that prevents significant blood loss following vascular injury. However, this finely tuned process serves to maintain the integrity of the circulatory system can go out of balance.

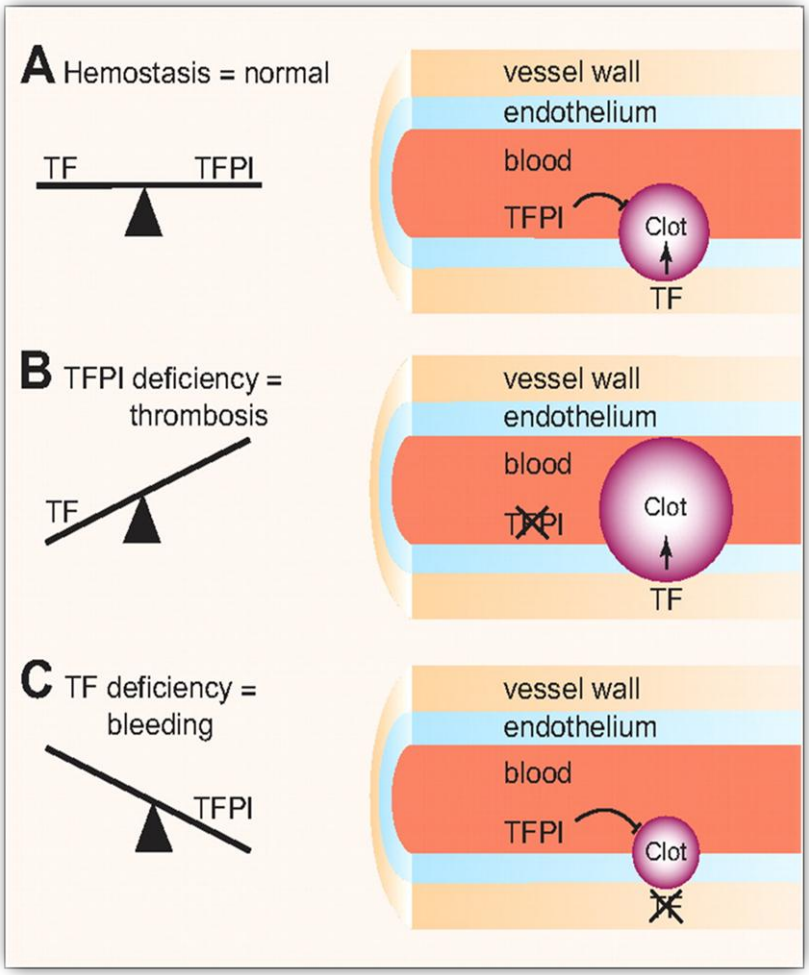

Figure 1. Balance clotting. (A) Levels of the procoagulant protein TF and the anticoagulant protein TFPI must be balanced to maintain normal hemostasis. **(B)** TFPI deficiency in the endothelium results in increased clot formation. **(C)** TF deficiency in the vessel wall results in decreased clot formation. (TF, tissue factor; TFPI, tissue factor pathway inhibitor)
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Tissue factor (TF) in complex with factor (F) VIIa functions as the primary initiator of blood coagulation cascade in vivo [1] and plays a major role in both hemostasis and thrombosis [2]. Following vascular injury, TF expressed by extravascular cells activates the clotting cascade to prevent the blood loss [2]. On the other hand, pathologic TF expression can trigger arterial and venous thrombosis [2]. However, the coagulation cascade is tightly regulated by various endogenous anticoagulants (Tissue factor pathway inhibitor (TFPI), the protein C pathway, and antithrombin) that act at different stages in the pathway [3], where TFPI acts as the primary inhibitor of the TF/FVIIa complex in a FXa-dependent manner [4]. An imbalance between procoagulant and anticoagulant pathways can result in either thrombosis or hemorrhage [5]. The consequences of imbalance between TF and TFPI are shown in Figure 1 [6].

1.1 Models of blood coagulation

1.1.1 Cascade model of coagulation

In 1964, two groups simultaneously proposed a "cascade or waterfall model" of blood coagulation that consisted of a series of steps in which activation of each coagulation factor led to the activation of another finally resulting in thrombin generation and fibrin clot formation [7,8]. Each clotting factor existed as a proenzyme that could be converted to an active enzyme. This concept was later modified when it was found that clotting factors previously thought to be enzymes were in fact cofactors and did not possess enzymatic activity (FVIIIa was found to be a cofactor for FIXa, and FVa for FXa) [9]. This model divides clotting sequences into two pathways, the intrinsic pathway (in which all the components to initiate the coagulation cascade are present in the blood) and the extrinsic pathway (in which extravascular cell membrane protein (TF) was required in addition to circulating components to initiate coagulation). However, the initiation of either pathway results in the formation of FXa and the eventual formation of a fibrin clot through a common pathway [10].

The intrinsic pathway is activated by negatively charged surfaces in vitro, (such as glass) and involves factors XII, XI, IX, VIII, and X as well as prekallikrein (PK), high molecular weight kininogen (HK), Ca^{2+}, and phospholipids (PL). This pathway commences with the contact phase, in which factors PK, HK, XII and XI are exposed to a negatively charged activating surface. FXII is activated to FXIIa at a limited speed with the help of HK

as cofactor. Once a small amount of FXIIa accumulates, it converts PK to kallikrein, with HK as an anchor. The newly produced kallikrein accelerates the conversion of FXII to FXIIa by positive feedback. FXIIa along with HK activates FXI to FXIa and then FXIa in the presence of Ca^{2+} activates FIX to FIXa. Finally, FIXa and FVIIIa together with Ca^{2+} and FX form the tenase complex and converts FX to FXa (Figure 2).

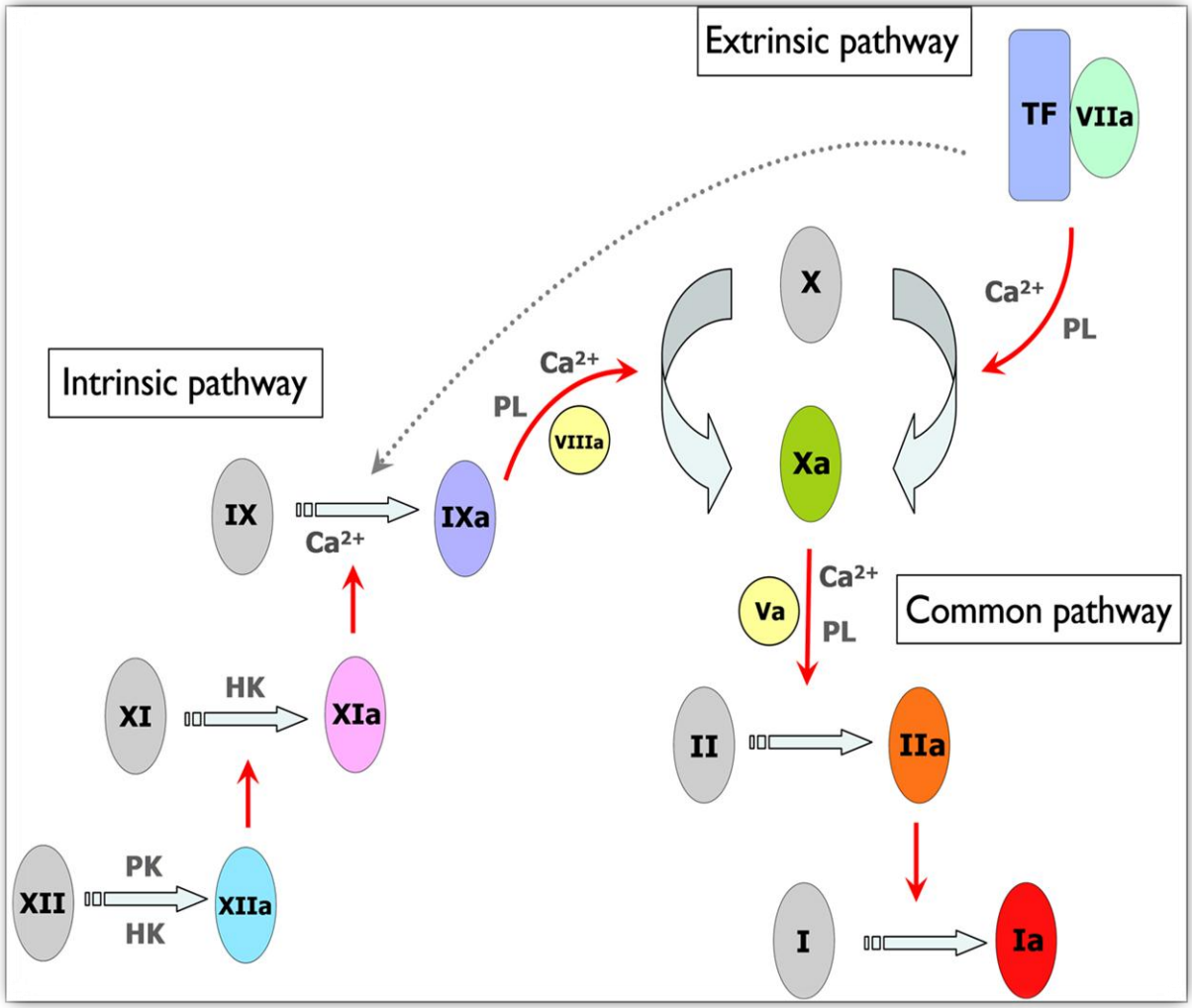

Figure 2. Cascade model of coagulation. Both the intrinsic and extrinsic pathways converge with the activation of FX and proceeds along the common pathway. TF/FVIIa complex activate not only FX in the extrinsic pathway, but also FIX in the intrinsic pathway. (HK, high molecular weight kininogen; PK, prekallikrein; PL, phospholipids)

The extrinsic pathway is initiated at the site of tissue injury with the exposure of TF on extravascular cells and involves TF (FIII), FVII, FX and Ca^{2+} (FIV). Injury to endothelium followed by expression of TF allows plasma containing FVII/FVIIa to come into contact with TF and forming TF/FVIIa complex [11]. FXa, FIXa, FXIIa and, to a lesser extent, thrombin

are capable of activating FVII to FVIIa [11]. Finally, TF/FVIIa complex together with Ca^{2+} and FX forms a complex similar to tenase complex and converts FX to FXa (Figure 2).

The common pathway begins once the FXa is produced by either the intrinsic or the extrinsic or both pathways. FXa in the presence of FVa and Ca^{2+} forms prothrombinase complex with FII (prothrombin) and converts FII to its active form, FIIa (thrombin). Thrombin then catalyzes the proteolysis of the soluble plasma protein fibrinogen (FI) to form fibrin monomers (FIa) that remain soluble (Figure 2). Fibrin monomers then polymerize to form a gel of fibrin polymers that trap blood cells. Thrombin also activates FXIII to FXIIIa, which in turn mediates the covalent cross-linking of the fibrin polymers to form a stable fibrin clot.

Even though cascade model served for many years as a useful concept, recent clinical and experimental observations highlighted that the cascade model does not completely reflect the events of hemostasis in vivo [12]. First, deficiency of FXII, PK or HK was not associated with hemorrhage, even though deficiencies in any one of these factors markedly prolong surface-activated coagulation assays for hemostasis, in vitro [12]. Second, cascade model does not explain the reason for severe bleeding in FVIII or FIX deficiency, even though the extrinsic pathway would be expected to bypass the need for FVIII and FIX [12]. A key observation was that the TF/FVIIa complex can activate not only FX but also FIX [13], explains how the extrinsic and intrinsic pathways might be interdependent in vivo.

1.1.2 Cell-based model of coagulation

A significant development over the past 15 years was the discovery of a cell-based model of coagulation [12], which emphasizes the interaction between coagulation factors and specific cell surfaces. This model was developed in part from cell-based experimental models that use monocytes [14,15,16] or fibroblasts [12] as the source of TF and activated platelets as a surface for thrombin generation [17]. In this model, coagulation occurs in 3 distinct, but overlapping phases: initiation, amplification and propagation [12]. The process requires the participation of 2 types of cells: TF-bearing cells and platelets. The initiation phase occurs on a TF-bearing cell, then in the amplification phase, platelets and cofactors are activated in order to prepare for large-scale thrombin generation. Finally, propagation phase occurs on the surface of activated platelets, and results in the production of large amounts of thrombin [12]. A key means of regulating cell-based model of coagulation is to keep the TF-bearing cells

and platelets separated from each other until an injury makes activation of coagulation desirable (hemostasis) [18].

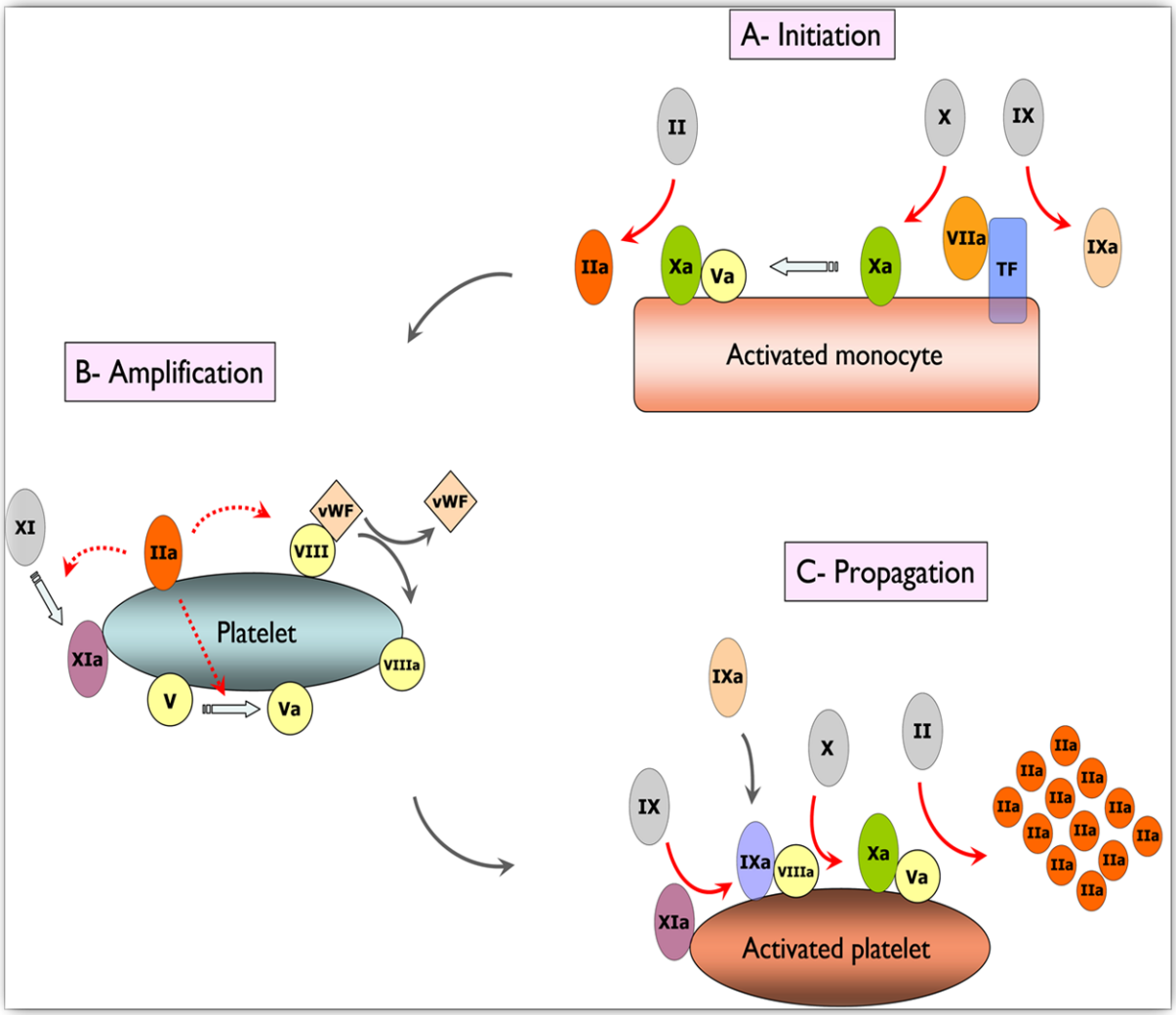

Figure 3. Cell-based model of coagulation. In this model coagulation occurs in 3 phases: **(A)** initiation phase occurs on TF-bearing cell (activated monocytes, as in the process of thrombosis), **(B)** amplification phase occurs on the platelet as it becomes activated, and **(C)** propagation phase occurs on the activated platelet surface. (vWF, von Willebrand factor)

The initiation phase is confined to cells that express TF, which are normally outside the vasculature and therefore, extrinsic to the blood (as in the process of hemostasis); hence this TF pathway may still be referred to as an extrinsic pathway [18]. In contrast, pathological conditions lead to induced TF expression by a variety of vascular cells, and this TF plays an important role in thrombosis [19], with or without damage to the vessel wall as occurs in arterial thrombosis and venous thrombosis respectively [20]. Once circulating blood come in contact with TF-bearing cells, circulating FVII binds tightly to TF and is rapidly activated

[21] by coagulation proteases [22] and by noncoagulation proteases [23], depending on the cellular location of the TF. The TF/FVIIa complex activates small amounts of FX as well as FIX (Figure 3A). Even though activated at the same site, FIXa and FXa play different and distinct roles in subsequent coagulation reactions [17]. Plasma FV is activated by FXa [24] or by noncoagulation proteases [25] on the TF-bearing cell. FXa then associates with FVa, to form prothrombinase complex and produce small amounts of thrombin (IIa) (Figure 3A). In the initiation phase, FXa is rapidly inhibited by TFPI or antithrombin (AT) if it leaves the protected environment of the TF-bearing cell surface. Thus, these inhibitors effectively localize FXa activity to the TF-bearing cell surface. By contrast, FIXa can leave the TF-bearing cell and move to a nearby platelet, as FIXa is not inhibited by TFPI, but only inhibited much slowly by AT.

The small amounts of thrombin generated on TF-bearing cells in initial phase amplify the initial procoagulant signal by enhancing platelet adhesion [26], and activation [15]. Thrombin also activates FXI [27], cofactors, FV [24] and FVIII [12] (Figure 3B). During activation, platelets release FV from α granules onto their surface in a partially activated form, where it gets fully activated by thrombin [28]. Thrombin cleaves von Willebrand factor (vWF) bound FVIII, to activate FVIII and releasing it from vWF [12] (Figure 3B). By the end of the amplification phase, activated platelets with bound FXIa and cofactors, FVa and FVIIIa set the stage for large-scale thrombin generation in the propagation phase. The propagation phase occurs on activated platelet surface (Figure 3C). During this phase FIXa activated during initiation phase binds to FVIIIa on the platelet surface. In addition, FIXa is also provided by platelet-bound FXIa. The FIXa/FVIIIa (tenase) complexe activate FX on the platelet surface and the resulting FXa can move directly into a complex with its cofactor, FVa. The platelet surface FXa/FVa (prothrombinase) complexes can now produce the burst of thrombin in sufficient magnitude to clot fibrinogen [12].

In the case of hemostasis, soon after fibrin clot formation at the site of injury, the coagulation process must be limited to prevent the clot from extending. Vascular endothelial cells possess specialized anti-coagulant features and prevent clot extension on the intact endothelium. Proteoglycans on the endothelial membrane bear heparin sulphate that binds and enhances the protease inhibiting activity of AT [12]. Thrombin that escapes into the circulation from the site of injury is either inhibited by AT [9] in the plasma or binds to thrombomodulin (TM) (a receptor for thrombin), which is expressed at high levels on

endothelial cells. Upon binding to TM, the specificity of thrombin is changed [29], and is no longer able to clot fibrinogen or activate platelets. Thrombin/TM complex activates protein C bound to endothelial cell protein C receptor (EPCR), expressed on the endothelium [30]. Then the activated protein C (APC) dissociates from EPCR and forms a complex with its cofactor protein S, this APC/ protein S complex cleaves and inactivates any FVa and FVIIIa that has been activated on endothelial cell surfaces [31]. This prevents the formation of additional procoagulant enzymes at sites where a healthy, intact endothelial lining layer is present.

Compared to the cascade model, the cell-based model of coagulation clearly explains pathophysiological mechanisms leading to hemophilia. Like the cascade model, the cell-based model also suggests the presence of both intrinsic and extrinsic pathways in the coagulation process. However, in the cell-based model, the extrinsic pathway comprising of TF/FVIIa complex and FXa/FVa complex operates on TF-bearing cell to initiate the coagulation. Whereas, the intrinsic pathway comprising of FXIa (but not FXII or HK or PK), the FIXa/FVIIIa complex, and the FXa/FVa complex operates on activated platelet surfaces. In hemophilia, initiation phase occurs normally on TF-bearing cell and a small amount of thrombin is generated. However, in propagation phase, FX activation on platelet surface by FIXa/FVIIIa is abolished and, therefore, thrombin generation on platelet surface fails. The TF/FVIIa complex cannot effectively bypass the need for FIXa/FVIIIa complex, because it produces FXa on TF-bearing cell and not on the platelet surface. Any FXa diffuse from TF-bearing cell is readily inhibited by TFPI and AT before it reaches the platelet surface.

1.2 Tissue factor

TF (also known as FIII or CD142 or thromboplastin) is a 45-47 kDa transmembrane glycoprotein and is the major cellular initiator of blood coagulation in vivo [1]. It was so named after it was noted that a component of tissue, when added to plasma, enhanced coagulation [32]. The primary role of TF is to maintain hemostasis. Upon vessel damage, plasma FVII/FVIIa [33] binds to TF expressed by extravascular cells and forms a complex, TF/FVIIa. This complex activates FX and FIX of the intrinsic pathway [13] leading to thrombin generation and ultimately results in the insoluble cross-linked fibrin clot formation. However, apart from hemostasis, the TF/FVIIa complex has both procoagulant and signaling activities and plays an important role in many biological processes, including thrombosis, inflammation, angiogenesis and tumor growth [34].

1.2.1 Gene, mRNA and protein structure of human TF

The TF cDNA sequence was published independently by four different groups in 1987 [35-38]. However, the complete 12.4 kb-long TF gene sequence was published two years later [39]. The TF gene is located on chromosome 1, at locus p21–p22 [40], and it contains six exons separated by five introns [39] (Figure 4). The exon 1 of the TF gene encodes the N-terminal signal sequence that is removed by proteolytic cleavage during transport of TF to the plasma membrane, exons 2 to 5 encode the extracellular domain, while exon 6 provide the transmembrane and cytoplasmic domains of the TF protein and a long 3' untranslated region [39].

Transcription of the TF gene results in a 2.2-2.3 kb mRNA [39], however, larger and less abundant transcripts were suggested to result from incomplete processing of introns [41]. Recently, it has been shown that human TF gene has two mRNA products suggesting an alternative splicing [42]. This alternatively spliced TF (asTF) lacks the exon 5, and that the exon 4 is spliced directly to the exon 6.

The translational product of TF open reading frame (ORF) consists of 295 amino acids, of which N-terminal 32 amino acids serve as a signal peptide, and the remaining 263 amino acids form the mature form of TF (Figure 4). On the other hand, the ORF of asTF mRNA is predicted to encode a truncated TF protein of 206 amino acids, of which residues 1–166 are identical to the extracellular domain of full length TF, whereas residues 167–206

correspond to a unique C-terminus [43], and it lacks transmembrane domain, making asTF soluble.

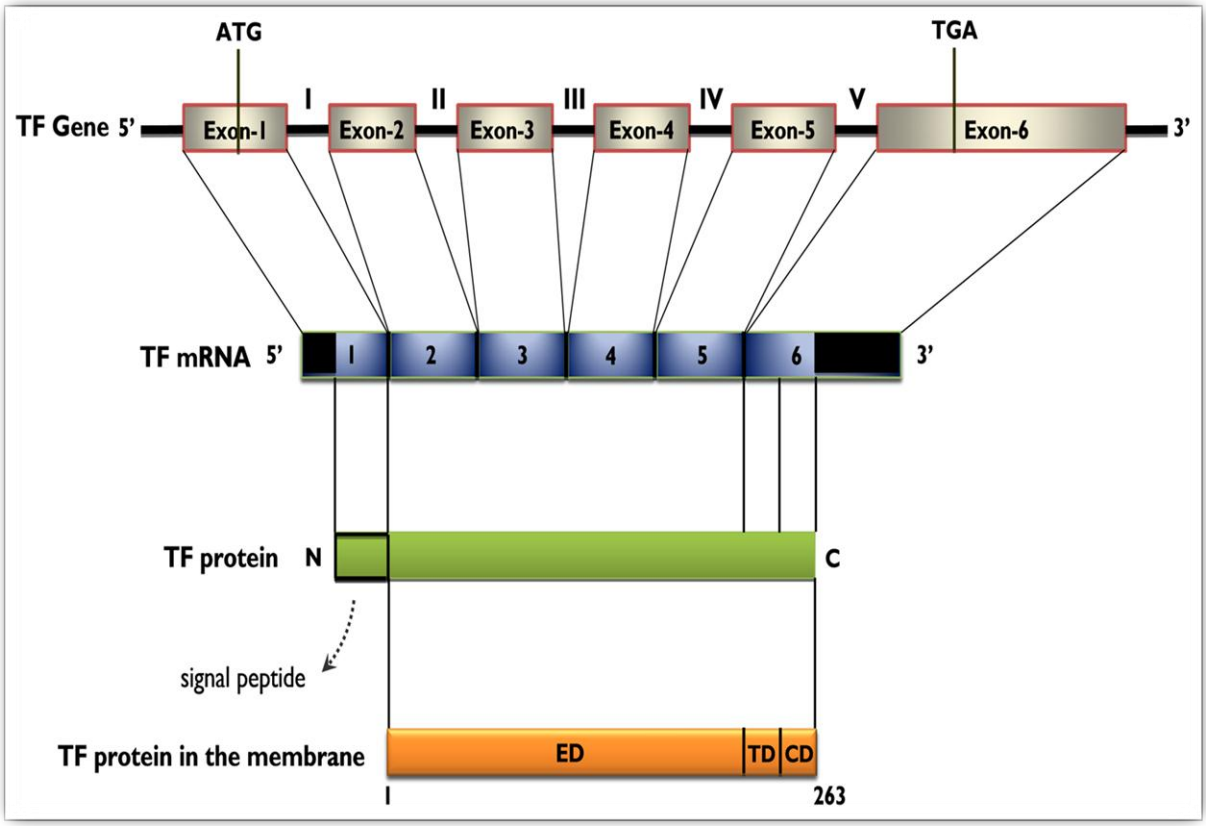

Figure 4. Schematic illustration of human TF gene, mRNA and protein. In TF gene, exons are labeled (1-6) and introns (I-V). In TF mRNA, black areas represent untranslated regions (UTR). In TF protein, N-terminal 32 amino acids serve as a signal peptide and the remaining 263 amino acids form the mature form of TF, made up of 3 domains.
(ED, extracellular domain; TD, transmembrane domain; CD, cytoplasmic domain)

The primary amino acid sequence of TF suggests 3 distinct domains, a hydrophilic extracellular domain (219 amino acids, 1–219), hydrophobic transmembrane domain (23 amino acids, 220–242), and a cytoplasmic domain (21amino acids, 243–263) [36] (Figure 5). Based on structural homology to the interferon-γ receptor, TF is classified as a class-II-type cytokine receptor [44]. The crystal structure of the extracellular domain showed that this domain consists of two fibronectin-like type III modules, joined by an inter-domain hinge region [45] that contributes to the binding of FVIIa [46]. The extracellular domain of TF is necessary and sufficient for procoagulant function, since recombinant variants lacking either the cytoplasmic domain or both the cytoplasmic and transmembrane domains retain full procoagulant activities [47].

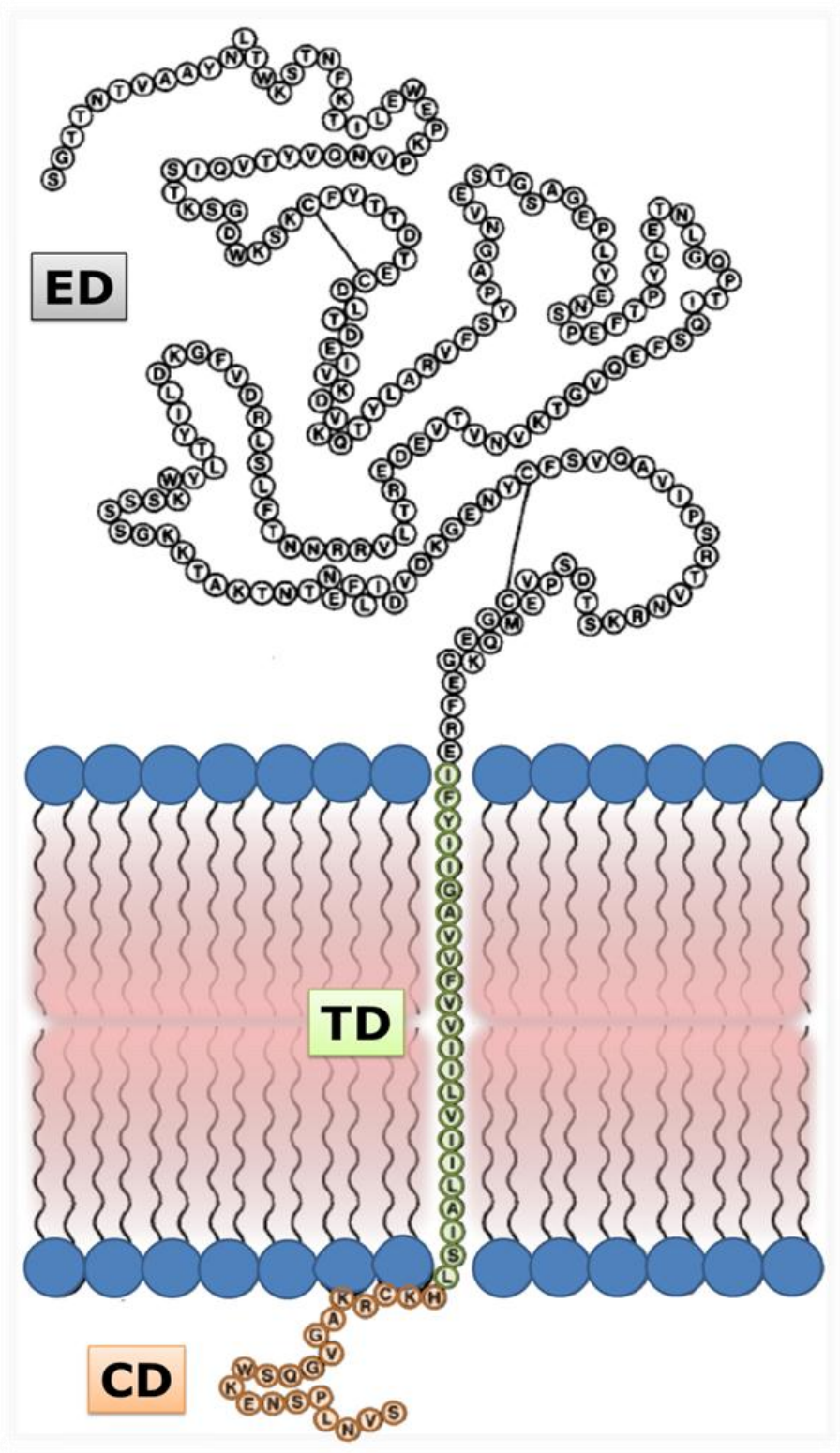

Figure 5. Model of human TF embedded in the membrane. The mature TF protein in the cell membrane (263 amino acids) made up of three domains, an extracellular domain (ED) (219 amino acids), a transmembrane domain (TD) (23 amino acids), and a cytoplasmic domain (CD) (21 amino acids).

(Reproduced from *O'Brien DP. The molecular biology and biochemistry of tissue factor. Baillieres Clin Haematol 1989;2:801-20*, with permission from Elsevier)

1.2.2 Distribution of TF

Under physiologic conditions, TF protein is absent in the cells and tissues located on the interface with circulating blood. However, TF is constitutively expressed by adventitial fibroblasts and vascular smooth muscle cells (SMCs) [48], which have no direct contact with blood. The distinct tissue-specific expression of TF is characterized by high levels of TF protein in brain, heart, lungs, placenta, and uterus [2]. The cells that produce high levels of TF in these organs are astrocytes in the brain, cardiac myocytes in the heart, alveolar cells in the lung, trophoblasts in the placenta, and epithelial cells in the uterine wall [2]. The high level of TF in these vital organs may provide additional hemostatic protection [49]. The cellular identity of TF sources among circulating white blood cells is still a matter of debate [50]. Previous studies have reported TF expression in platelets [50], neutrophils [50], and eosinophils [51] under diverse conditions. In contrast, other studies have failed to detect TF expression in platelets [50,52,53], neutrophils [50,53,54], and eosinophils [55]. However, based on the available data blood monocytes represent the predominant source of TF in the circulation and they even constitutively express little TF under basal conditions [50,53,54]. Recently, presence of blood-born TF or circulating TF was demonstrated [56], and the majority of this TF is associated with microparticles (MPs). However, presence of TF-positive MPs in healthy individuals is a matter of debate, as some studies failed to detect TF antigen and activity in MPs isolated from healthy individuals [53].

1.2.3 TF-dependent activation of coagulation

TF functions as the receptor and cofactor for FVII/FVIIa, and is considered to be the physiological trigger of the blood coagulation system in normal hemostasis and in most thrombotic diseases [57]. Although the majority of FVII circulates in the zymogen form, normal individuals have been found to contain trace amounts of FVIIa in their plasma [33]. Once bound to TF, FVII is rapidly converted to FVIIa via limited proteolysis [21]. Thus, there are two possible ways to form TF/FVIIa complex, either by direct capture of circulating FVIIa by TF, or through capture of FVII by TF followed by conversion of bound FVII to FVIIa [57]. In addition, TF/VIIa complex can itself catalyze the activation of FVII bound to TF, via auto activation [58]. While FVII is a single-chain protein, FVIIa is composed of an N-terminal light chain and a C-terminal heavy chain held together by a disulfide bond [57]. The light chain contains the γ-carboxyglutamic acid-rich domain (Gla) and two epidermal

growth factor- like domains (EGF1 and EGF2), whereas the heavy chain contains the serine protease domain [57] (Figure 6).

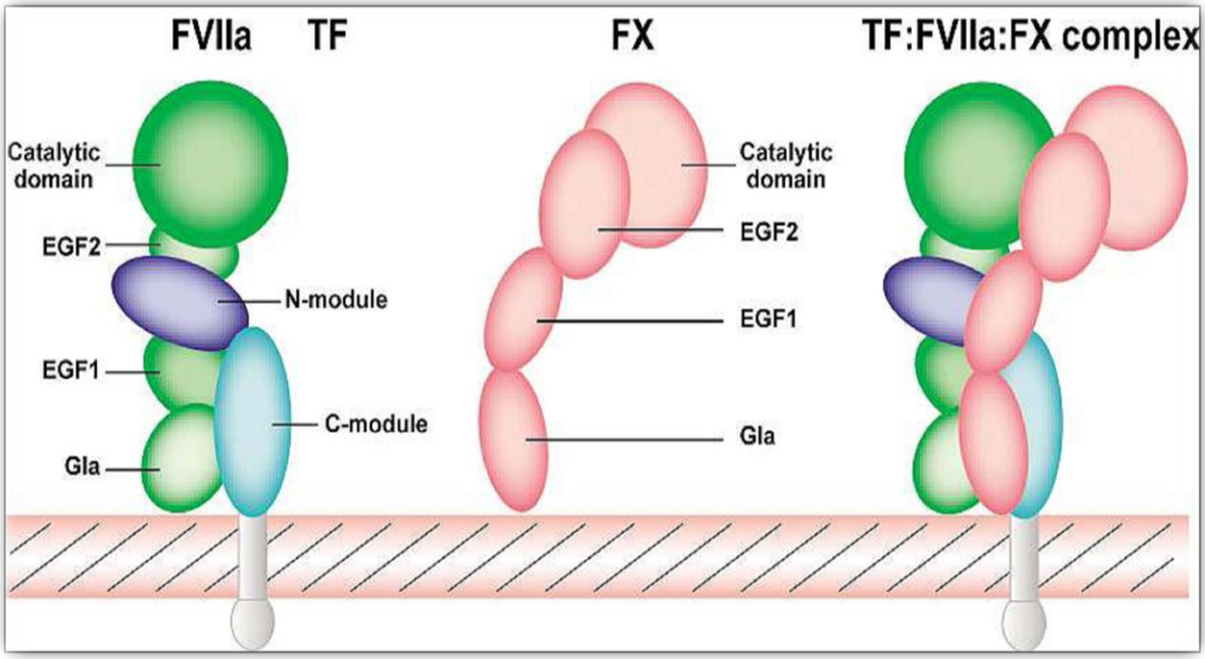

Figure 6. Model of the formation of the TF/FVIIa/FX complex on a cell surface. The extracellular domains of TF are shown in light and dark blue and the transmembrane and cytoplasmic domain in gray. FVIIa is shown is green and FX in pink. The various domains of each protein are shown. (Gla, γ-carboxyglutamic acid-rich domain; EGF1 and EGF2, epidermal growth factor- like domains 1 and 2)
(Reproduced from reference 43, with permission from the journal- 'Thrombosis and Haemostasis')

Vitamin K is involved in the carboxylation of glutamate residues to form Gla-residues. The Gla domain confers the ability to bind in a reversible and calcium-dependent manner, to membranes containing negatively charged phospholipids [59]. Assembly of the TF/FVIIa on cellular surfaces catalyzes the activation of small amounts of FX via limited proteolysis. The FXa then interacts with its cofactor Va to convert a small amount of prothrombin to thrombin.

1.2.4 Role of TF in hemostasis

TF is essential for life, most likely because of its central role in hemostasis. TF is expressed in a tissue-specific manner with high levels in vital organs, such as the brain, heart, lungs, placenta and uterus. This suggests that TF would provide additional hemostatic

protection to these vital organs [49]. Even though TF-deficient humans have not been identified, TF deficiency causes embryonic lethality in the mouse. Mice deficient in TF, FVII, FX, FV, and FII survive embryonic development, but die in the perinatal period because of spontaneous hemorrhages resulting from impaired hemostasis [2]. The hemostatic role of TF in various physiological processes was examined using low-TF mice (murine $TF^{-/-}$, human TF^{+}) [60] and low-FVII mice [2]. Defects in low-TF and low-FVII mice include fatal hemorrhages in the brain, lung, intestine, and uterus [2].

1.2.5 Role of TF in thrombosis

Pathologic TF expression can trigger arterial thrombosis, venous thrombosis and disseminated intravascular coagulation (DIC) [61]. In arterial thrombosis, atherosclerotic plaques contain large amounts of TF, and this triggers thrombosis after plaque rupture [48,62]. Higher TF levels have also been shown in atheroma from patients with unstable angina compared with patients with stable angina [63]. Inhibition of the TF/FVIIa complex in animal models reduces or prevents arterial thrombosis [64]. Selective deletion of the TF gene in vascular SMCs is resulted in reduced carotid arterial thrombosis in mice [65]. In contrast to arterial thrombosis, venous thrombosis typically occurs in the absence of gross vessel wall disruption, but elevated levels of circulating TF may be a predominant initiator of VTE. Several groups have demonstrated that patients with VTE have increased levels of TF antigen and activity [66,67]. Excessive intravascular TF expression may lead to septic shock, which can be complicated with DIC, multiorgan failure [61].

1.2.6 Role of TF in signaling

TF-dependent signaling regulates a broad range of cellular responses, including gene transcription, protein translation, apoptosis and cytoskeletal reorganization [68]. FVIIa in complex with TF causes several intracellular effects [69], such as mobilization of intracellular calcium stores [70] and transient phosphorylation of intracellular proteins [71]. Mitogen-activated protein kinase (MAPK) is one such protein activated by TF/FVIIa signaling [72]. Phosphorylated MAPK enters the cell nucleus and activates several transcription factors, and its actions are implicated in tumor metastasis [73]. The TF/VIIa complex also plays a role in tumor angiogenesis and metastasis, by enhancing cellular migration in both vascular SMCs [74], and pancreatic cancer cells [75]. Even though precise pathway of intracellular signaling activated by the TF/FVIIa complex and its effect on the

target cell is not fully understood, it is likely that members of G-protein-coupled, protease-activated receptors (PARs) family are involved in this signal transduction [76]. Direct or indirect cell signaling by TF/FVIIa or downstream coagulation proteases have been shown to activate PARs [68]. TF/FVIIa has been shown to primarily activate PAR2, while the TF/VIIa/FXa complex can activate both PAR1 and PAR2 [77].

1.2.7 Role of TF in inflammation

Previous studies have shown that TF contributes to inflammation in a variety of disease models, including endotoxemia, sepsis and ischemia-reperfusion (I/R) [34]. It is well established that an inflammatory response can activate coagulation by inducing TF expression in monocytes [78]. Activation of the coagulation system, in turn, leads to the release of inflammatory cytokines that further stimulate the coagulation system. TF plays a major role in the cross-talk between coagulation and inflammation as the main initiator of blood coagulation and as a modulator of inflammation [78]. Inflammation may enhance activation of TF in diseases such as atherosclerosis and sepsis. Inhibition of the TF/FVIIa complex in a baboon model of sepsis has been shown to reduce the inflammatory response as measured by a reduction in interleukin-6 (IL-6) and IL-8 levels in circulation [79]. Anti-TF interventions have been shown to have beneficial effects in diseases where the combination of coagulation and inflammation plays a major role [78].

1.3 Tissue factor pathway inhibitor

TFPI is a multivalent Kunitz-type proteinase inhibitor and is the principal inhibitor of TF-induced coagulation, which binds and inhibits TF/FVIIa complex in a FXa-dependent manner [80]. By targeting the TF/FVIIa/Xa complex, TFPI ensures that a small procoagulant stimulus does not elicit an uncontrolled burst of thrombin generation [4]. Consequently, a natural threshold must be passed, (the FXa generated must exceed the inhibitory potential of TFPI) before TF/FVIIa-initiated coagulation is allowed to proceed.

1.3.1 Historical perspective of TFPI

Experiments performed in 1940s demonstrated the presence of an endogenous inhibitor of TF-initiated coagulation activation [81-83]. Subsequently, Hjort in 1957 reported that convertin, now known as TF/FVIIa catalytic activity, was inhibited by a component present in serum [84] and named the inhibitor, anticonvertin. The nature of this inhibition remained obscure until 25 years later, when a chromogenic substrate assay system for the determination of the inhibitor was published [85]. In 1985, Sanders et al. reported that inhibition of TF/FVIIa requires the presence of FX [86], and it was quickly shown that FXa rather than FX was responsible for the inhibition of TF/FVIIa. The inhibitor was isolated in 1987, by two independent groups [87,88], and was cloned and characterized in 1988 [89]. The name of the inhibitor varied during the years [anithromboplastin, anticonvertin, TF/FVIIa inhibitor, TF inhibitor, extrinsic pathway inhibitor (EPI) and lipoprotein-associated coagulation inhibitor (LACI)], until a consensus meeting of the Scientific and Standardization Committee of the ISTH in 1991 agreed on the name, TFPI.

1.3.2 Gene, mRNA and protein structure of human TFPI

Initially the human TFPI gene was reported to consist of 9 exons separated by 8 introns that span ~70 kb on the long arm of chromosome 2, at locus q31-q32.1 [90-92] and encodes for TFPI (TFPIα). Because a new exon specific for TFPIβ (alternatively spliced form of TFPI) was found between exon 7 and 8 [93], the new exon was designated exon 8 and the original exons 8 and 9 were designated exons 9 and 10, respectively, in the Figure 7. The presence of TFPIβ was first found in the mouse gene [94] and then confirmed in the human gene [93]. The first two exons are not translated. TFPIα is expressed from exons 3, 4, 5, 6, 7, 9, and 10; whereas, TFPIβ is expressed from exons 3, 4, 5, 6, 7, and 8 (Figure 7). Exon 3

encodes the signal peptide and the acidic N-terminal region of the mature protein, in both TFPIα and TFPIβ. Exons 4, 6, and 9 encode the three Kunitz (K)-domains (K1, K2, and K3) of TFPIα, and exons 5 and 7 encode the peptide sequences located between the three K-domains. Exon 10 encodes the highly basic C-terminal region of TFPIα. On the other hand, exons 4 and 6 encode the first two K-domains (K1 and K2) of TFPIβ, and exon 5 encode the peptide sequence located between the two K-domains. Whereas, exon 8 encodes a totally new C-terminal region that contains a glycosylphosphatidylinositol (GPI) anchor attachment signal and replaces the third K-domain and basic C-terminal region of TFPIα (Figure 7).

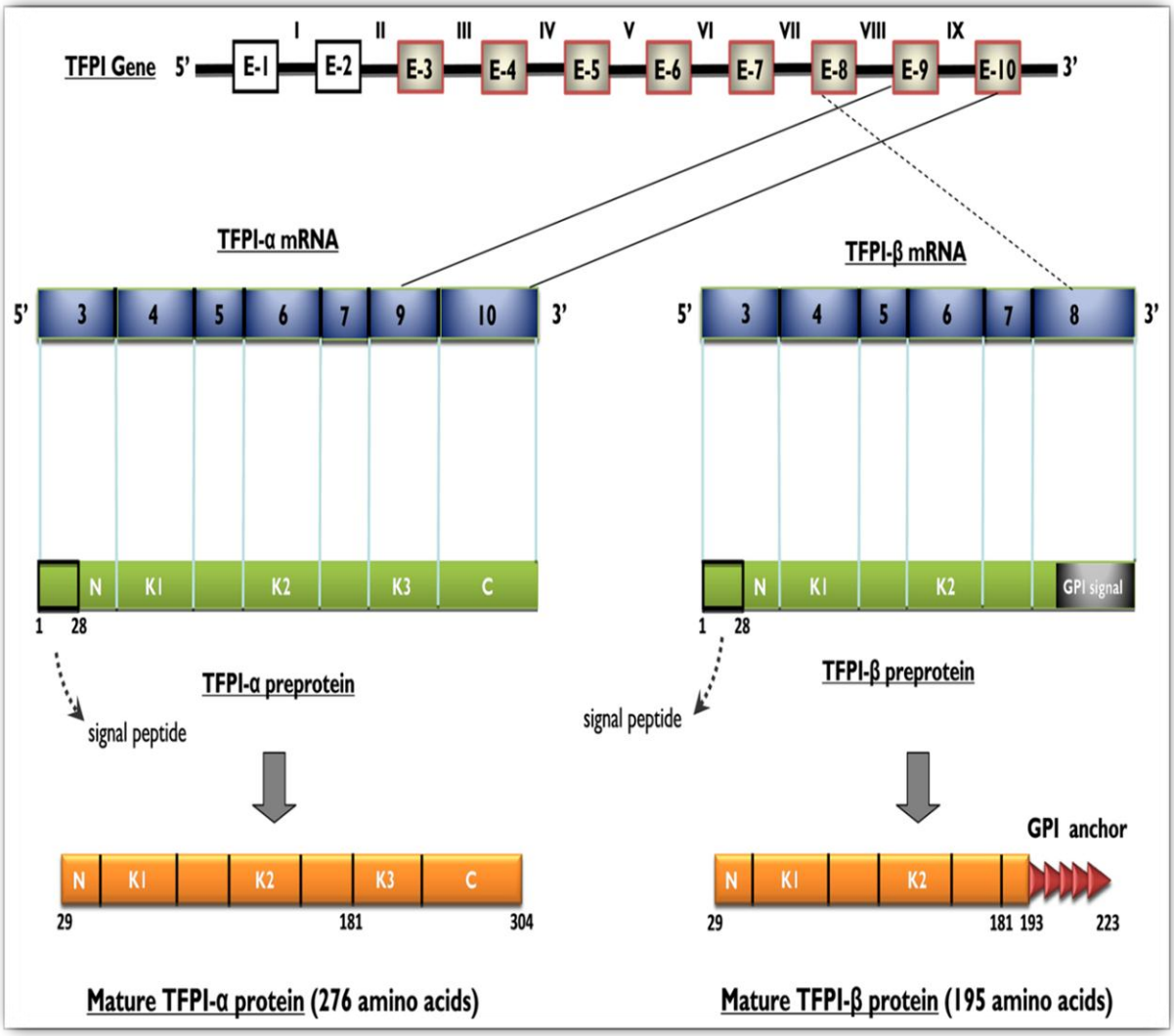

Figure 7. Schematic illustration of human TFPI gene, mRNA and protein. In TFPI gene, exons are labeled (E1-10) and introns (I-IX). In TFPI mRNA, exon 9 and 10 are specific to TFPIα and exon 8 is specific to TFPIβ. (N, acidic N-terminal; K, Kunitz domain; C, basic C-terminal)

Transcription of TFPI yields 2 major sets of mRNA species of ~1.4 kb and 4 kb, respectively [95]. This size difference is primarily attributable to the use of alternative 3'-polyadenylation signals. Further heterogeneity also arises through multiple alternative transcriptional start sites. Differential splicing of the short exon 2, which spans part of the 5'-untranslated region, also contributes to size differences between TFPI transcripts. Despite this variability in mRNA size and sequence, the primary product is the well characterized TFPI protein that is translated from the TFPIα transcript [4].

The mature TFPIα protein consists of 276 amino acids and is the soluble form of TFPI. It is a ~46 kDa glycoprotein and consists of an acidic N-terminal region followed by three tandem, K-type protease inhibitor domains and a basic C-terminal region [89] (Figure 7). The K1 and K2 domains are responsible for TFPI binding and inhibition of the TF/FVIIa complex and FXa, respectively [96]. The K3 domain (which lacks proteinase inhibitory activity) and the C-terminus of TFPIα have been shown to be involved in its cell-surface localization [97]. Recently, it was observed that, in the presence of Ca^{2+} and phospholipids, direct TFPIα inhibition of FXa is significantly enhanced by protein S [98], and this interaction between protein S and TFPIα requires the K3 domain of TFPIα [99]. The C-terminus also plays an important role in inhibitory functions of TFPIα, as C-terminal truncated forms are less potent inhibitors of TF/FVIIa [100] and FXa [101]. Studies of cultured endothelial cells [102] and human placenta [103] have demonstrated that TFPIα associates with the cell surface through a GPI-anchor in a manner that is not dependent on glycosaminoglycans or altered by heparin. Since TFPIα does not contain a C-terminal GPI-anchor attachment sequence, it has been hypothesized that it indirectly associates with the cell surface by tightly binding to an unidentified, TFPI binding protein [93,104].

The mature TFPIβ protein consists of 195 amino acids is an alternatively spliced form of TFPI in which the K3 domain and the C-terminal region of TFPIα are replaced with an unrelated C-terminal region (amino acids 182-193) followed by a GPI-anchor attachment sequence (amino acids 194-223). Thus, TFPIβ associates with cell surfaces exclusively via this direct GPI-anchor [93]. Based on protein mass, TFPIβ (28 kDa) is considerably smaller than TFPIα (36 kDa), but both migrate with the same apparent molecular mass (~46 kDa) on SDS-PAGE, suggesting a difference in post-translational modifications [105]. Although TFPIβ accounts for only 20% of total surface TFPI, it is responsible for most of the TF/FVIIa inhibitory activity, suggesting a potential alternative role for cell-surface TFPIα [105].

Truncated forms of TFPI also exist in the circulation and they lack most of the C-terminus [106]. Although it is not known how the truncated forms are generated physiologically, in vitro data have demonstrated that TFPI is cleaved into degraded forms by various proteases that TFPI might encounter physiologically [107-110].

1.3.3 Distribution of TFPI

The major site of TFPI production is in endothelial cells [111], which constitutively express both α and β forms of the protein under normal conditions [105]. Monocytes are also shown to constitutively express TFPI [112, 113], and contain both α and β forms of TFPI [114]. Whereas, megakaryocytes/platelets are capable of synthesizing TFPIα, but not β form, and they express TFPI on their surface only following dual activation with collagen and thrombin [115]. In addition, TFPI is also normally expressed by vascular SMCs [116] and cardiomyocytes [117]. The majority of TFPI in plasma (70–80%) is truncated and bound to low-density lipoproteins (LDL). Only 10% of plasma TFPI is considered to be free full-length TFPI (TFPIα) [118].

1.3.4 Inhibition of TF-induced coagulation by TFPI

TFPI exerts its anticoagulant function by neutralizing the catalytic activity of FXa, and by feedback inhibition of the TF/FVIIa complex, in the presence of FXa [119,120]. By targeting these serine proteases, TFPI directly inhibits the initiation phase of coagulation. Although the requirement for FXa is not absolute for the inhibition of TF/FVIIa, it is unlikely that TFPI significantly inactivates TF/FVIIa physiologically in the absence of FXa or before its production. Therefore, TFPI may only impart its inhibitory function once the initial stages of the TF-pathway have been allowed to proceed. In the first stage, TFPI inhibits FXa in a 1:1 stoichiometric complex by binding at or close to the active site serine of FXa. This occurs via the ionic binding of FXa to either full-length plasma TFPI (TFPIα), or cell-associated TFPI (TFPIα and/or β) (Figure 8). This interaction involves the P1 residue (Arg 107) in TFPI K-2 domain [96]. This binding is reversible and does not require Ca^{2+} ions. However, Ca^{2+} does augment the potency of FXa inhibition by TFPI in the presence of phospholipid surfaces [121]. In the second stage, TFPI in complex with FXa binds and inhibits the TF/FVIIa complex. During this step, the P1 residue (Lys 36) in TFPI K-1 domain interacts with the active site of FVIIa [96]. Unlike the binding/inhibition of FXa, this step is Ca^{2+}-dependent

and leads to the formation of an inactive quaternary complex, TFPI/FXa/TF/FVIIa on the plasma membrane (Figure 8).

However, kinetic data favor a model in which TFPI interacts with FXa that has not yet been released from the activating TF/FVIIa complex [122] (Figure 8). This quaternary complex has no catalytic activity toward either FX or FIX and results in the marked dampening of TF-induced coagulation.

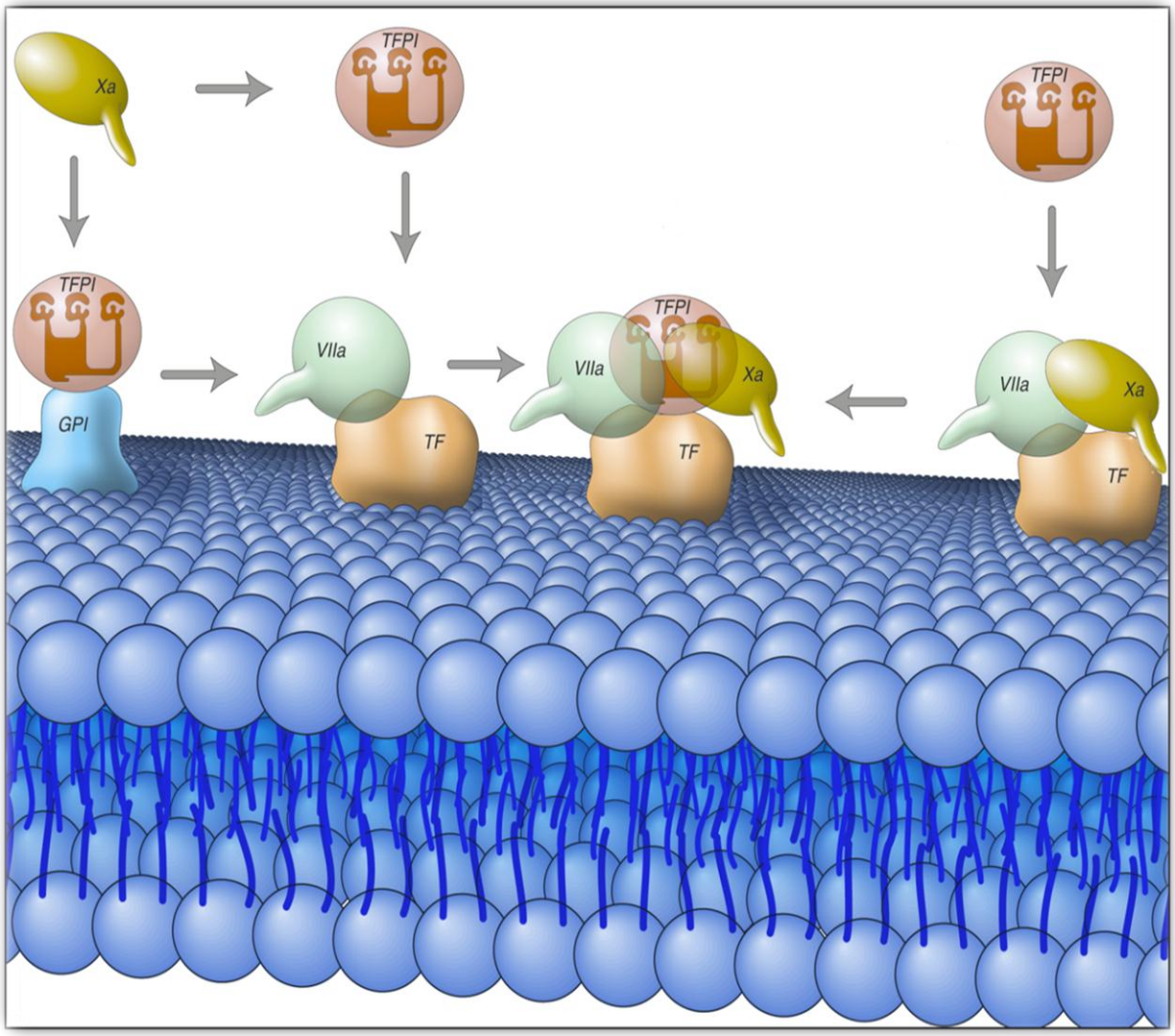

Figure 8. Schematic illustration of the inhibition of TF-dependent coagulation by TFPI. **(A)** After binding to TF/FVIIa complex, FX gets activated and it may dissociate from the activation complex. This FXa become inactivated by cell-associated (TFPIα and/or β) or plasma TFPI (TFPIα) via its K2 domain. This TFPI, in complex with FXa can now bind to the TF/FVIIa complex, with the K1 domain binding to the active site of FVIIa. **(B)** However, kinetic studies favor a model whereby TFPI binds and inactivates TF/FVIIa/FXa before FXa dissociation. In both cases, the resulting final inactive quaternary complex is the same.

1.3.5 Consequences of TFPI deficiency

Similar to TF, TFPI is essential for survival and its deficiency has not been reported in humans. However, complete deficiency of TFPI in mice is embryonically lethal [123]. The physiological importance of TFPI is clearly demonstrated by the lethal phenotype exhibited by homozygous TFPI gene deletion in mice ($TFPI^{-/-}$). TFPI-null mouse embryos die in utero because of intrauterine coagulopathy and vascular disintegrity [123]. However, consistent with its primary role as the inhibitor of the TF-induced coagulation, this embryonic lethality can be overcome by dramatically reducing levels of TF, highlighting the importance of maintaining a balance between TF and TFPI levels [5] to allow hemostasis and prevent thrombosis (Figure 1). Furthermore in rabbits, immunodepletion of TFPI dramatically lowers the TF threshold required to initiate coagulation [124]. Whereas, administration of recombinant TFPI can increase the TF threshold needed to initiate coagulation, thereby preventing thrombosis [125-127].

1.4 Carotid atherosclerosis and arterial thrombosis

Arterial thrombosis, characterized by atherosclerotic plaque disruption with superimposed thrombosis, is the main cause of stroke. Stroke is the second most common cause of death and major cause of disability worldwide [128]. Most strokes are ischemic and the result of carotid atherosclerotic disease. The spectrum of symptoms related to carotid artery disease includes transient ischemic attack, amaurosis fugax, and outright cerebrovascular accidents [129]. Atherosclerosis is a process that affects medium and large-sized arteries, initiated by endothelial dysfunction and is characterized by patchy intramural thickening of the subintima that protrudes into the arterial lumen. Each vascular bed may be affected by this process; the etiology, treatment and clinical impact of atherosclerosis varies from one vascular bed to another [130].

Atherosclerosis proceeds through a series of pathological stages: intima-media thickening, fatty streak formation, and development of the fibrous atherosclerotic plaque (the hallmark of established atherosclerosis), which may grow and cause narrowing of the vessel [131]. Ultimately the plaque may accumulate large amounts of lipids and inflammatory cells, and often becomes unstable and plaque may finally rupture. The latter frequently results in acute occlusion of the artery, downstream of the lesion by embolization with plaque debris; or most importantly, release of TF and ultimately formation of thrombus [132]. Various risk factors associated with the development of atherosclerosis, some constitutional and therefore immutable and others are acquired and potentially controllable [133]. The constitutional risk factors are age, sex, race and genetic disposition. There are four major acquired risk factors: hyperlipidemia, hypertension, smoking and diabetes mellitus [133], that can cause injury to the endothelial cells. Repeated injury to the endothelial cells can cause increased endothelial permeability for lipids, increased expression of adhesion molecules for leukocytes, monocytes and platelets, resulting in increased migration of monocytes into subendothelial space where they transform into macrophages and avidly engulf lipoproteins [134].

1.4.1 Stages in the atherosclerotic plaque formation

The consensus report by the Committee on Vascular Lesions of American Heart Association classifies the different stages of atherosclerotic plaque development into three types of early lesions (plaque types I to III) and three types of mature late lesions (plaque types IV to VI) according to their histopathological appearance [135]. Type I is an initial

lesion, which contains sufficient atherogenic lipoprotein to elicit accumulation of monocyte-derived macrophages within the vascular wall, which are also transformed into few foam cells. Subsequently, the changes are more marked in locations of arteries with adaptive intimal thickening, which are present at constant locations and do not obstruct the lumen. These adaptive thickenings represent a mere adaptation to the mechanical forces applied to these regions of the vessels. Type II lesions primarily consist of the layers of foam cells and lipid-laden SMCs, co-localizing with the fatty streaks. Type III lesions are the intermediate stages between type II and type IV. In addition to the lipid-laden cells in the lesion of type II, type III lesions contain a varying number of extracellular lipid droplets and particles that disrupt the coherence of some intimal SMCs. These lipid droplets are the precursors of the larger, confluent, and more disruptive core of extracellular lipid that is characteristic to type IV lesions. Lesions that usually have a lipid core may also contain thick layers of fibrous connective tissue (type V lesion) and/or fissure, hematoma, and thrombus (type VI lesion). Some type V lesions are largely calcified (type Vb), and some consist of fibrous connective tissue with little or no accumulated lipid or calcium (type Vc).

An alternative pathohistological staging of an atherosclerotic plaque can be graded into the seven relative stages of development. First, LDL moves into the subendothelium and is oxidized by monocytes/macrophages and vascular SMCs (stages 1 and 2). During stages 3 and 4, the release of growth factors and cytokines attracts additional monocytes. Foam cells accumulation and proliferation of vascular SMCs result in growth of the plaque (stages 5, 6, and 7) [130]. At these later stages, gradually enlarging plaques may clinically manifest as a chronic stable angina if coronary artery is affected or myocardial infarction, which may often occur even if the artery has a relatively unremarkable narrowing. This happens due to the plaque erosions and formation of blood thrombus, which narrows the lumen of an artery [136,137]. Apoptosis of endothelial cells facilitates the exposure of the thrombogenic plaque material to the coagulation factors present in circulating blood, especially, when the large amounts of TF expressed by plaque monocytes/macrophages and activated circulating monocytes are released [137]. Furthermore, it is well recognized that inflammatory mediators, like TNF-α, interleukins, interferons and chemoattractants, which are pathognomonic to atherosclerosis, all can activate monocyte TF expression [138-141].

1.4.2 Plaque composition and thrombosis

Plaque morphology in terms of echogenicity (reflectance of the emitted ultrasound signal) can be classified into four grades: echolucent, predominantly echolucent, predominantly echogenic and echogenic [142]. Plaques that appear echolucent are characterized by high content of lipids and inflammatory cells and covered by a thin fibrous cap, making them vulnerable to rupture. On the other hand, echogenic plaques have higher content of fibrous tissue (that appears to stabilize the plaque) and/or calcification and are covered by a thicker fibrous cap [143]. It is known that the frequency of thrombosis in the sites of calcified plaques is significantly lower than in lipid-rich lesions [144,145]. The postmortem analysis of coronary plaque morphology in suddenly died men revealed that in 70% of cases of acute coronary thrombosis one or more arteries contained lipid-rich vulnerable plaques [146].

1.5 Venous thromboembolism

Deep vein thrombosis (DVT) and pulmonary embolism (PE) are collectively referred to as venous thromboembolism (VTE), is the cause of substantial morbidity and mortality worldwide. DVT is the formation of a blood clot in the deep veins, usually affecting the large veins of the leg or thigh, but can also occur in other parts of the body. PE occurs when a blood clot dislodges from its original site and embolize to the arterial blood supply of the lungs. The annual incidence of VTE is 1-2 per 1000 inhabitants [147], and it is the third most common cardiovascular disease after myocardial infarction and stroke [148]. VTE is a multifactorial disease including genetic, acquired and environmental risk factors. Genetic risk factors include major hereditary prothrombotic conditions associated with reduced levels of the anticoagulant proteins (TFPI, AT, protein C, and protein S), increased levels or function of the coagulation factors (APC resistance, factor FV Leiden mutation, prothrombin gene G20210A mutation) and elevated levels of FVIII, FIX, and FXI [149]. Acquired and environmental risk factors include aging, obesity, surgery, trauma, hospitalization, acute medical conditions, malignancy, immobilization, pregnancy, use of estrogens and the lupus anticoagulant [150,151]. Despite knowledge of several risk factors for VTE, 30-50 % of the VTE events still occur in the absence of predisposing factors [147].

In contrast to arterial thrombosis which appears to be primarily caused by TF exposed by atherosclerotic plaque rupture, venous thrombosis typically occurs in the absence of gross

vessel wall disruption [152]. Under normal conditions due to the presence of various proteins, such as TFPI, TM, and EPCR, the endothelial surface inhibits blood coagulation [153,154]. However, due to physical (e.g., vascular damage) or functional (e.g., hypoxia) alterations of the venous endothelium, thrombosis is initiated as a result of reduced expression of anticoagulants and the induction of TF expression [154]. In 1856, the German pathologist Rudolph Virchow proposed a triad of causes for venous thrombosis: alterations in blood flow (e.g., stasis), alterations in the vessel wall (activation of the endothelium), and hypercoagulability of blood [155].

Studies have shown that venous stasis plays a significant role in the pathogenesis of VTE [156,157]. Venous thrombosis is believed to start in regions of stasis of blood flow, often in the valve pockets of deep venous valves [158]. Alteration in blood flow allows the accumulation of prothrombotic substances, such as TF-positive MPs and thrombin within the valve pockets, which under normal conditions washed downstream and are inactivated in the capillary beds of the lungs [159]. In addition, hemoglobin in the resident erythrocytes undergoes rapid desaturation under static conditions, leading to hypoxic responses in leukocytes, platelets, and endothelial cells [160]. Activation of these cells can trigger coordinated inflammatory and hemostatic responses. Hypoxia stimulates TF expression on monocytes within a time-course relevant to venous thrombosis [161] and concomitantly induces the release of TF-positive MPs [162]. In addition, exposure to hypoxic conditions results in surface P-selectin expression [163], and up-regulation of procoagulant activity on endothelium [164-166], which creates a potential docking site for nascent TF-positive MPs, as well as for platelets, leukocytes, and leukocyte- or tumor-derived MPs expressing P-selectin glycoprotein ligand-1 (PSGL-1) [167,168]. Since these TF-positive MPs have varying concentrations of negatively charged phosphatidylserine on the outer leaflets of their membranes [162], once bind to P-selectin on endothelial cells, they provide both (i) a suitable negatively charged membrane surface for binding coagulation proteins and (ii) TF to initiate the coagulation cascade. In addition, TF-bearing MPs bound to platelets fuse with the platelet membrane, resulting in a phenotype transfer [169]. Studies utilizing inhibitors of P-selectin shed more light on the role of the PSGL-1 and P-selectin binding interaction in the genesis of venous thrombosis [170-172]. The supposition is that by inhibiting P-selectin binding, TF-positive MPs are unable to bind to target cells and to initiate localized thrombus formation. TF-positive MPs may also contribute to the hypercoagulable state associated with disease conditions (e.g., cancer) with an increased risk of DVT [173].

2 Aims of the study

TF expression in human monocytes both at mRNA and protein levels is well documented, but little is known about the expression and functional role of monocyte TFPI. In addition, little is known about the relationship between monocyte TF and TFPI, and its association with the risk of arterial and venous thrombosis. The main purpose of this study was to investigate the expression levels of TF and TFPI at their gene, protein levels and their possible impact on the risk of arterial and venous thrombosis.

Specific aims of the present study:

1. To investigate the relationship between monocyte TF and TFPI at their gene and protein levels in circulating peripheral blood mononuclear cells (PBMCs) obtained from healthy subjects, as well as impact of monocyte surface presented TFPI on monocyte TF-mediated thrombogenicity.

2. TF expression in blood cells other than monocytes has been disputed, possibly due to methodological variations and usage of less characterized TF-antibodies. The aim of this study was to investigate the specificity and sensitivity of four commercially available TF antibodies towards detection of TF antigen on platelets, monocytes and microparticles, using flow cytometry.

3. To investigate the possible imbalance between TF and TFPI in circulating monocytes obtained from asymptomatic subjects with echolucent or echogenic carotid plaques and healthy controls without carotid atherosclerosis, as well as in lipid-rich and calcified carotid plaque specimens obtained from patients with advanced atherosclerosis.

4. To investigate the impact of blood monocyte count on the future risk of incident VTE, and to study procoagulant functions of circulating blood monocytes obtained from patients with a previous history of unprovoked VTE and sex- and age matched healthy controls recruited from the general population.

3 Summary of papers

3.1

Paper I: The role of TFPI in regulation of TF-induced thrombogenicity on the surface of human monocytes.

Although the procoagulant activity of monocytes largely depends on expression and cell surface presentation of TF, little is known about TFPI expression in monocytes and its impact on regulation of TF procoagulant activity on the monocyte surface. In this study, we investigated the relationship between monocyte TF and TFPI at their gene and protein levels in circulating peripheral blood mononuclear cells (PBMCs) obtained from healthy subjects, as well as impact of monocyte surface presented TFPI on monocyte TF-mediated thrombogenicity.

Unstimulated PBMCs expressed 2-fold higher TFPIα than TF mRNA. In addition, unstimulated CD14+ monocytes constitutively expressed surface TFPI antigen, but not TF. LPS-stimulation caused an immediate upregulation of TF, TFPIα and TFPIβ mRNA, which peaked after 6 h. TFPIα and TFPIβ exhibited similar profiles of mRNA expression and both remained upregulated during 24 h of LPS-stimulation, whereas, TF mRNA expression declined gradually from 6 to 24 h. Similar to mRNA, LPS-stimulation caused a marked increase in the TF+, CD14+ cell population, which remained unchanged after reaching the peak after 6 h, whereas, TF-MFI declined gradually after 6 h. In contrast, TFPI-MFI increased gradually during 24 h LPS stimulation. Pre-incubation of unstimulated PBMCs with anti-TFPI IgG caused a pronounced increase in their surface TF activity, which indicates that 40-50% of the TF activity in these cells is neutralized by endogenous TFPI. In CAT assay, PBMCs pre-treated with anti-TFPI IgG enhanced their ability to induce thrombin generation, measured by shortening of the lag time, increased peak thrombin generation and elevated endogenous thrombin potential (ETP). Likewise, pre-treatment of LPS-stimulated PBMCs with anti-TFPI IgG enhanced their ability to induce thrombin generation compared to LPS-stimulated PBMCs.

Our findings support the concept that surface bound TFPI is an important regulator of procoagulant reactivity of human monocytes.

3.2

Paper II: Differential impact of tissue factor antibody clones on tissue factor antigen expression in blood cells and microparticles.

Under normal conditions, cells in contact with blood do not express physiologically active TF [174]. But, monocytes are able to express TF upon activation under certain pathological conditions [175]. However, its presence in other blood cells has been disputed. The discrepancies in the various reports on TF localization in different blood cells are possibly due to the methodological variations and the usage of TF antibodies that do not meet stringent criteria for monospecificity. To explore this hypothesis, we investigated the specificity and sensitivity of 4 commercially available TF antibodies (HTF-1, TF9-10H10, CLB/TF-5 and VD8) in flow cytometry.

HTF-1 detected TF antigen only on LPS-stimulated monocytes, whereas, TF9-10H10 and VD8 detected TF associated with MPs in addition to LPS-stimulated monocytes. Surprisingly, CLB/TF-5 detected TF on platelets, monocytes and MPs, where the percentage of TF positive events detected was ~1.8% for unstimulated platelets, ~11% for A23187-stimulated platelets, ~7.7 % for unstimulated monocytes and ~7.5% for plasma derived MPs, possibly due to non-specific binding. Further, to assess inhibitory effect of TF antibodies on cell surface TF activity, LPS stimulated monocytes and MPs shed from monocytes during LPS stimulation (MMPs) were incubated with purified TF antibodies at different antibody concentrations prior to TF activity measurement. At a final concentration of 10 μg/mL, HTF-1, CLB/TF-5 and VD8 inhibited monocytes TF activity by 81-84% and MMPs TF activity by 92-96%. In contrast, TF9-10H10 had no inhibitory effect.

Our results show a possible non-specific binding by the CLB/TF-5 antibody in a flow cytometry test system that may explain the discrepancies in some of the reports, where presence of TF on different blood cells, particularly on platelets and MPs was documented.

3.3

Paper III: Circulating monocytes mirror the imbalance in TF and TFPI expression in carotid atherosclerotic plaques with lipid-rich and calcified morphology.

Thrombogenicity of atherosclerotic plaque largely depends on plaque morphology and their TF and TFPI content. In this study, we investigated whether lipid-rich (echolucent) or calcified (echogenic) morphology of carotid atherosclerotic plaques is associated with differences in TF and TFPI expression in circulating blood monocytes and within carotid atherosclerotic plaques. We studied levels of monocyte TF and TFPI mRNA, protein and TF activity in PBMCs obtained from asymptomatic subjects with echolucent (n=20) or echogenic (n=20) carotid plaques, or controls without carotid atherosclerosis (n=20). Further, sections of lipid-rich or calcified carotid plaques obtained from symptomatic patients were assessed for TF and TFPI antigen expression.

Individuals with echolucent carotid plaques (echolucent group) expressed higher levels of monocyte-TF mRNA, TF and TFPI antigen, TF/TFPI ratio and TF activity compared to echogenic group or healthy controls ($P<0.05$). In contrast, the levels of monocyte-TFPIα mRNA were lower in echolucent group, compared to echogenic group or healthy controls ($P<0.05$). Interestingly, levels of monocyte-TFPIβ mRNA were lower in echolucent and echogenic groups, compared to healthy controls ($P<0.05$). The immunostaining of lipid-rich and calcified carotid plaque sections against TF and TFPI antigens showed nearly 2.5 fold higher TF and 1.5 fold higher TFPI protein levels in lipid-rich plaques relative to calcified lesions ($P<0.05$), also yielding a higher TF/TFPI ratio.

Our findings indicate that the imbalance found between TF and TFPI expression in circulating monocytes from echolucent group reflect changes found within advanced carotid atherosclerotic plaques obtained from symptomatic patients. Although, TF expression within atherosclerotic lesion is a major mediator of plaque thrombogenicity, we believe that circulating blood monocytes could participate in thrombus formation on the surface of lipid-rich plaques contributing to a highly inflammatory milieu of a lipid-rich lesion. This scenario induces high levels of procoagulant TF within the plaque, which leads to formation of unstable lesion prone to thrombosis.

3.4

Paper IV: Monocyte count and procoagulant functions are associated with risk of venous thromboembolism: the Tromsø study

Monocytes are important regulators of blood thrombogenicity through expression of TF on their surface under various pathological conditions. It has been speculated that monocytes are involved in the pathogenesis of VTE. In this paper, we investigated the impact of monocyte count on the risk of VTE in a population based cohort study, and monocytes procoagulant functions were assessed in a population based case-control study. Monocyte counts were available in 25127 subjects at baseline and incident VTE events were registered through the end of follow-up. Monocyte TF mRNA, antigen and TF activity were studied in a subsequent study of patients with unprovoked VTE (n=20) and control subjects (n=20).

There were 429 incident VTE events during a median of 12.5 years of follow-up. The impact of monocyte count on risk of VTE changed significantly over time (*P*=0.004), with a moderately increased risk of VTE during the first 5 years of follow-up. Subjects with monocyte count $\geq$0.7 x10^9/L, had a 2.5-fold increased risk of VTE as compared with subjects with monocyte count $\leq$0.4x10^9/L during the first year. The risk estimates became attenuated throughout the study period, and by the end of follow-up there was no association between monocyte count and VTE. In the case-control study, patients with unprovoked VTE expressed higher levels of monocyte surface TF antigen (*P*=0.036) and TF activity (*P*=0.015) under resting conditions. LPS-stimulation of monocytes caused two-fold higher TF mRNA (P<0.01) expression and shedding of microparticles with higher TF activity (P=0.011) among VTE patients.

Our findings support the concept that monocyte count and procoagulant functions are involved in the pathogenesis of venous thrombosis.

4 Methodological considerations

4.1 Isolation of PBMCs and pure monocytes

To study functional and morphological characteristics of monocytes/macrophages, isolated human blood PBMCs/pure monocytes or monocytic cell lines (e.g., THP-1) can be used [176]. Blood monocytes can be isolated and purified by different methods, such as adhesion of blood monocytes [177], or isolation of PBMCs by density gradient centrifugation followed by monocyte purification by flow cytometry cell sorting [178], or by counter-current elutriation [179] or by negative/positive selection with magnetic bead labeling [180].

In all four papers, for the isolation of PBMCs by density gradient centrifugation (Lymphoprep), EDTA anticoagulated whole blood was used, since EDTA has been shown to results in minimal monocyte-platelet aggregates [181], and less cytotoxicity [182]. In our preliminary experiments, EDTA also results in higher PBMC yield and lower monocyte TF activity in unstimulated PBMCs compared to citrate and heparin. PBMCs were isolated using lymphoprep by centrifuging at 800 x g for 20 min. The PBMC interface was carefully collected and washed with endotoxin-free PBS without Ca^{2+} and Mg^{2+} twice at 150 x g and once at 250 x g. With these low speed centrifugation steps, most of the platelets were easily eliminated. All centrifugation steps were conducted at room temperature (25 ± 3 ^{o}C). Temperature is also a critical factor, as it has been shown that monocytes aggregate spontaneously [183], and platelets get activated [184] at lower temperatures. PBMCs were then resuspended in RPMI-1640 medium and cryopreserved at -152 ^{o}C after adding 10% heat inactivated fetal bovine serum (FBS) and 7.5% DMSO. Prior to cryopreservation, we always subjected PBMCs to a standard routine testing of flow cytometric viability (staining for both Annexin V and 7-AAD) and also monocyte activation status by measuring TF activity. Previously it has been shown that these cryopreserved PBMCs retain their functionalities, such as procoagulant activity, phagocytosis, maturation and viability, comparable to freshly isolated cells [179, 185-188]. No platelets were detected in purified PBMCs by cell counting (ABX micros 60-CT) before cryopreservation and after thawing.

We have used cryopreserved PBMCs isolated by density gradient centrifugation in paper I and III. In paper-IV, we purified CD14-positive monocytes from cryopreserved PBMCs, by positive selection using ‘anti human CD14 magnetic particles-DM’ (BD Biosciences). Because of the limited PBMCs available from VTE patients, we used positive

selection, which results in pure monocytes with bound CD14 magnetic particles. However, we found that the presence of magnetic particles on purified monocytes had no effect at least on monocyte LPS stimulation and MPs generation. In addition, APC-conjugated CD14 antibody was readily detected these monocytes in flow cytometry analysis. In paper-II, we prepared untouched pure monocytes from freshly isolated PBMCs, by negative selection using 'human monocyte enrichment cocktail' (contains biotin-conjugated mouse anti-human antibodies- CD3, CD45RA, CD19, CD56, and CD235a) (BD Biosciences) and 'streptavidin particles plus-DM' (magnetic nanoparticles that have streptavidin covalently conjugated to their surface). Surprisingly, when we use an additional antibody against platelets (biotin mouse anti-human CD41a) to remove any remaining platelets, it resulted in relatively low monocyte yield, explains the presence of platelets (activated platelets) in the form of monocyte-platelet aggregates. This is one of the known limitations of working with blood or isolated blood cells ex vivo, as washing steps can activate platelets present in the PBMC fraction and thereby allow them to aggregate with monocytes through interactions between P-selectin (on activated platelets) and PSGL-1 (on monocytes) [181], making it very hard to isolate pure platelet free monocytes.

As seen in paper-II, pure platelet-free monocytes can be isolated by negative selection, with the use of an additional antibody against platelets, where all free platelets and monocytes aggregated with platelets can be removed. However, this approach leads to very low monocyte yield, making it difficult to use in clinical studies, where a limited amount of patient blood samples are available. Since we have not used this isolation approach in our papers-I, III, and IV, there is a possibility that very few platelets may still be present in our PBMC samples. Despite of possible low platelet contamination in our PBMCs, available data shows that platelets may have no contribution in TF [52] and TFPI [115] expression. Even though, controversy is still going on about TF expression in platelets, many studies have found no detectable TF antigen or TF activity in platelets [52,53,189]. On the other hand, although TFPI is produced by megakaryocytes, it is neither expressed on the platelet surface, nor stored within platelet α granules [190]. However, TFPI is expressed on the surface of coated-platelets, a sub-population of platelets observed only following dual stimulation with thrombin and convulxin [190]. TFPIβ is however, absent in human platelets [115,190].

4.2 Lipopolysaccharide

LPS (endotoxin) is the main element of the outer cell membrane in the Gram-negative bacteria [191] and is the major initiator of innate immune response to Gram-negative bacterial infection. Human monocytes are highly sensitive to even minute amounts of LPS since they represent one of the major cellular components of the innate immune system evolved to recognize constituents of common infectious agents. Human monocytes respond to LPS by expressing TF and inflammatory mediators such as cytokines (TNF-α, IL-1, IL-6) and chemokines (IL-8, Monocyte chemoattractant protein-1) [191]. LPS can be sensed by monocytes via CD14 and TLR4 (Toll-like receptor 4), which both are receptors on the surface of the cell membrane. LPS forms complexes with LPS-binding protein (LBP) in plasma and is delivered to the cell surface receptor CD14. Due to its lack of a transmembrane domain, CD14 cannot transfer the signal across the cell membrane. However, CD14 can transfer LPS to the transmembrane signaling receptor (TLR4) and its accessory protein (MD2) [191]. Stimulation of human monocytes with LPS activates a variety of intracellular signaling pathways and three MAPK pathways. These signaling pathways in turn activate several transcription factors which coordinate the induction of many genes encoding inflammatory mediators [191].

Different concentrations of LPS was used in various studies to stimulate human monocytes in vitro, ranging from physiologically relevant concentrations (~10 pg/mL) [192] to supraphysiological concentrations (1 μg/mL) [189], making it difficult to decide optimal LPS concentration. However, Henriksson et al has convincingly shown that LPS concentration above 1 ng/mL was not followed by a concomitant increase in monocyte TF-expression [193]. Addition to this, with increasing LPS concentrations up to 1000 ng/mL, increased number of monocytes positive for Annexin V was observed. By using flow cytometry they revealed that many of these Annexin V positive monocytes were dying or dead cells, as the cells were double positive for both Annexin V and 7-AAD (7-Amino-Actinomycin D). With this background, we utilized LPS concentrations ranging from 1 to 100 ng/mL in our preliminary experiments. No cell death was observed in our experiments with LPS up to 5 ng/mL, as majority of the monocytes were negative for 7-AAD. Thus, we have considered using 5 ng/mL LPS as the optimal concentration to study monocyte TF without significant cell death.

4.3 RT-qPCR

RT-qPCR (reverse transcription quantitative real-time PCR) was used in papers I, III and IV for the quantification of TF, TFPIα and TFPIβ mRNAs. A two-step RT-qPCR was used in all three papers, which includes reverse transcription of total RNA into cDNA using reverse transcriptase (RT) and then this cDNA is transferred to a separate tube for the qPCR reaction. Total RNA was isolated from human PBMCs or pure CD14+ monocytes using Absolutely RNA RT-PCR kit (Stratagene) and cDNA was synthesized using Superscript III RT (Invitrogen). Quantity and quality of RNA and cDNA was assessed using NanoDrop 1000 spectrophotometer. Real-time PCR was conducted using the Mx3000P real-time platform (Stratagene) to amplify and simultaneously quantify the cDNA sequences corresponding to TF, TFPIα, TFPIβ and GUS (β-glucuronidase). As the name suggests, specific sequences of amplified cDNA accumulated in the reaction was quantified in real time after each amplification cycle. In this way, RT-qPCR allows both detection and quantification of TF, TFPIα, TFPIβ and GUS specific sequence in each cDNA sample.

In paper-I and III, total RNA was isolated from human PBMCs, whereas in paper-IV total RNA was isolated from CD14+ monocytes. Next, for cDNA synthesis from total RNA, total RNA was primed with oligo$(dT)_{20}$ primers instead of using random hexamers or gene specific primers. This is because, oligo(dT) is used to hybridize to 3' poly(A) tails, which are found in the vast majority of eukaryotic mRNAs. Since poly $(A)^+$ RNA constitutes approximately 1 to 2% of total RNA, the amount and complexity of cDNA is considerably less than with random hexamers. Even though, using gene specific primers results in amplification of cDNA specific to gene of interest, for every gene of interest a separate cDNA reaction should be used. Since we were interested to study four genes, we have used oligo (dT) primers to synthesis cDNA from total RNA.

For quantification of gene specific cDNA sequence in the PCR reaction different fluorescence-based detection technologies are available; DNA-binding dyes (SYBR Green I), primer-based and probe-based detection systems. We have used probe-based detection system in all 3 papers as this system provides highly sensitive and specific detection of DNA, compared to DNA-binding dyes. In DNA-binding dye technology the dye binds indiscriminately to all double-stranded DNA formed during real-time PCR, and not just to the target DNA. Hydrolysis probes (TaqMan probes) require a pair of PCR primers in addition to a probe with both a reporter and a quencher dye attached. The probe is designed to bind to

sequence amplified by the primers. During qPCR, the probe is cleaved by the 5' nuclease activity of *Taq* DNA polymerase; this releases the reporter dye and generates a fluorescent signal that increases with each cycle. Using Beacon designer software (Stratagene) we designed primers and probes complementary to human cDNA sequences of TF, TFPIα, TFPIβ (sequences are presented in Paper-I) and GUS. The TFPIα and TFPIβ assays share a common forward primer and probe, but different reverse primers, specifically amplifying TFPIα isoform or TFPIβ isoform.

Further, for the quantification of results from qPCR experiment there are two basic quantification methods are in use; absolute or relative quantification. In absolute quantification, samples of known quantity are serially diluted and then amplified to generate a standard curve. An unknown sample can then be quantified by interpolating their quantity from the standard curve. Using absolute quantification, one can calculate absolute gene copy numbers in a given cDNA sample. Whereas in relative quantification method, the gene of interest in one sample (i.e., treated) is compared to the same gene in another sample (i.e., control). The results are expressed as fold up- or down-regulation of the treated in relative to the untreated. Recently, Mälarstig et al [194] have reported very sensitive absolute quantification method to detect low amounts of TF (down to 10^2 copies). We have used the same quantification method in all three papers.

Finally, normalization is essential to neutralize the effects of variability in cDNA quality that can lead to misinterpretation of the results. The use of a normalizer gene (housekeeping gene) is the most thorough method of addressing almost every source of variability [195]. In quantitative PCR, the issue of housekeeping genes is indeed very important, in particular for experimental settings where LPS is used for the activation monocytes/PBMCs [196]. Therefore, we set to evaluate a series of potential housekeeping genes prior to setting up our assays for TF, TFPIα and TFPIβ. Using a commercial system for evaluation of 11 housekeeping genes (Taqman human endogenous control assay, Applied Biosystems) we performed repetitive runs with and without stimulation by LPS. GUS was clearly one of the genes least influenced by LPS. Our results are in line with a previous study by Löseke et al [197] confirming that GUS could be used as a suitable housekeeping gene for PCR quantification upon stimulation with LPS. Thus, GUS was selected as housekeeping gene because it had the smallest deviations from the calibrator baseline under LPS stimulation.

4.4 Quantification of TF activity

TF activity of the test sample [i.e., intact PBMCs (paper-I and III), intact monocytes (paper-II and IV), or monocyte derived MPs (paper-II and IV)] was measured in a two-stage amidolytic assay based on the ability of TF to accelerate the activation of FX by FVIIa [198], using bovine FV/Va (Enzyme Research Laboratories) and barium citrate eluate containing FVII [199], FX, and FII [200] purified from human plasma. The reaction was initiated by adding $CaCl_2$ (4.0 mM final concentration). The thrombin generated was then quantified by the addition of substrate 'Pefachrome TH' (Pentapharm). The purified clotting factors were combined to obtain an extremely sensitive assay for TF activity in the following way: to FVII (8.8 nM), FX (91.68 nM), FV/Va (15.15 nM), and FII (500 nM), all final concentrations, we added cephalin (as phospholipid source) and the test sample (TF source). Eight dilutions of a standard TF preparation from rabbit brain (Nycomed Pharma) were used to make a standard curve. TF standard and test samples were diluted in ½ TBS/BSA buffer (0.05 M Tris, 0.15 M NaCl, pH 7.5 was diluted 1:1 with sterile distilled water and then added 1 mg/ml BSA). The substrate 'Pefachrome TH' dissolved in sterile distilled water at a concentration of 2.5 mM was mixed 1:1 with TBS/HCl, pH 9.5, before use.

60 µl of preincubation mixture (50 µl barium citrate eluate containing FVII, FX and FII, 75 µl bovine FV/Va, 5 µl cephalin, and 4 ml ½ TBS-BSA buffer) was mixed with 15 µl of diluted test sample or TF standard. After addition of $CaCl_2$, the mixture was incubated at 37 ^{o}C for 4 min. The thrombin generated was then quantified by adding 30 µl of 'Pefachrome TH' substrate and after 4 min the reaction was stopped with 120 µl of 50% acetic acid. TF activity was directly proportional to the amount of the colored product *para*-nitroanilide dihydroacetate (pNa) generated, which was determined at 405 nm spectrophotometrically. TF activity in our undiluted standard rabbit brain preparation was defined as 1 U, thus; TF activity measured in our test sample was expressed as mU/cell count.

In contrast to measuring thrombin as explained, the most commonly used TF activity assay evaluates FXa generation, based on the ability of TF to promote the activation of FX, in the presence of FVIIa and $CaCl_2$ [189]. Same principle is used in commercial TF activity assay (Actichrome TF, American Diagnostica) in which FVIIa, FX, and a chromogenic substrate for FXa are added to the sample. Even though measuring FXa is straight forward procedure compared to measuring thrombin, this procedure should be used carefully in order to measure FXa generated specifically from TF/VIIa complex, and not by FVIIa alone.

Supraphysiological concentrations of FVIIa (10 nM) are used in many studies [201-204], exceeding those circulating FVIIa in vivo (~ 0.1 nM) [33] by 100 times. At this high concentration, FVIIa can convert FX to FXa in the absence of TF [205,206], making quantification of TF activity unreliable. Furthermore, Østerud et al. clearly demonstrated that platelets with increased FV activity after freezing and thawing significantly influence the measurement of FXa, but not thrombin [28]. On the other hand, a recent study found that FXa generation in the 'Actichrome TF activity assay' may not depend on exogenous FVIIa or endogenous TF [207]. It was also found that results can be influenced by the color of the plasma in this commercial assay [207]. Since our frozen-thawed PBMCs used in paper-I, III and IV may still possibly contain few platelets and thereby influence the FXa measurement, we measured thrombin in our TF activity assay, instead of measuring FXa.

4.5 Microparticles

MPs are submicron-size vesicles released from cell membranes in response to activation or apoptosis [208]. They are generally heterogeneous in size (0.1-1μm) and mostly express phosphatidylserine and membrane antigens representative of their cellular origin [209]. MPs differ from exosomes (which are smaller <0.1 μm) with respect to their origin, protein content, and size distribution [209]. Elevated number of MPs originated from different cells was reported in various clinical conditions [210]. However, standardization of pre-analytical and analytical methods for the measurement of MPs remains a challenge [211].

4.5.1 Isolation of MPs from plasma and cell-supernatants

Different pre-analytical approaches may lead to different results concerning the number of MPs and their procoagulant activity, making any comparison difficult between different studies [211,212]. Standardized pre-analytical methods: blood collection, plasma isolation, storage, and MP isolation are prerequisite in order to get accurate results in further analytical approach [211]. Even though direct measurement of MPs in plasma is ideal to prevent loss of MPs and to preserve their morphology during the isolation procedure, the advantages of using isolated MPs are less interference of plasma proteins and an increase in MP concentration. In paper-II, MPs were isolated from 'platelet free plasma' and also from the supernatant of LPS stimulated monocytes (MMPs). In paper-IV, MMPs were isolated from monocytes supernatant. In both papers, we have used 20,000 x g as a standard centrifugation speed to successfully pellet MPs, as it has been shown that at this speed more

than 90% of the MPs are recovered [213]. However in many previous studies centrifugation speed up to 100,000 x g were used to pellet MPs [214], which possibly pellet exosomes along with MPs [215]. Prior to further analysis, we avoided freeze/thawing procedures that may affect the morphology of MPs and also can lead to loss of TF activity. Contrasting data has been reported concerning the impact of freeze/thawing procedures on MPs antigen expression levels [211,215,216].

4.5.2 MP detection and characterization

Several different experimental approaches have been used to analyze MPs [211]. However, flow cytometry is the commonly used method, as it is fast and allows both the enumeration of MPs and the assessment of their cellular origin [214]. Flow cytometers measure light that is scattered at two different angles: forward and side. The enumeration of MPs is based on the light that is scattered by each MP in forward direction (proportional to their size) and in side direction (dependent on to granularity and structural complexity). However, forward scatter is in fact influenced more by refraction, and therefore any inference as to particle size must be made cautiously. In general, beads of 1 μm diameter are used to define the upper limit of the MP population [214]. Another possibility is to use a mixture of fluorescent beads with diameters of 0.5, 0.9, and 3 μm (Megamix beads) to cover the MP (0.5 and 0.9 μm) and platelet populations (0.9 and 3 μm) ranges [217]. However, forward scatter characteristics of a synthetic bead and a cell-derived MP may differ considerably. Another limitation of using flow cytometry is that, the lower detection limit of most of the modern flow cytometers is around 0.3 μm, making it impossible to detect MPs size range between 0.1-0.3 μm. Even though presence of multiple antigens on MPs can be analyzed in a single sample using flow cytometry, antibody clones, antibody concentration and proper usage of isotype control is very important [218].

In paper-II and IV, MPs were analyzed using FACSAria I flow cytometer (BD Bioscience) and MP size specific gate was determined using ‘megamix beads’ (Biocytex). In paper-II, we compared four commercially available TF antibody clones to assess their specificity against MP-associated TF antigen. Clones TF9-10H10 and VD8 are able to detect TF antigen on MPs, whereas, clone HTF-1 was unable to detect MP-TF, may be due to the reason that the site required for HTF-1 binding is already bound with FVII/FVIIa [219]. But, clone CLB/TF-5 tends to bind with MPs very strongly probably due to non-specific binding. In paper-IV, MMPs were analyzed to measure percentage of TF-positive MPs.

5 Discussion

Activation of tissue factor (TF) system in circulating monocytes plays a major role in pathogenesis of thrombosis [220]. Thus, understanding the mechanisms of TF regulation in monocytes is of vital importance. Therefore, a search for mechanistic explanations of TF-related clinical phenomena and for the novel factors involved in TF regulation in monocytes has a definite impact in development of novel, potent anti-thrombotic therapies.

Although TF is normally absent in cells in contact with circulating blood [174], monocytes are capable of expressing TF upon activation under certain pathological conditions [175], representing as a major source of TF in human circulation. In addition, monocytes are shown to contain little TF under normal conditions, even in healthy individuals [221]. Even though it is proposed that TF in resting blood monocytes is encrypted and TF activity is too low to play any thrombogenic role [50], it is not yet clear about the presence of possible inhibition that will not allow low amount of TF to induce thrombin generation in the blood. In addition, only little is known about TFPI expression in monocytes and its inhibitory action against monocyte procoagulant activity [112-114]. To address this, we have undertaken a study aiming at characterizing the monocyte TFPI expression levels in PBMCs isolated from healthy individuals. We found that the human monocytes constitutively express cell surface TFPI, and upregulation of cell surface antigen and both isoforms of TFPI mRNA was detected during time course of LPS stimulation. Using blocking antibody against surface presented monocyte TFPI in unstimulated PBMCs, significantly increased TF activity and thrombin generation (shortened lag-time) were observed. This indicates that TFPI present on monocyte surface regulates monocyte TF-dependent initiation of coagulation (Paper I).

In the first study (Paper I), our data are consistent with the earlier reports regarding presence of TFPI protein and mRNA in monocytes [112-114,222,223] but are in contrast to some [114,222], but not all [112] reports where they failed to find significant increase in TFPI expression in LPS stimulated monocytes. These discrepancies could be arising from the methodological variations and the sensitivity of assays used to measure TFPI expression in different studies. In contrast to LPS effect on monocyte TFPI, Bajaj et al. [114] noted significant upregulation in TFPI mRNA and antigen levels in fibronectin-adherent monocytes along with monocyte TF, which in turn explains the ability of monocytes to express higher levels of TFPI in response to increased levels of TF activity. Further studies are needed to understand how various other stimuli influence TFPI expression in monocytes. Even though

we are able to clearly demonstrate the presence of both isoforms of TFPI mRNA (α and β) in human monocytes, we were not able to characterize which of these two TFPI isoforms is predominantly expressed on monocyte surface, as TFPI antibody utilized in flow cytometry and confocal microscopy is specifically against K1 domain of TFPI, that present in both TFPIα and β isoforms. This limitation is due to the lack of well characterized TFPI antibodies raised specifically against K3 domain (for TFPIα) and against unique C-terminal region in TFPIβ (i.e., amino acids 182-193). However, it has been reported that approximately 70% of the monocyte surface bound TFPI is GPI-anchored, as assessed by flow cytometry measurement of TFPI antigen on monocytes treated with phosphatidylinositol-specific phospholipase C (PI-PLC) [223]. Further, pre-treatment of unstimulated monocytes with anti-TF IgG did not significantly affect thrombin generation, however, inhibiting cell-surface TFPI by anti-TFPI IgG led to a substantial, over 49% shortening of lag time and augmentation of the propagation phase of coagulation, assessed by a 53% increase in endogenous thrombin potential (ETP). This explains the inability of TF present on resting monocytes to exert thrombogenic effect, as half of the TF activity is neutralized by TFPI present on monocyte surface.

Numerous studies in search of TF expression in blood cells show contradicting results [50]. While monocytes are the only blood cells convincingly demonstrated to synthesize and express TF, its presence in other blood cells has been disputed [50]. The discrepancies in the various reports on TF localization in different blood cells are possibly due to the methodological variations and the usage of TF antibodies that do not meet stringent criteria for monospecificity [50]. To explore this hypothesis, we compared four commercially available TF antibody clones (HTF-1, TF9-10H10, CLB/TF-5 and VD8), for detection of TF antigen on platelets, monocytes and MPs. Using flow cytometry, we found that these four TF clones considerably differ in their sensitivity. TF antigen present on MPs can be identified using clones-TF9-10H10 or VD8, but not with HTF-1. All these three TF clones failed to identify TF antigen on platelets. In contrast, clone CLB/TF-5 readily binds to all, including platelets and monocytes, even under unstimulated conditions, possibly due to non-specific binding (Paper II).

In our second study (Paper II), our data clearly shows non-specific binding of clone-CLB/TF-5 on platelets, monocytes and MPs. However, a recent study has shown significantly higher TF expression on platelet derived MPs (PDMPs) isolated from collagen stimulated

platelet rich plasma using CLB/TF-5 [224]. In addition, two fold higher TF expression in PDMPs isolated from type-1 diabetes patients compared to healthy control also reported in the same study. Clone HTF-1 failed to detect TF antigen on MPs. Our observation is in line with the previous report by Key et al. [219], who reported successful detection of TF on MPs with clone-VIC7, which binds to the TF/VIIa complex, but not with clone HTF-1 that competes with FVII for binding to TF. Results from their study suggest that TF on circulating MPs is bound to FVII/FVIIa [219]. On the contrary, recent studies have reported elevated TF-positive MPs in cancer patients using HTF-1 [173,225,226]. Furthermore, clones TF9-10H10 and VD8 successfully detected MP bound TF in our study. Unlike CLB/TF-5, we found no significant levels of TF9-10H10 and VD8 positive platelets. In contrast, a significant amount of TF expression (clone-VD8) on resting platelets, isolated from healthy people have been reported by Siddiqui et al. [227], but recently challenged by Bouchard et al. [52].

Arterial thrombosis is one of the most common complications of atherosclerosis. The formation of blood thrombi originates on the sites of vascular lesions, which contain abundance of TF-expressing monocytes and macrophages [228]. In atherosclerosis, a proinflammatory milieu of blood also activates TF synthesis in circulating monocytes, which further aggravates blood thrombogenicity [229]. Thrombogenicity of atherosclerotic plaques varies with plaque morphology [230]. In contrast to stable calcified atherosclerotic plaques, thin-capped lesions with lipid-rich core enriched with inflammatory cells and high TF content [231] are vulnerable to rupture, making them responsible for approximately 75% of coronary and 90% of carotid thrombi in acute myocardial infarction and ischemic strokes, respectively [144-146,230]. It is well demonstrated that along with TF, monocyte-derived macrophages express TFPI in atherosclerotic plaques [232]. The presence of TFPI was shown to be associated with reduced TF activity [233], and preincubation of the sections with anti-TFPI IgG enhanced TF-mediated coagulation activation [234]. In addition, altered TF/TFPI balance is shown to be associated with higher plaque thrombotic potential [235]. Although most studies relate plaque thrombogenicity to an imbalance of intramural expression of TF and TFPI, no previous study has examined the relationship between morphological composition of plaque and expression levels of TF and TFPI in circulating blood monocytes. To explore this relationship we measured the levels of TF and TFPI in circulating monocytes obtained from asymptomatic subjects with echolucent or echogenic carotid plaques, and within lipid-rich and calcified carotid plaque sections obtained from patients with advanced atherosclerosis. Our findings suggest that subjects with asymptomatic echolucent lipid-rich

carotid plaques had an imbalance in TF and TFPI expression in circulating monocytes similar to that observed in sections from advanced carotid atherosclerosis of lipid-rich morphology (Paper III).

In our third study (paper-III), we noted significantly higher expression of TF gene, TF and TFPI surface presentation, surface TF/TFPI ratio, and TF activity, in monocytes obtained from subjects with echolucent plaques. On the other hand, the levels of monocyte TFPIα and TFPIβ mRNAs were significantly lower in subjects with echolucent plaques. These data are in line with previously reported TF/TFPI imbalance in patients with acute coronary syndromes [236]. Furthermore, studies report that increased TF/TFPI ratio contributes to the progression of atherosclerosis in patients with peripheral vascular disease [237]. Thus, higher TF/TFPI ratio in monocytes of patients with lipid-rich atherosclerosis could reflect insufficient inhibition of TF by TFPI placing these individuals at a greater risk for acute vascular events. Sections from lipid-rich carotid plaques contained 2.5-fold higher TF and 1.5-fold higher TFPI antigens relative to calcified lesions, also yielding a higher TF/TFPI ratio. Our finding complement the data by Hutter et al, who reported higher levels of mRNA encoding for TF in lipid-rich atherosclerotic plaques than in fibrous lesions [238]. Our results are consistent with previous studies that found that lipid-rich plaques contained more TFPI antigen than plaques with predominantly calcified morphology [232]. Recently, patients with severe dyslipidemia were shown to have altered TF/TFPI expression levels in atherosclerotic plaques [235]. Although, cholesterol may increase TF expression in monocytes [239], the exact molecular mechanisms altering TF/TFPI ratio in monocytes and within the lipid-rich plaques remain unknown. Data from paper-III offer a novel link between morphology and thrombogenicity of carotid atherosclerotic plaques. Although TF present within atherosclerotic lesion is a major mediator of plaque thrombogenicity [240-242], circulating blood monocytes could participate in thrombus formation on the surface of lipid-rich plaques contributing to a highly inflammatory milieu of the lipid-rich lesion [145,146]. This scenario induces high levels of procoagulant TF within the plaque, which is not counteracted by balanced expression of TFPI, and may results in formation of unstable lesion prone to thrombosis.

Unlike arterial thrombosis, venous thrombosis occurs in the absence of gross vessel wall disruption [152]. However, it has been suggested that circulating TF is more likely to play a role in venous thrombosis that is not associated with vessel damage. Several studies

have identified TF-bearing MPs to be associated with increased risk of venous thrombosis in cancer patients [243]. On the other hand, association between elevated platelet and leukocyte counts with VTE was reported in cancer patients [244]. However, no association between platelet count and risk of VTE was found in another study [245]. Recent studies have reported that elevated white blood cell count is associated with incident arterial and venous thrombosis in patients with polycythemia vera [246], and recurrent VTE in cancer patients [247]. Likewise, cross-sectional studies have reported that increased monocyte count is associated with peripheral arterial disease [248] and carotid plaque formation [249]. However, no previous studies have investigated the association between monocyte count and future risk of incident VTE. Since monocytes are the major source of TF in blood [54], we investigated the possible association between monocyte count and future risk of incident VTE in a prospective population-based cohort and further studied procoagulant functions of monocytes isolated from patients with unprovoked VTE in a subsequent case-control study. Monocyte count was associated with a moderately increased risk of VTE during the first 5 years of follow-up. Monocytes from patients with unprovoked VTE expressed higher levels of cell surface TF antigen and TF activity under resting conditions, and they tend to shed MPs with higher TF activity after LPS-stimulation, ex vivo. These findings suggest that monocytes may be involved in the pathogenesis of VTE (Paper IV).

In our fourth study (paper-IV), subjects with monocyte count, $\geq 0.7 \times 10^9$/L had a 2.5 fold increased risk of VTE as compared with those with monocyte count, $\leq 0.4 \times 10^9$/L during the first year, and monocyte count was associated with a moderately increased risk of VTE during the first 5-years of follow-up. The risk estimates became attenuated throughout the study period, and by the end of follow-up there was no association between monocyte count and VTE. To the best of our knowledge, our prospective cohort study is the first to investigate the association between monocyte count and future risk of VTE in a general population. The reason(s) why monocyte count weaken as a predictor of VTE over time is not known, but large intra-individual variability in the measurement over time will dilute the estimated gradient towards the null, a phenomenon known as regression dilution bias. Little is known about intra-individual long-term variability of monocyte counts in blood, but high variability has been reported in healthy subjects [250]. Thus, the observed temporal attenuation of the association between monocytes and VTE in our study, is likely explained by regression dilution or other unrecognized confounders that have changed during the observation time. The low number of VTE cases during the first years of observation limited

the statistical power of the risk estimates for continuous and categorized analyses at these time-points. The ability of monocyte count to predict VTE in a short time frame may support the hypothesis of a direct stimulating impact of circulating monocytes on the coagulation cascade. This concept is supported by the fact that monocytes are the major source of TF in blood [54]. Experimental studies in mice have demonstrated that deletion of the TF gene reduced the formation of venous thrombosis [251]. Recently, a randomized placebo-controlled study (the JUPITER study) showed that statin treatment was associated with a 43% reduction in VTE events independent of the cholesterol-lowering effects [252]. Previous experimental studies reported that statin treatment inhibited synthesis and cell surface expression of TF in monocyte-derived macrophages [253], and that simvastatin treatment blunted resting and endotoxin-induced expression of TF in circulatory monocytes of healthy individuals in vivo [254]. An alternative explanation for the temporary association between monocyte count and risk of VTE may be an indirect elevation of monocyte count, secondary to an inflammatory process triggering the coagulation cascade. Despite strong evidence for an impact of inflammation pathways on the coagulation cascade [255], including the ability of C-reactive protein (CRP) to induce synthesis and expression of TF in blood monocytes [256], most [257-259] but not all [260,261] prospective studies have not identified associations between inflammatory mediators and future risk of VTE.

The concept that circulating TF is possibly involved in the pathogenesis of venous thrombosis is supported by our findings in paper-IV, where monocytes isolated from patients with unprovoked VTE, more than a year after the acute event, expressed higher TF on the cell surface under resting conditions than age- and sex-matched controls. Monocyte TF expression is known to express high inter-individual but low intra-individual variability over time [262] under stable unprovoked conditions. Along with a rather long time interval between the acute VTE event and assessment of monocyte TF expression, it may be conceivable that the enhanced monocyte TF expression in patients with unprovoked VTE might have existed already prior to development of thrombosis. Despite higher TF mRNA expression, cell surface TF expression in LPS-stimulated monocytes from VTE patients was similar to healthy controls, most probably explained by elevated shedding of TF-bearing MPs with higher TF activity than controls. Previous studies have reported monocyte activation in the acute phase of venous thrombosis [263], and higher TF gene and antigen expression of blood monocytes isolated from patients with acute deep venous thrombosis [67,264] and cerebral venous thrombosis [265]. Furthermore, predisposing factors for venous thrombosis

such as total knee arthroplasty [266] and endotoxinemia [267] was associated with a transient increase in monocyte TF activity that preceded the median time of VTE diagnosis, thereby suggesting potential involvement of monocytes in the pathogenesis of VTE. Enhanced ability of monocytes from VTE patients to generate TF-bearing MPs under experimental conditions ex vivo supports the concept that monocyte derived TF-positive MPs may play an important role in the pathogenesis of venous thrombosis. However, further studies are needed to elucidate the underlying mechanism(s).

Taken together, this thesis summarizes the collection of studies which characterize-
(i) The role of monocyte TFPI in regulation of monocyte procoagulant activity,
(ii) Differential impact of four TF antibody clones, and
(iii) Explain possible monocyte role in the pathogenesis of arterial and venous thrombosis.

6 Conclusions

1. Human monocytes constitutively express both TFPIα and TFPIβ mRNAs, along with cell surface TFPI antigen, but not TF; which indicates no possible activation of monocytes due to freezing and thawing procedure. By blocking TFPI on monocyte surface, we found that monocyte surface presented TFPI functions as an important regulator of monocyte procoagulant activity.

2. We found that TF clone CLB/TF-5 binds to platelets, monocytes and MPs, possibly due to non-specificity. Clones TF9-10H10 and VD8 are found to be suitable for the detection of TF on MPs. Even though, clone HTF-1 binds to TF on LPS-stimulated monocytes with high specificity, it is not suitable for MP bound TF detection.

3. Monocytes from individuals with echolucent carotid plaques exhibited higher cell surface TF and TFPI expression, surface TF/TFPI ratio, and TF activity. Likewise, lipid-rich carotid plaques contained 2.5-fold higher TF and 1.5-fold higher TFPI proteins, also yielding a higher TF/TFPI ratio. These findings indicate that the imbalance between TF and TFPI expression in circulating monocytes from individuals with echolucent carotid plaques, and within lipid-rich atherosclerotic plaques may contribute to increased thrombogenicity of lipid-rich atherosclerotic lesions.

4. Monocyte count was found to be associated with a moderately increased risk of VTE during first 5 years of follow-up. However, the association was diluted over time, most probably due to regression dilution bias. Monocytes from patients with unprovoked VTE expressed higher cell surface TF antigen and TF activity compared to healthy controls. Further, monocytes from VTE patients shed MPs with higher TF activity than that of healthy controls during 6h of LPS-stimulation. These findings suggest possible monocyte involvement in the pathogenesis of VTE.

7 Future avenues

This thesis mainly focused on monocyte procoagulant function and relationship between monocyte TF and TFPI and their possible role in the development of arterial and venous thrombosis. Monocyte bound TF plays a major role in the pathogenesis of many thrombotic disorders [229]. We have shown that monocytes constitutively express TFPI and its role as a potent regulator of procoagulant activity of monocytes. In order to control the elevated levels of monocyte TF activity in thrombotic disorders, further studies are needed to develop novel drugs that are targeted to shift the balance between TF and TFPI expression in monocytes. For instance, novel drugs that are specifically targeted against monocytes to induce TFPI expression, in order to balance elevated monocyte TF expression can regulate procoagulant activity of monocytes without causing bleeding by interfere with hemostasis.

In recent years, the role of procoagulant activity associated with cell surfaces has been extended to include circulating procoagulant activity in the form of MPs. Many studies have found the association between increased MPs procoagulant activity and their elevated number in many thrombotic disorders [268]. However, the study of MPs is hampered by the methodological limitations mainly due to their smaller size (0.1 to 1µm). MPs isolation, detection and characterization still need to overcome many technical challenges [211,269] in order to uncover their actual role in thrombosis. Even though several methods are reported for MP analysis [211], flow cytometry is the widely used method which allows detection of MPs origin and quantification simultaneously. Depending on the laser settings of a particular flow cytometer, presence of several antigens on MPs can be analyzed, including TF. In our paper-II and IV, we have successfully studied MPs using FACSAria flow cytometer and identified that TF antibody clones TF9-10H10 and VD8 are suitable for the detection of TF associated with MPs. This allows us further to study the role of MPs in the pathophysiology of carotid atherosclerosis and VTE, by analyzing MP origin, TF expression and quantification in plasma samples collected from the patients and healthy controls enrolled in paper- III and IV, respectively.

8 References

1. Bach RR. Initiation of coagulation by tissue factor. CRC Crit Rev Biochem 1988;23:339-68.

2. Mackman N. Role of tissue factor in hemostasis, thrombosis, and vascular development. Arterioscler Thromb Vasc Biol 2004;24:1015-22.

3. Mann KG, Butenas S, Brummel K. The dynamics of thrombin formation. Arterioscler Thromb Vasc Biol 2003;23:17-25.

4. Crawley JT, Lane DA. The haemostatic role of tissue factor pathway inhibitor. Arterioscler Thromb Vasc Biol 2008;28:233-42.

5. Pedersen B, Holscher T, Sato Y, Pawlinski R, Mackman N. A balance between tissue factor and tissue factor pathway inhibitor is required for embryonic development and hemostasis in adult mice. Blood 2005;105:2777-82.

6. Mackman N. Balancing clotting. Blood 2010;116:1634-5.

7. Macfarlane RG. An Enzyme Cascade in the Blood Clotting Mechanism, and its Function as a Biochemical Amplifier. Nature 1964;202:498-9.

8. Davie EW, Ratnoff OD. Waterfall Sequence for Intrinsic Blood Clotting. Science 1964;145:1310-2.

9. Roberts HR, Monroe DM, Escobar MA. Current concepts of hemostasis: implications for therapy. Anesthesiology 2004;100:722-30.

10. Luchtman-Jones L, Broze GJ, Jr. The current status of coagulation. Ann Med 1995;27:47-52.

11. Sabharwal AK, Birktoft JJ, Gorka J, Wildgoose P, Petersen LC, Bajaj SP. High affinity Ca(2+)-binding site in the serine protease domain of human factor VIIa and its role in tissue factor binding and development of catalytic activity. J Biol Chem 1995;270:15523-30.

12. Hoffman M, Monroe DM, 3rd. A cell-based model of hemostasis. Thromb Haemost 2001;85:958-65.

13. Osterud B, Rapaport SI. Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation. Proc Natl Acad Sci U S A 1977;74:5260-4.

14. Monroe DM, Roberts HR, Hoffman M. Platelet procoagulant complex assembly in a tissue factor-initiated system. Br J Haematol 1994;88:364-71.

15. Monroe DM, Hoffman M, Roberts HR. Transmission of a procoagulant signal from tissue factor-bearing cell to platelets. Blood Coagul Fibrinolysis 1996;7:459-64.

16 Kjalke M, Monroe DM, Hoffman M, Oliver JA, Ezban M, Roberts HR. Active site-inactivated factors VIIa, Xa, and IXa inhibit individual steps in a cell-based model of tissue factor-initiated coagulation. Thromb Haemost 1998;80:578-84.

17 Monroe DM, Hoffman M, Roberts HR. Platelets and thrombin generation. Arterioscler Thromb Vasc Biol 2002;22:1381-9.

18. Monroe DM, Hoffman M. What does it take to make the perfect clot? Arterioscler Thromb Vasc Biol 2006;26:41-8.

19. Mackman N, Tilley RE, Key NS. Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. Arterioscler Thromb Vasc Biol 2007;27:1687-93.

20. Mackman N. Triggers, targets and treatments for thrombosis. Nature 2008;451:914-8.

21. Nemerson Y, Repke D. Tissue factor accelerates the activation of coagulation factor VII: the role of a bifunctional coagulation cofactor. Thromb Res 1985;40:351-8.

22. Wildgoose P, Kisiel W. Activation of human factor VII by factors IXa and Xa on human bladder carcinoma cells. Blood 1989;73:1888-95.

23. Kazama Y, Hamamoto T, Foster DC, Kisiel W. Hepsin, a putative membrane-associated serine protease, activates human factor VII and initiates a pathway of blood coagulation on the cell surface leading to thrombin formation. J Biol Chem 1995;270:66-72.

24. Monkovic DD, Tracy PB. Activation of human factor V by factor Xa and thrombin. Biochemistry 1990;29:1118-28.

25. Allen DH, Tracy PB. Human coagulation factor V is activated to the functional cofactor by elastase and cathepsin G expressed at the monocyte surface. J Biol Chem 1995;270:1408-15.

26. Diaz-Ricart M, Estebanell E, Lozano M, et al. Thrombin facilitates primary platelet adhesion onto vascular surfaces in the absence of plasma adhesive proteins: studies under flow conditions. Haematologica 2000;85:280-8.

27. Oliver JA, Monroe DM, Roberts HR, Hoffman M. Thrombin activates factor XI on activated platelets in the absence of factor XII. Arterioscler Thromb Vasc Biol 1999;19:170-7.

28. Osterud B, Rapaport SI, Lavine KK. Factor V activity of platelets: evidence for an activated factor V molecule and for a platelet activator. Blood 1977;49:819-34.

29. Ye J, Esmon NL, Esmon CT, Johnson AE. The active site of thrombin is altered upon binding to thrombomodulin. Two distinct structural changes are detected by fluorescence, but only one correlates with protein C activation. J Biol Chem 1991;266:23016-21.

30. Stearns-Kurosawa DJ, Kurosawa S, Mollica JS, Ferrell GL, Esmon CT. The endothelial cell protein C receptor augments protein C activation by the thrombin thrombomodulin complex. Proc Natl Acad Sci U S A 1996;93:10212-6.

31. Dahlback B, Villoutreix BO. Regulation of blood coagulation by the protein C anticoagulant pathway: novel insights into structure-function relationships and molecular recognition. Arterioscler Thromb Vasc Biol 2005;25:1311-20.

32. Mackman N, Taubman M. Tissue factor: past, present, and future. Arterioscler Thromb Vasc Biol 2009;29:1986-8.

33. Morrissey JH, Macik BG, Neuenschwander PF, Comp PC. Quantitation of activated factor VII levels in plasma using a tissue factor mutant selectively deficient in promoting factor VII activation. Blood 1993;81:734-44.

34. Mackman N. The many faces of tissue factor. J Thromb Haemost 2009;7 Suppl 1:136-9.

35. Morrissey JH, Fakhrai H, Edgington TS. Molecular cloning of the cDNA for tissue factor, the cellular receptor for the initiation of the coagulation protease cascade. Cell 1987;50:129-35.

36. Spicer EK, Horton R, Bloem L, et al. Isolation of cDNA clones coding for human tissue factor: primary structure of the protein and cDNA. Proc Natl Acad Sci U S A 1987;84:5148-52.

37. Scarpati EM, Wen D, Broze GJ, Jr., et al. Human tissue factor: cDNA sequence and chromosome localization of the gene. Biochemistry 1987;26:5234-8.

38. Fisher KL, Gorman CM, Vehar GA, O'Brien DP, Lawn RM. Cloning and expression of human tissue factor cDNA. Thromb Res 1987;48:89-99.

39. Mackman N, Morrissey JH, Fowler B, Edgington TS. Complete sequence of the human tissue factor gene, a highly regulated cellular receptor that initiates the coagulation protease cascade. Biochemistry 1989;28:1755-62.

40. Carson SD, Henry WM, Shows TB. Tissue factor gene localized to human chromosome 1 (1pter----1p21). Science 1985;229:991-3.

41. van der Logt CP, Reitsma PH, Bertina RM. Alternative splicing is responsible for the presence of two tissue factor mRNA species in LPS stimulated human monocytes. Thromb Haemost 1992;67:272-6.

42. Bogdanov VY, Balasubramanian V, Hathcock J, Vele O, Lieb M, Nemerson Y. Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. Nat Med 2003;9:458-62.

43. Mackman N. Alternatively spliced tissue factor - one cut too many? Thromb Haemost 2007;97:5-8.

44. Carmeliet P, Collen D. Tissue factor. Int J Biochem Cell Biol 1998;30:661-7.

45. Harlos K, Martin DM, O'Brien DP, et al. Crystal structure of the extracellular region of human tissue factor. Nature 1994;370:662-6.

46. Ruf W, Edgington TS. Structural biology of tissue factor, the initiator of thrombogenesis in vivo. FASEB J 1994;8:385-90.

47. Paborsky LR, Caras IW, Fisher KL, Gorman CM. Lipid association, but not the transmembrane domain, is required for tissue factor activity. Substitution of the transmembrane domain with a phosphatidylinositol anchor. J Biol Chem 1991;266:21911-6.

48. Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. Proc Natl Acad Sci U S A 1989;86:2839-43.

49. Drake TA, Morrissey JH, Edgington TS. Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis. Am J Pathol 1989;134:1087-97.

50. Osterud B, Bjorklid E. Sources of tissue factor. Semin Thromb Hemost 2006;32:11-23.

51. Moosbauer C, Morgenstern E, Cuvelier SL, et al. Eosinophils are a major intravascular location for tissue factor storage and exposure. Blood 2007;109:995-1002.

52. Bouchard BA, Mann KG, Butenas S. No evidence for tissue factor on platelets. Blood 2010;116:854-5.

53. Osterud B, Olsen JO, Bjorklid E. What is blood borne tissue factor? Thromb Res 2009;124:640-1.

54. Egorina EM, Sovershaev MA, Olsen JO, Osterud B. Granulocytes do not express but acquire monocyte-derived tissue factor in whole blood: evidence for a direct transfer. Blood 2008;111:1208-16.

55. Sovershaev MA, Lind KF, Devold H, et al. No evidence for the presence of tissue factor in high-purity preparations of immunologically isolated eosinophils. J Thromb Haemost 2008;6:1742-9.

56. Giesen PL, Rauch U, Bohrmann B, et al. Blood-borne tissue factor: another view of thrombosis. Proc Natl Acad Sci U S A 1999;96:2311-5.

57. Morrissey JH. Tissue factor: an enzyme cofactor and a true receptor. Thromb Haemost 2001;86:66-74.

58. Nakagaki T, Foster DC, Berkner KL, Kisiel W. Initiation of the extrinsic pathway of blood coagulation: evidence for the tissue factor dependent autoactivation of human coagulation factor VII. Biochemistry 1991;30:10819-24.

59. O'Hara PJ, Grant FJ, Haldeman BA, et al. Nucleotide sequence of the gene coding for human factor VII, a vitamin K-dependent protein participating in blood coagulation. Proc Natl Acad Sci U S A 1987;84:5158-62.

60. Parry GC, Erlich JH, Carmeliet P, Luther T, Mackman N. Low levels of tissue factor are compatible with development and hemostasis in mice. J Clin Invest 1998;101:560 9.

61. Owens AP, 3rd, Mackman N. Tissue factor and thrombosis: The clot starts here. Thromb Haemost 2010;104:432-9.

62. Marmur JD, Thiruvikraman SV, Fyfe BS, et al. Identification of active tissue factor in human coronary atheroma. Circulation 1996;94:1226-32.

63. Annex BH, Denning SM, Channon KM, et al. Differential expression of tissue factor protein in directional atherectomy specimens from patients with stable and unstable coronary syndromes. Circulation 1995;91:619-22.

64. Speidel CM, Thornton JD, Meng YY, Eisenberg PR, Edgington TS, Abendschein DR. Procoagulant activity on injured arteries and associated thrombi is mediated primarily by the complex of tissue factor and factor VIIa. Coron Artery Dis 1996;7:57-62.

65. Wang L, Miller C, Swarthout RF, Rao M, Mackman N, Taubman MB. Vascular smooth muscle-derived tissue factor is critical for arterial thrombosis after ferric chloride-induced injury. Blood 2009;113:705-13.

66. Himber J, Kling D, Fallon JT, Nemerson Y, Riederer MA. In situ localization of tissue factor in human thrombi. Blood 2002;99:4249-50.

67. Kamikura Y, Wada H, Nobori T, et al. Elevated levels of leukocyte tissue factor mRNA in patients with venous thromboembolism. Thromb Res 2005;116:307-12.

68. Versteeg HH, Ruf W. Emerging insights in tissue factor-dependent signaling events. Semin Thromb Hemost 2006;32:24-32.

69. Petersen LC, Freskgard P, Ezban M. Tissue factor-dependent factor VIIa signaling. Trends Cardiovasc Med 2000;10:47-52.

70. Rottingen JA, Enden T, Camerer E, Iversen JG, Prydz H. Binding of human factor VIIa to tissue factor induces cytosolic Ca2+ signals in J82 cells, transfected COS-1 cells, Madin-Darby canine kidney cells and in human endothelial cells induced to synthesize tissue factor. J Biol Chem 1995;270:4650-60.

71. Masuda M, Nakamura S, Murakami T, Komiyama Y, Takahashi H. Association of tissue factor with a gamma chain homodimer of the IgE receptor type I in cultured human monocytes. Eur J Immunol 1996;26:2529-32.

72. Poulsen LK, Jacobsen N, Sorensen BB, et al. Signal transduction via the mitogen-activated protein kinase pathway induced by binding of coagulation factor VIIa to tissue factor. J Biol Chem 1998;273:6228-32.

73. Reddy KB, Nabha SM, Atanaskova N. Role of MAP kinase in tumor progression and invasion. Cancer Metastasis Rev 2003;22:395-403.

74. Sato Y, Asada Y, Marutsuka K, Hatakeyama K, Kamikubo Y, Sumiyoshi A. Tissue factor pathway inhibitor inhibits aortic smooth muscle cell migration induced by tissue factor/factor VIIa complex. Thromb Haemost 1997;78:1138-41.

75. Taniguchi T, Kakkar AK, Tuddenham EG, Williamson RC, Lemoine NR. Enhanced expression of urokinase receptor induced through the tissue factor-factor VIIa pathway in human pancreatic cancer. Cancer Res 1998;58:4461-7.

76. Riewald M, Ruf W. Orchestration of coagulation protease signaling by tissue factor. Trends Cardiovasc Med 2002;12:149-54.

77. Rao LV, Pendurthi UR. Tissue factor-factor VIIa signaling. Arterioscler Thromb Vasc Biol 2005;25:47-56.

78. Levi M, van der Poll T, ten Cate H. Tissue factor in infection and severe inflammation. Semin Thromb Hemost 2006;32:33-9.

79. Taylor FB, Chang AC, Peer G, Li A, Ezban M, Hedner U. Active site inhibited factor VIIa (DEGR VIIa) attenuates the coagulant and interleukin-6 and -8, but not tumor necrosis factor, responses of the baboon to LD100 Escherichia coli. Blood 1998;91:1609-15.

80. Bajaj MS, Birktoft JJ, Steer SA, Bajaj SP. Structure and biology of tissue factor pathway inhibitor. Thromb Haemost 2001;86:959-72.

81. Thomas L. Studies on the intravascular thromboplastic effect of tissue suspensions in mice: a factor in normal rabbit serum which inhibits the thromboplastic effect of the sedimentable tissue component. Bull Johns Hopkins Hosp 1947;81:26-42.

82. Schneider CL. The active principle of placental toxin; thromboplastin; its inactivator in blood; antithromboplastin. Am J Physiol 1947;149:123-9.

83. Biggs R, Mac FR. The reaction of haemophilic plasma to thromboplastin. J Clin Pathol 1951;4:445-59.

84. Hjort PF. Intermediate reactions in the coagulation of blood with tissue thromboplastin; convertin, accelerin, prothrombinase. Scand J Clin Lab Invest 1957;9:1-183.

85. Dahl PE, Abildgaard U, Larsen ML, Tjensvoll L. Inhibition of activated coagulation factor VII by normal human plasma. Thromb Haemost 1982;48:253-6.

86. Sanders NL, Bajaj SP, Zivelin A, Rapaport SI. Inhibition of tissue factor/factor VIIa activity in plasma requires factor X and an additional plasma component. Blood 1985;66:204-12.

87. Warn-Cramer BJ, Maki SL, Zivelin A, Rapaport SI. Partial purification and characterization of extrinsic pathway inhibitor (the factor Xa-dependent plasma inhibitor of factor VIIa/tissue factor). Thromb Res 1987;48:11-22.

88. Broze GJ, Jr., Warren LA, Girard JJ, Miletich JP. Isolation of the lipoprotein associated coagulation inhibitor produced by HepG2 (human hepatoma) cells using bovine factor Xa affinity chromatography. Thromb Res 1987;48:253-9.

89. Wun TC, Kretzmer KK, Girard TJ, Miletich JP, Broze GJ, Jr. Cloning and characterization of a cDNA coding for the lipoprotein-associated coagulation inhibitor shows that it consists of three tandem Kunitz-type inhibitory domains. J Biol Chem 1988;263:6001-4.

90. Girard TJ, Eddy R, Wesselschmidt RL, et al. Structure of the human lipoprotein-associated coagulation inhibitor gene. Intro/exon gene organization and localization of the gene to chromosome 2. J Biol Chem 1991;266:5036-41.

91. van der Logt CP, Reitsma PH, Bertina RM. Intron-exon organization of the human gene coding for the lipoprotein-associated coagulation inhibitor: the factor Xa dependent inhibitor of the extrinsic pathway of coagulation. Biochemistry 1991;30:1571-7.

92. Enjyoji K, Emi M, Mukai T, et al. Human tissue factor pathway inhibitor (TFPI) gene: complete genomic structure and localization on the genetic map of chromosome 2q. Genomics 1993;17:423-8

93. Zhang J, Piro O, Lu L, Broze GJ, Jr. Glycosyl phosphatidylinositol anchorage of tissue factor pathway inhibitor. Circulation 2003;108:623-7.

94. Chang JY, Monroe DM, Oliver JA, Roberts HR. TFPIbeta, a second product from the mouse tissue factor pathway inhibitor (TFPI) gene. Thromb Haemost 1999;81:45-9.

95. Girard TJ, Warren LA, Novotny WF, Bejcek BE, Miletich JP, Broze GJ, Jr. Identification of the 1.4 kb and 4.0 kb messages for the lipoprotein associated coagulation inhibitor and expression of the encoded protein. Thromb Res 1989;55:37-50.

96. Girard TJ, Warren LA, Novotny WF, et al. Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor. Nature 1989;338:518-20.

97. Piro O, Broze GJ, Jr. Role for the Kunitz-3 domain of tissue factor pathway inhibitor-alpha in cell surface binding. Circulation 2004;110:3567-72.

98. Hackeng TM, Sere KM, Tans G, Rosing J. Protein S stimulates inhibition of the tissue factor pathway by tissue factor pathway inhibitor. Proc Natl Acad Sci U S A 2006;103:3106-11.

99. Ndonwi M, Tuley EA, Broze GJ, Jr. The Kunitz-3 domain of TFPI-alpha is required for protein S-dependent enhancement of factor Xa inhibition. Blood 2010;116:1344-51.

100. Lindhout T, Franssen J, Willems G. Kinetics of the inhibition of tissue factor-factor VIIa by tissue factor pathway inhibitor. Thromb Haemost 1995;74:910-5.

101. Wesselschmidt R, Likert K, Girard T, Wun TC, Broze GJ, Jr. Tissue factor pathway inhibitor: the carboxy-terminus is required for optimal inhibition of factor Xa. Blood 1992;79:2004-10.

102. Ott I, Miyagi Y, Miyazaki K, et al. Reversible regulation of tissue factor-induced coagulation by glycosyl phosphatidylinositol-anchored tissue factor pathway inhibitor. Arterioscler Thromb Vasc Biol 2000;20:874-82.

103. Mast AE, Acharya N, Malecha MJ, Hall CL, Dietzen DJ. Characterization of the association of tissue factor pathway inhibitor with human placenta. Arterioscler Thromb Vasc Biol 2002;22:2099-104.

104. Sevinsky JR, Rao LV, Ruf W. Ligand-induced protease receptor translocation into caveolae: a mechanism for regulating cell surface proteolysis of the tissue factor-dependent coagulation pathway. J Cell Biol 1996;133:293-304.

105. Piro O, Broze GJ, Jr. Comparison of cell-surface TFPIalpha and beta. J Thromb Haemost 2005;3:2677-83.

106. Novotny WF, Palmier M, Wun TC, Broze GJ, Jr., Miletich JP. Purification and properties of heparin-releasable lipoprotein-associated coagulation inhibitor. Blood 1991;78:394-400.

107. Higuchi DA, Wun TC, Likert KM, Broze GJ, Jr. The effect of leukocyte elastase on tissue factor pathway inhibitor. Blood 1992;79:1712-9.

108. Petersen LC, Bjorn SE, Nordfang O. Effect of leukocyte proteinases on tissue factor pathway inhibitor. Thromb Haemost 1992;67:537-41.

109. Li A, Wun TC. Proteolysis of tissue factor pathway inhibitor (TFPI) by plasmin: effect on TFPI activity. Thromb Haemost 1998;80:423-7.

110. Salemink I, Franssen J, Willems GM, et al. Factor Xa cleavage of tissue factor pathway inhibitor is associated with loss of anticoagulant activity. Thromb Haemost 1998;80:273-80.

111. Bajaj MS, Kuppuswamy MN, Saito H, Spitzer SG, Bajaj SP. Cultured normal human hepatocytes do not synthesize lipoprotein-associated coagulation inhibitor: evidence

that endothelium is the principal site of its synthesis. Proc Natl Acad Sci U S A 1990;87:8869-73.

112. McGee MP, Foster S, Wang X. Simultaneous expression of tissue factor and tissue factor pathway inhibitor by human monocytes. A potential mechanism for localized control of blood coagulation. J Exp Med 1994;179:1847-54.

113. Ott I, Andrassy M, Zieglgansberger D, Geith S, Schomig A, Neumann FJ. Regulation of monocyte procoagulant activity in acute myocardial infarction: role of tissue factor and tissue factor pathway inhibitor-1. Blood 2001;97:3721-6.

114. Bajaj MS, Ghosh M, Bajaj SP. Fibronectin-adherent monocytes express tissue factor and tissue factor pathway inhibitor whereas endotoxin-stimulated monocytes primarily express tissue factor: physiologic and pathologic implications. J Thromb Haemost 2007;5:1493-9.

115. Maroney SA, Mast AE. Expression of tissue factor pathway inhibitor by endothelial cells and platelets. Transfus Apher Sci 2008;38:9-14.

116. Caplice NM, Mueske CS, Kleppe LS, Peterson TE, Broze GJ, Jr., Simari RD. Expression of tissue factor pathway inhibitor in vascular smooth muscle cells and its regulation by growth factors. Circ Res 1998;83:1264-70.

117. Kereveur A, Enjyoji K, Masuda K, Yutani C, Kato H. Production of tissue factor pathway inhibitor in cardiomyocytes and its upregulation by interleukin-1. Thromb Haemost 2001;86:1314-9.

118. Hackeng TM, Maurissen LF, Castoldi E, Rosing J. Regulation of TFPI function by protein S. J Thromb Haemost 2009;7 Suppl 1:165-8.

119. Broze GJ, Jr., Lange GW, Duffin KL, MacPhail L. Heterogeneity of plasma tissue factor pathway inhibitor. Blood Coagul Fibrinolysis 1994;5:551-9.

120. Broze GJ, Jr., Warren LA, Novotny WF, Higuchi DA, Girard JJ, Miletich JP. The lipoprotein-associated coagulation inhibitor that inhibits the factor VII-tissue factor complex also inhibits factor Xa: insight into its possible mechanism of action. Blood 1988;71:335-43.

121. Huang ZF, Wun TC, Broze GJ, Jr. Kinetics of factor Xa inhibition by tissue factor pathway inhibitor. J Biol Chem 1993;268:26950-5.

122. Lu G, Broze GJ, Jr., Krishnaswamy S. Formation of factors IXa and Xa by the extrinsic pathway: differential regulation by tissue factor pathway inhibitor and antithrombin III. J Biol Chem 2004;279:17241-9.

123. Huang ZF, Higuchi D, Lasky N, Broze GJ, Jr. Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. Blood 1997;90:944-51.

124. Sandset PM, Warn-Cramer BJ, Rao LV, Maki SL, Rapaport SI. Depletion of extrinsic pathway inhibitor (EPI) sensitizes rabbits to disseminated intravascular coagulation

induced with tissue factor: evidence supporting a physiologic role for EPI as a natural anticoagulant. Proc Natl Acad Sci U S A 1991;88:708-12.

125. Kaiser B, Fareed J. Recombinant full-length tissue factor pathway inhibitor (TFPI) prevents thrombus formation and rethrombosis after lysis in a rabbit model of jugular vein thrombosis. Thromb Haemost 1996;76:615-20.

126. Ragni M, Golino P, Cirillo P, et al. Endogenous tissue factor pathway inhibitor modulates thrombus formation in an in vivo model of rabbit carotid artery stenosis and endothelial injury. Circulation 2000;102:113-7.

127. Golino P, Cirillo P, Calabro P, et al. Expression of exogenous tissue factor pathway inhibitor in vivo suppresses thrombus formation in injured rabbit carotid arteries. J Am Coll Cardiol 2001;38:569-76.

128. Donnan GA, Fisher M, Macleod M, Davis SM. Stroke. Lancet 2008;371:1612-23.

129. Bamford J, Sandercock P, Dennis M, Burn J, Warlow C. Classification and natural history of clinically identifiable subtypes of cerebral infarction. Lancet 1991;337:1521-6.

130. Faxon DP, Fuster V, Libby P, et al. Atherosclerotic Vascular Disease Conference: Writing Group III: pathophysiology. Circulation 2004;109:2617-25.

131. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993;362:801-9.

132. Libby P. Inflammation in atherosclerosis. Nature 2002;420:868-74.

133. Glasser SP, Selwyn AP, Ganz P. Atherosclerosis: risk factors and the vascular endothelium. Am Heart J 1996;131:379-84.

134. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation 2002;105:1135-43.

135. Stary HC, Chandler AB, Dinsmore RE, et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1995;92:1355-74.

136. Brener SJ. Insights into the pathophysiology of ST-elevation myocardial infarction. Am Heart J 2006;151:S4-10.

137. Davies MJ. Stability and instability: two faces of coronary atherosclerosis. The Paul Dudley White Lecture 1995. Circulation 1996;94:2013-20.

138. Salgado A, Boveda JL, Monasterio J, et al. Inflammatory mediators and their influence on haemostasis. Haemostasis 1994;24:132-8.

139. Carson SD, Johnson DR, Tracy SM. Tissue factor and the extrinsic pathway of coagulation during infection and vascular inflammation. Eur Heart J 1993;14 Suppl K:98-104.

140. Conkling PR, Greenberg CS, Weinberg JB. Tumor necrosis factor induces tissue factor-like activity in human leukemia cell line U937 and peripheral blood monocytes. Blood 1988;72:128-33.

141. Wiesel ML, Faradji A, Grunebaum L, et al. Hemostatic changes in human adoptive immunotherapy with activated blood monocytes or derived macrophages. Ann Hematol 1992;65:75-8.

142. Gray-Weale AC, Graham JC, Burnett JR, Byrne K, Lusby RJ. Carotid artery atheroma: comparison of preoperative B-mode ultrasound appearance with carotid endarterectomy specimen pathology. J Cardiovasc Surg (Torino) 1988;29:676-81.

143. Falk E, Shah PK, Fuster V. Coronary plaque disruption. Circulation 1995;92:657-71.

144. Virmani R, Burke AP, Kolodgie FD, Farb A. Pathology of the thin-cap fibroatheroma: a type of vulnerable plaque. J Interv Cardiol 2003;16:267-72.

145. Virmani R, Burke AP, Farb A, Kolodgie FD. Pathology of the unstable plaque. Prog Cardiovasc Dis 2002;44:349-56.

146. Burke AP, Farb A, Malcom GT, Liang YH, Smialek J, Virmani R. Coronary risk factors and plaque morphology in men with coronary disease who died suddenly. N Engl J Med 1997;336:1276-82.

147. Cushman M, Tsai AW, White RH, et al. Deep vein thrombosis and pulmonary embolism in two cohorts: the longitudinal investigation of thromboembolism etiology. Am J Med 2004;117:19-25.

148. Glynn RJ, Rosner B. Comparison of risk factors for the competing risks of coronary heart disease, stroke, and venous thromboembolism. Am J Epidemiol 2005;162:975-82.

149. Bertina RM. The role of procoagulants and anticoagulants in the development of venous thromboembolism. Thromb Res 2009;123 Suppl 4:S41-5.

150. Ageno W, Squizzato A, Garcia D, Imberti D. Epidemiology and risk factors of venous thromboembolism. Semin Thromb Hemost 2006;32:651-8.

151. Heit JA, Silverstein MD, Mohr DN, Petterson TM, O'Fallon WM, Melton LJ, 3rd. Risk factors for deep vein thrombosis and pulmonary embolism: a population-based case-control study. Arch Intern Med 2000;160:809-15.

152. Sevitt S. The structure and growth of valve-pocket thrombi in femoral veins. J Clin Pathol 1974;27:517-28.

153. Wakefield TW, Myers DD, Henke PK. Mechanisms of venous thrombosis and resolution. Arterioscler Thromb Vasc Biol 2008;28:387-91.

154. Becker BF, Heindl B, Kupatt C, Zahler S. Endothelial function and hemostasis. Z Kardiol 2000;89:160-7.

155. Bagot CN, Arya R. Virchow and his triad: a question of attribution. Br J Haematol 2008;143:180-90.

156. Gibbs NM. Venous thrombosis of the lower limbs with particular reference to bed-rest. Br J Surg 1957;45:209-36.

157. Warlow C, Ogston D, Douglas AS. Deep venous thrombosis of the legs after strokes. Part I--incidence and predisposing factors. Br Med J 1976;1:1178-81.

158. Brooks EG, Trotman W, Wadsworth MP, et al. Valves of the deep venous system: an overlooked risk factor. Blood 2009;114:1276-9.

159. Lopez JA, Chen J. Pathophysiology of venous thrombosis. Thromb Res 2009;123 Suppl 4:S30-4.

160. Hamer JD, Malone PC, Silver IA. The PO2 in venous valve pockets: its possible bearing on thrombogenesis. Br J Surg 1981;68:166-70.

161. Lawson CA, Yan SD, Yan SF, et al. Monocytes and tissue factor promote thrombosis in a murine model of oxygen deprivation. J Clin Invest 1997;99:1729-38.

162. Nomura S, Ozaki Y, Ikeda Y. Function and role of microparticles in various clinical settings. Thromb Res 2008;123:8-23.

163. Closse C, Seigneur M, Renard M, et al. Influence of hypoxia and hypoxia-reoxygenation on endothelial P-selectin expression. Thromb Res 1997;85:159-64.

164. Ogawa S, Gerlach H, Esposito C, Pasagian-Macaulay A, Brett J, Stern D. Hypoxia modulates the barrier and coagulant function of cultured bovine endothelium. Increased monolayer permeability and induction of procoagulant properties. J Clin Invest 1990;85:1090-8.

165. Shreeniwas R, Ogawa S, Cozzolino F, et al. Macrovascular and microvascular endothelium during long-term hypoxia: alterations in cell growth, monolayer permeability, and cell surface coagulant properties. J Cell Physiol 1991;146:8-17.

166. Yan SF, Mackman N, Kisiel W, Stern DM, Pinsky DJ. Hypoxia/Hypoxemia-Induced activation of the procoagulant pathways and the pathogenesis of ischemia-associated thrombosis. Arterioscler Thromb Vasc Biol 1999;19:2029-35.

167. Falati S, Liu Q, Gross P, et al. Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. J Exp Med 2003;197:1585-98.

168. Thomas GM, Panicot-Dubois L, Lacroix R, Dignat-George F, Lombardo D, Dubois C. Cancer cell-derived microparticles bearing P-selectin glycoprotein ligand 1 accelerate thrombus formation in vivo. J Exp Med 2009;206:1913-27.

169. Del Conde I, Shrimpton CN, Thiagarajan P, Lopez JA. Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. Blood 2005;106:1604-11.

170. Myers DD, Jr., Rectenwald JE, Bedard PW, et al. Decreased venous thrombosis with an oral inhibitor of P selectin. J Vasc Surg 2005;42:329-36.

171. Myers DD, Jr., Wrobleski SK, Longo C, et al. Resolution of venous thrombosis using a novel oral small-molecule inhibitor of P-selectin (PSI-697) without anticoagulation. Thromb Haemost 2007;97:400-7.

172. Ramacciotti E, Myers DD, Jr., Wrobleski SK, et al. P-selectin/ PSGL-1 inhibitors versus enoxaparin in the resolution of venous thrombosis: a meta-analysis. Thromb Res 2010;125:e138-42.

173. Campello E, Spiezia L, Radu CM, et al. Endothelial, platelet, and tissue factor-bearing microparticles in cancer patients with and without venous thromboembolism. Thromb Res 2011;127:473-7.

174. Butenas S, Mann KG. Active tissue factor in blood? Nat Med 2004;10:1155-6.

175. Lwaleed BA, Bass PS, Cooper AJ. The biology and tumour-related properties of monocyte tissue factor. J Pathol 2001;193:3-12.

176. Whatling C, Bjork H, Gredmark S, Hamsten A, Eriksson P. Effect of macrophage differentiation and exposure to mildly oxidized LDL on the proteolytic repertoire of THP-1 monocytes. J Lipid Res 2004;45:1768-76.

177. Islam LN, Wilkinson PC. Evaluation of methods for isolating human peripheral blood monocytes. Studies on chemotactic locomotion and other functional characteristics. J Immunol Methods 1989;121:75-84.

178. de Gruijter M, van Rijn MA, Verkerk A, Jongkind JF. Isolation of human and porcine monocytes and lymphocytes by three parameter flow sorting. Zentralbl Veterinarmed A 1990;37:585-91.

179. Lund PK, Joo GB, Westvik AB, Ovstebo R, Kierulf P. Isolation of monocytes from whole blood by density gradient centrifugation and counter-current elutriation followed by cryopreservation: six years' experience. Scand J Clin Lab Invest 2000;60:357-65.

180. Gelinas L, Falkenham A, Oxner A, Sopel M, Legare JF. Highly purified human peripheral blood monocytes produce IL-6 but not TNF{alpha} in response to angiotensin II. J Renin Angiotensin Aldosterone Syst 2011.

181. Harding SA, Din JN, Sarma J, et al. Flow cytometric analysis of circulating platelet-monocyte aggregates in whole blood: methodological considerations. Thromb Haemost 2007;98:451-6.

182. Son BK, Roberts RL, Ank BJ, Stiehm ER. Effects of anticoagulant, serum, and temperature on the natural killer activity of human peripheral blood mononuclear cells stored overnight. Clin Diagn Lab Immunol 1996;3:260-4.

183. Mentzer SJ, Guyre PM, Burakoff SJ, Faller DV. Spontaneous aggregation as a mechanism for human monocyte purification. Cell Immunol 1986;101:312-9.

184. Oliver AE, Tablin F, Walker NJ, Crowe JH. The internal calcium concentration of human platelets increases during chilling. Biochim Biophys Acta 1999;1416:349-60.

185. Kierulf P, Andersen AB. Lipopolysaccharide-instructed, cryopreserved, human monocytes sequentially convert plasma fibrinogen to fibrin and lyse the fibrin formed. Pathobiology 1991;59:197-9.

186. Osnes LT, Westvik AB, Kierulf P. Procoagulant and profibrinolytic activities of cryopreserved human monocytes. Thromb Res 1994;76:373-83.

187. Hansen JB, Halvorsen DS, Haldorsen BC, Olsen R, Sjursen H, Kierulf P. Retention of phagocytic functions in cryopreserved human monocytes. J Leukoc Biol 1995;57:235-41.

188. Seager Danciger J, Lutz M, Hama S, et al. Method for large scale isolation, culture and cryopreservation of human monocytes suitable for chemotaxis, cellular adhesion assays, macrophage and dendritic cell differentiation. J Immunol Methods 2004;288:123-34.

189. Butenas S, Bouchard BA, Brummel-Ziedins KE, Parhami-Seren B, Mann KG. Tissue factor activity in whole blood. Blood 2005;105:2764-70.

190. Maroney SA, Haberichter SL, Friese P, et al. Active tissue factor pathway inhibitor is expressed on the surface of coated platelets. Blood 2007;109:1931-7.

191. Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal 2001;13:85-94.

192. Meszaros K, Aberle S, Dedrick R, et al. Monocyte tissue factor induction by lipopolysaccharide (LPS): dependence on LPS-binding protein and CD14, and inhibition by a recombinant fragment of bactericidal/permeability-increasing protein. Blood 1994;83:2516-25.

193. Henriksson CE, Klingenberg O, Ovstebo R, Joo GB, Westvik AB, Kierulf P. Discrepancy between tissue factor activity and tissue factor expression in endotoxin-induced monocytes is associated with apoptosis and necrosis. Thromb Haemost 2005;94:1236-44.

194. Malarstig A, Tenno T, Jossan S, Aberg M, Siegbahn A. A quantitative real-time PCR method for tissue factor mRNA. Thromb Res 2003;112:175-83.

195. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55:611-22.

196. Piehler AP, Grimholt RM, Ovstebo R, Berg JP. Gene expression results in lipopolysaccharide-stimulated monocytes depend significantly on the choice of reference genes. BMC Immunol 2010;11:21.

197. Loseke S, Grage-Griebenow E, Wagner A, Gehlhar K, Bufe A. Differential expression of IFN-alpha subtypes in human PBMC: evaluation of novel real-time PCR assays. J Immunol Methods 2003;276:207-22.

198. Osterud B. Platelet activating factor enhancement of lipopolysaccharide-induced tissue factor activity in monocytes: requirement of platelets and granulocytes. J Leukoc Biol 1992;51:462-5.

199. Rao LV, Bajaj SP. Purification of human factor VII utilizing O-(diethylaminoethyl)-Sephadex and Sulfopropyl-Sephadex chromatography. Anal Biochem 1984;136:357-61.

200. Bajaj SP, Rapaport SI, Prodanos C. A simplified procedure for purification of human prothrombin, factor IX and factor X. Prep Biochem 1981;11:397-412.

201. Iakhiaev A, Pendurthi UR, Voigt J, Ezban M, Vijaya Mohan Rao L. Catabolism of factor VIIa bound to tissue factor in fibroblasts in the presence and absence of tissue factor pathway inhibitor. J Biol Chem 1999;274:36995-7003.

202. Balasubramanian V, Vele O, Nemerson Y. Local shear conditions and platelet aggregates regulate the incorporation and activity of circulating tissue factor in ex-vivo thrombi. Thromb Haemost 2002;88:822-6.

203. Dietzen DJ, Page KL, Tetzloff TA. Lipid rafts are necessary for tonic inhibition of cellular tissue factor procoagulant activity. Blood 2004;103:3038-44.

204. Mandal SK, Pendurthi UR, Rao LV. Cellular localization and trafficking of tissue factor. Blood 2006;107:4746-53.

205. Telgt DS, Macik BG, McCord DM, Monroe DM, Roberts HR. Mechanism by which recombinant factor VIIa shortens the aPTT: activation of factor X in the absence of tissue factor. Thromb Res 1989;56:603-9.

206. Bom VJ, Bertina RM. The contributions of Ca2+, phospholipids and tissue-factor apoprotein to the activation of human blood-coagulation factor X by activated factor VII. Biochem J 1990;265:327-36.

207. Bogdanov VY, Cimmino G, Tardos JG, Tunstead JR, Badimon JJ. Assessment of plasma tissue factor activity in patients presenting with coronary artery disease: limitations of a commercial assay. J Thromb Haemost 2009;7:894-7.

208. Nieuwland R, Sturk A. Why do cells release vesicles? Thromb Res 2010;125 Suppl 1:S49-51.

209. Toth B, Lok CA, Boing A, et al. Microparticles and exosomes: impact on normal and complicated pregnancy. Am J Reprod Immunol 2007;58:389-402.

210. Piccin A, Murphy WG, Smith OP. Circulating microparticles: pathophysiology and clinical implications. Blood Rev 2007;21:157-71.

211. Yuana Y, Bertina RM, Osanto S. Pre-analytical and analytical issues in the analysis of blood microparticles. Thromb Haemost 2011;105:396-408.

212. Jy W, Horstman LL, Jimenez JJ, et al. Measuring circulating cell-derived microparticles. J Thromb Haemost 2004;2:1842-51.

213. Jayachandran M, Litwiller RD, Owen WG, et al. Characterization of blood borne microparticles as markers of premature coronary calcification in newly menopausal women. Am J Physiol Heart Circ Physiol 2008;295:H931-H8.

214. Shet AS, Aras O, Gupta K, et al. Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. Blood 2003;102:2678-83.

215. Shet AS. Characterizing blood microparticles: technical aspects and challenges. Vasc Health Risk Manag 2008;4:769-74.

216. Shah MD, Bergeron AL, Dong JF, Lopez JA. Flow cytometric measurement of microparticles: pitfalls and protocol modifications. Platelets 2008;19:365-72.

217. Robert S, Poncelet P, Lacroix R, et al. Standardization of platelet-derived microparticle counting using calibrated beads and a Cytomics FC500 routine flow cytometer: a first step towards multicenter studies? J Thromb Haemost 2009;7:190-7.

218. Trummer A, De Rop C, Tiede A, Ganser A, Eisert R. Isotype controls in phenotyping and quantification of microparticles: a major source of error and how to evade it. Thromb Res 2008;122:691-700.

219. Key NS, Mackman N. Tissue factor and its measurement in whole blood, plasma, and microparticles. Semin Thromb Hemost 2010;36:865-75.

220. Eilertsen KE, Osterud B. Tissue factor: (patho) physiology and cellular biology. Blood Coagul Fibrinolysis 2004;15:521-38.

221. Egorina EM, Sovershaev MA, Bjorkoy G, et al. Intracellular and surface distribution of monocyte tissue factor: application to intersubject variability. Arterioscler Thromb Vasc Biol 2005;25:1493-8.

222. Nguyen P, Broussas M, Cornillet-Lefebvre P, Potron G. Coexpression of tissue factor and tissue factor pathway inhibitor by human monocytes purified by leukapheresis and elutriation. Response of nonadherent cells to lipopolysaccharide. Transfusion 1999;39:975-82.

223. Paysant J, Soria C, Cornillet-Lefebvre P, et al. Long-term incubation with IL-4 and IL-10 oppositely modifies procoagulant activity of monocytes and modulates the surface expression of tissue factor and tissue factor pathway inhibitor. Br J Haematol 2005;131:356-65.

224. Mobarrez F, Antovic J, Egberg N, et al. A multicolor flow cytometric assay for measurement of platelet-derived microparticles. Thromb Res 2010;125:e110-6.

225. Hron G, Kollars M, Weber H, et al. Tissue factor-positive microparticles: cellular origin and association with coagulation activation in patients with colorectal cancer. Thromb Haemost 2007;97:119-23.

226. Trappenburg MC, van Schilfgaarde M, Bredewold EO, et al. Elevated numbers and altered subsets of procoagulant microparticles in breast cancer patients using endocrine therapy. Thromb Res 2011;127:363-9.

227. Siddiqui FA, Desai H, Amirkhosravi A, Amaya M, Francis JL. The presence and release of tissue factor from human platelets. Platelets 2002;13:247-53.

228. Moreno PR, Bernardi VH, Lopez-Cuellar J, et al. Macrophages, smooth muscle cells, and tissue factor in unstable angina. Implications for cell-mediated thrombogenicity in acute coronary syndromes. Circulation 1996;94:3090-7.

229. Shantsila E, Lip GY. The role of monocytes in thrombotic disorders. Insights from tissue factor, monocyte-platelet aggregates and novel mechanisms. Thromb Haemost 2009;102:916-24.

230. Thim T, Hagensen MK, Bentzon JF, Falk E. From vulnerable plaque to atherothrombosis. J Intern Med 2008;263:506-16.

231. Taubman MB, Fallon JT, Schecter AD, et al. Tissue factor in the pathogenesis of atherosclerosis. Thromb Haemost 1997;78:200-4.

232. Drew AF, Davenport P, Apostolopoulos J, Tipping PG. Tissue factor pathway inhibitor expression in atherosclerosis. Lab Invest 1997;77:291-8.

233. Caplice NM, Mueske CS, Kleppe LS, Simari RD. Presence of tissue factor pathway inhibitor in human atherosclerotic plaques is associated with reduced tissue factor activity. Circulation 1998;98:1051-7.

234. Crawley J, Lupu F, Westmuckett AD, Severs NJ, Kakkar VV, Lupu C. Expression, localization, and activity of tissue factor pathway inhibitor in normal and atherosclerotic human vessels. Arterioscler Thromb Vasc Biol 2000;20:1362-73.

235. Zawadzki C, Susen S, Richard F, et al. Dyslipidemia shifts the tissue factor/tissue factor pathway inhibitor balance toward increased thrombogenicity in atherosclerotic plaques: evidence for a corrective effect of statins. Atherosclerosis 2007;195:e117-25.

236. Ott I. Tissue factor in acute coronary syndromes. Semin Vasc Med 2003;3:185-92.

237. Blann AD, Amiral J, McCollum CN, Lip GY. Differences in free and total tissue factor pathway inhibitor, and tissue factor in peripheral artery disease compared to healthy controls. Atherosclerosis 2000;152:29-34.

238. Hutter R, Valdiviezo C, Sauter BV, et al. Caspase-3 and tissue factor expression in lipid-rich plaque macrophages: evidence for apoptosis as link between inflammation and atherothrombosis. Circulation 2004;109:2001-8.

239. Liu ML, Reilly MP, Casasanto P, McKenzie SE, Williams KJ. Cholesterol enrichment of human monocyte/macrophages induces surface exposure of phosphatidylserine and the release of biologically-active tissue factor-positive microvesicles. Arterioscler Thromb Vasc Biol 2007;27:430-5.

240. Toschi V, Gallo R, Lettino M, et al. Tissue factor modulates the thrombogenicity of human atherosclerotic plaques. Circulation 1997;95:594-9.

241. Badimon JJ, Lettino M, Toschi V, et al. Local inhibition of tissue factor reduces the thrombogenicity of disrupted human atherosclerotic plaques: effects of tissue factor pathway inhibitor on plaque thrombogenicity under flow conditions. Circulation 1999;99:1780-7.

242. Reininger AJ, Bernlochner I, Penz SM, et al. A 2-step mechanism of arterial thrombus formation induced by human atherosclerotic plaques. J Am Coll Cardiol 2010;55:1147-58.

243. Manly DA, Boles J, Mackman N. Role of tissue factor in venous thrombosis. Annu Rev Physiol 2011;73:515-25.

244. Khorana AA, Connolly GC. Assessing risk of venous thromboembolism in the patient with cancer. J Clin Oncol 2009;27:4839-47.

245. Braekkan SK, Mathiesen EB, Njolstad I, Wilsgaard T, Stormer J, Hansen JB. Mean platelet volume is a risk factor for venous thromboembolism: the Tromso Study, Tromso, Norway. J Thromb Haemost 2010;8:157-62.

246. Landolfi R, Di Gennaro L, Barbui T, et al. Leukocytosis as a major thrombotic risk factor in patients with polycythemia vera. Blood 2007;109:2446-52.

247. Trujillo-Santos J, Di Micco P, Iannuzzo M, et al. Elevated white blood cell count and outcome in cancer patients with venous thromboembolism. Findings from the RIETE Registry. Thromb Haemost 2008;100:905-11.

248. Nasir K, Guallar E, Navas-Acien A, Criqui MH, Lima JA. Relationship of monocyte count and peripheral arterial disease: results from the National Health and Nutrition Examination Survey 1999-2002. Arterioscler Thromb Vasc Biol 2005;25:1966-71.

249. Johnsen SH, Fosse E, Joakimsen O, et al. Monocyte count is a predictor of novel plaque formation: a 7-year follow-up study of 2610 persons without carotid plaque at baseline the Tromso Study. Stroke 2005;36:715-9.

250. Maloney EM, Brown LM, Kurman CC, et al. Temporal variability in immunological parameters: peripheral blood mononuclear cell subsets, serum immunoglobulins, and soluble markers of immune system activation. J Clin Lab Anal 1997;11:190-5.

251. Day SM, Reeve JL, Pedersen B, et al. Macrovascular thrombosis is driven by tissue factor derived primarily from the blood vessel wall. Blood 2005;105:192-8.

252. Glynn RJ, Danielson E, Fonseca FA, et al. A randomized trial of rosuvastatin in the prevention of venous thromboembolism. N Engl J Med 2009;360:1851-61.

253. Colli S, Eligini S, Lalli M, Camera M, Paoletti R, Tremoli E. Vastatins inhibit tissue factor in cultured human macrophages. A novel mechanism of protection against atherothrombosis. Arterioscler Thromb Vasc Biol 1997;17:265-72.

254. Steiner S, Speidl WS, Pleiner J, et al. Simvastatin blunts endotoxin-induced tissue factor in vivo. Circulation 2005;111:1841-6.

255. Levi M, van der Poll T, Buller HR. Bidirectional relation between inflammation and coagulation. Circulation 2004;109:2698-704.

256. Cermak J, Key NS, Bach RR, Balla J, Jacob HS, Vercellotti GM. C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. Blood 1993;82:513-20.

257. Tsai AW, Cushman M, Rosamond WD, et al. Coagulation factors, inflammation markers, and venous thromboembolism: the longitudinal investigation of thromboembolism etiology (LITE). Am J Med 2002;113:636-42.

258. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. N Engl J Med 1997;336:973-9.

259. Christiansen SC, Naess IA, Cannegieter SC, Hammerstrom J, Rosendaal FR, Reitsma PH. Inflammatory cytokines as risk factors for a first venous thrombosis: a prospective population-based study. PLoS Med 2006;3:e334.

260. Folsom AR, Lutsey PL, Astor BC, Cushman M. C-reactive protein and venous thromboembolism. A prospective investigation in the ARIC cohort. Thromb Haemost 2009;102:615-9.

261. Quist-Paulsen P, Naess IA, Cannegieter SC, et al. Arterial cardiovascular risk factors and venous thrombosis: results from a population-based, prospective study (the HUNT 2). Haematologica 2010;95:119-25.

262. Ossterud B. The high responder phenomenon: enhancement of LPS induced tissue factor activity in monocytes by platelets and granulocytes. Platelets 1995;6:119-25.

263. Chirinos JA, Heresi GA, Velasquez H, et al. Elevation of endothelial microparticles, platelets, and leukocyte activation in patients with venous thromboembolism. J Am Coll Cardiol 2005;45:1467-71.

264. Vieira LM, Dusse LM, Fernandes AP, et al. Monocytes and plasma tissue factor levels in normal individuals and patients with deep venous thrombosis of the lower limbs: potential diagnostic tools? Thromb Res 2007;119:157-65.

265. Holschermann H, Haberbosch W, Terhalle HM, et al. Increased monocyte tissue factor activity in women following cerebral venous thrombosis. J Neurol 2003;250:631-2.

266. Johnson GJ, Leis LA, Bach RR. Tissue factor activity of blood mononuclear cells is increased after total knee arthroplasty. Thromb Haemost 2009;102:728-34.

267. Pernerstorfer T, Hollenstein U, Hansen JB, et al. Lepirudin blunts endotoxin-induced coagulation activation. Blood 2000;95:1729-34.

268. Leroyer AS, Tedgui A, Boulanger CM. Role of microparticles in atherothrombosis. J Intern Med 2008;263:528-37.

269. Lacroix R, Robert S, Poncelet P, Dignat-George F. Overcoming limitations of microparticle measurement by flow cytometry. Semin Thromb Hemost 2010;36:807-18.

Paper-I

Paper-II

Paper-III

Paper-IV