NEW CLINICAL AND INVESTIGATIVE TOOLS FOR EVALUATING THROMBOSIS AND HAEMOSTASIS

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Abstract

Haemostasis is maintained by a dynamic balance between pro- and anti-thrombotic mediators. Its dysregulation can lead to bleeding or thrombosis, and is a major cause of morbidity and mortality. Thus, elucidation of the mechanisms involved in maintaining or disrupting this balance have important implications in health and disease. Investigative tools enable characterization of the haemostatic system, but are often associated with limitations. For instance, haemostasis in animal models is often investigated by assessing bleeding responses in one particular vessel or tissue without a complete understanding of how the results translate to the regulation of haemostasis in other vascular beds. As a second example, microparticles (MPs) are a heterogeneous population of submicron-sized vesicles that may be important in thrombosis. With the exception of a few subtypes, MPs cannot be reliably characterized using widely accessible techniques. Finally, the thrombin generation assay (TGA), which measures *ex vivo* activation and inhibition of thrombin, is a promising tool for clinical assessment of thrombosis and haemostasis. However, characterization of thrombin generation in the general population, and the development of point of care testing are in their infancies. As a result, the TGA remains largely a research tool. The works described in this thesis specifically seek to address these three limitations in thrombosis and haemostasis research. The first isolated murine arterial bleeding model is presented and its characterization with respect to bleeding in other vascular tissues is described. In addition, a solid-phase capture assay for evaluating procoagulant, P-selectin-binding MPs, which are postulated to be mediators of thrombosis, was developed in order to determine

whether these MPs associate with risk of recurrent venous thromboembolism. Lastly, a 25 x 20 mm chip that performs four individual thrombin generation assays using ~10 µl of capillary blood was developed as a proof of concept for point of care thrombin generation testing.

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List of abbreviations

-a	Activated
ACD	Acid-citrate-dextrose
ADP	Adenosine diphosphate
AFM	Atomic force microscopy
ANOVA	Analysis of variance
APC	Activated protein C
APTT/aPTT	Activated partial thromboplastin time
AT	Antithrombin III
ATP	Adenosine triphosphate
AUC	Area under the curve
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cGMP	Cyclic guanosine monophosphate
CI	Confidence interval
CMFDA	5-chloromethylfluorescein diacetate
CV	Coefficient of variation
DLS	Dynamic light scattering
CTAD	Citrate-theophylline-adenosine-dipyridamole
DVT	Deep vein thrombosis
EC_{50}	Half maximal effective concentration
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor
ETP	Endogenous thrombin potential
F	Factor
FRET	Förster resonance energy transfer
FSC	Forward scatter
Gla	γ-carboxyglutamic
GP	Glycoprotein
HBSS	Hanks' buffered saline solution
HK	High molecular weight kininogen
IgG	Immunoglobulin G
INR	International normalized ratio
IP	Intraperitoneal

IVC	Inferior vena cava
Leo.H4	anti-αIIbβ3 antibody
LMWH	Low molecular weight heparin
LPS	Lipopolysaccharide
MP	Microparticle
NET	Neutrophil extracellular trap
NO	Nitric oxide
NTA	Nanoparticle tracking analysis
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease activated receptor
PC	Phosphatidylcholine
PDI	Protein disulfide isomerase
Peth	Phosphatidylethanolamine
PE	Pulmonary embolism
PGI_2	Prostacyclin
PK	Prekallikrein
PRP	Platelet-rich plasma
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein-1
PT	Prothrombin time
r-	recombinant
RBC	Red blood cell
RPS	Resistive pulse sensing
SEM	Standard error of the mean
SSC	Side scatter
TAFI	Thrombin-activatable fibrinolysis inhibitor
TBS	Tris-buffered saline
TEM	Transmission electron microscopy
TF	Tissue factor
TF^{low}	Low human tissue factor transgenic mice
TFPI	Tissue factor pathway inhibitor
TGA	Thrombin generation assay
TM	Thrombomodulin
TMEM16F	Transmembrane 16F
tPA	Tissue plasminogen activator
TTP	Time to lag
TXA_2	Thromboxane A_2
UFH	Unfractionated heparin

uPA	Urokinase plasminogen activator
VSMC	Vascular smooth muscle cell
VTE	Venous thromboembolism
vWF	von Willebrand factor

Chapter 1: General Introduction

1.1. Part 1: Haemostasis

Haemostasis is the physiological arrest of blood loss from injured vessels. It is governed through a complex homeostatic mechanism that keeps blood in a fluid state and confined to the vasculature. When a blood vessel is damaged, components of the haemostatic system rapidly activate to form a haemostatic plug that seals off the injury site and minimizes bleeding. In healthy vessels, these pathways are actively inhibited to prevent the formation of a thrombus, which can obstruct blood flow. Key physiological elements include platelets and coagulation factors, but regulation of this dynamic balance also involves other components.

1.1.1. Overview of haemostasis

Platelets are cell fragments formed from megakaryocytes in the bone marrow and released into the circulation. They circulate in a resting state, which is maintained through inhibitory signals present in blood, but can become locally activated in a damaged vessel upon contact with components of the subendothelium or soluble agonists (Jackson, 2007; Broos et al., 2011; Versteeg et al., 2013). Activated platelets adhere and aggregate at an injury site, resulting in the formation of a platelet plug that seals off the injury and limits bleeding. The cessation of blood loss mediated through this process is referred to as ‘primary haemostasis.’

The platelet plug is reinforced by a dense proteinaceous gel principally composed of fibrin, which is formed through the concomitant activation of the coagulation cascade

(Hoffman and Monroe, 2001; Versteeg et al., 2013). Like platelets, most coagulation factors circulate in a latent state and become activated after injury to the vessel wall, which exposes subendothelial tissue factor (TF), causing a procoagulant shift in the vascular microenvironment. TF triggers activation of the coagulation cascade, ultimately resulting in the formation of thrombin, which proteolytically converts soluble fibrinogen molecules into fibrin. These fibrin monomers, in turn, quickly polymerize and cross-link into a dense, insoluble mesh, further sealing off the injury site as part of a process termed 'secondary haemostasis.' In addition, thrombin enhances activation of nearby platelets and endothelial cells through the cleavage of surface receptors, and contributes to the amplification of the coagulation cascade as well as its eventual inhibition by activating other coagulation factors and inhibitors, respectively.

Clot dissolution is mediated by the fibrinolytic system (Chapin and Hajjar, 2015). Tissue-type and urokinase plasminogen activators (tPA and uPA, respectively) convert plasminogen into plasmin; the enzyme responsible for fibrin degradation. Fibrinolysis is negatively regulated by various inhibitors, including α_2-antiplasmin, thrombin-activatable fibrinolysis inhibitor (TAFI), and plasminogen activator inhibitor-1 (PAI-1). Additional components such as circulating cells, endothelial cells, vascular smooth muscle cells (VSMCs) and various inhibitors have auxiliary roles in the haemostatic process. The mechanisms that govern haemostasis involve perpetual cross-talk between all of these mediators, which continuously modulate pro- and anti-coagulant signals to prevent bleeding or thrombosis. Thus, haemostasis is a dynamic balance maintained by a

complex network of regulators, which together form a physiological barrier against blood loss and circulatory obstruction.

1.1.2. Platelets

Flow dynamics within the vessel cause small components such as platelets to circulate near the endothelium, where they can rapidly respond to vascular damage (Fogelson and Neeves, 2015). Blood-borne inhibitors, such as nitric oxide (NO) and prostacyclin (PGI_2), which are predominantly released by endothelial cells, normally suppress platelet activation through cyclic adenosine monophosphate (cAMP)- and cyclic guanosine monophosphate (cGMP)-mediated pathways (Moncada et al., 1976; Radomski et al., 1987; Broos et al., 2011). Endothelial injury however, exposes extracellular matrix (ECM) proteins of the vessel wall, such as collagen, which can bind various platelet membrane glycoproteins and platelet-binding proteins, causing platelets to adhere and activate (Jackson, 2007; Broos et al., 2011).

Adhesion to collagen under low shear conditions can be mediated by direct interactions with the platelet receptors, glycoprotein (GP) VI and the integrin $\alpha_2\beta_1$. High shear conditions initially require cross-linking by constitutively-expressed GPIb of the GPIb-IX-V complex via collagen-bound von Willebrand factor (vWF) (Savage et al., 1998; Ruggeri, 2009; Broos et al., 2011). Shear-induced changes in the structure of the multimeric collagen-bound vWF expose binding sites that mediate high on-rate interactions with GPIb (Siedlecki et al., 1996; Broos et al., 2011). Adhesion can also occur through interactions between various platelet membrane proteins and other ECM

constituents, such as fibronectin, laminin, and thrombospondin (Ruggeri, 2009; Broos et al., 2011).

Adhesion triggers intracellular signal transduction pathways that lead to platelet activation, resulting in, among other things, cytoskeletal remodeling, as well as synthesis and release of the platelet agonist thromboxane A_2 (TXA_2), and secretion of platelet alpha and dense granules (Li et al., 2010; Broos et al., 2011). TXA_2 and adenosine diphosphate (ADP), which is released from platelet dense granules, act on G protein-coupled receptors in an autocrine and paracrine fashion to further activate and recruit additional platelets to the growing platelet plug. In addition, thrombin, formed concomitantly through the coagulation cascade, is a potent platelet agonist which activates platelets by initiating signaling through the platelet G protein-coupled receptors, protease activated receptor (PAR) 1 and PAR4 in humans, and PAR3 and PAR4 in mice (Coughlin, 2000). In humans, thrombin-mediated cleavage of the N-termini of these receptors exposes new N-terminal sequences that function as tethered ligands, triggering receptor autoactivation and intracellular signaling. Platelet activation also results in the conversion of the $\alpha_{IIb}\beta_3$ integrin from a low-affinity to a high-affinity binding state, by inducing a conformational change through an "inside-out" signaling mechanism (Li et al., 2010; Broos et al., 2011). Binding of this activated integrin to fibrinogen, or other soluble molecules such as vWF or fibronectin, promotes cross-linking with adjacent activated platelets, thereby strongly contributing to platelet aggregation (Jackson, 2007). This binding also induces $\alpha_{IIb}\beta_3$-mediated outside-in signaling, which is associated with platelet spreading, retraction,

enhanced adhesion as well as further secretion of granular contents (Li et al., 2010; Broos et al., 2011). An overview of thse processes is presented in Figure 1.1.

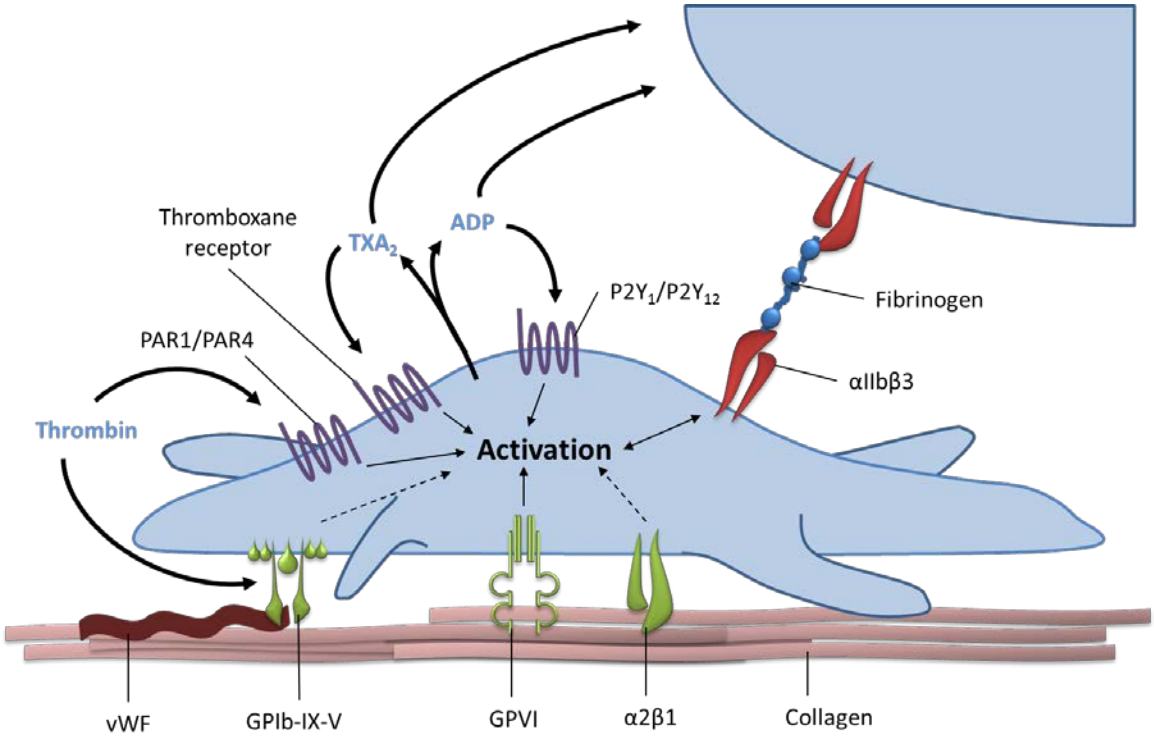
Platelets may regulate fibrin clot growth in several ways. The activated platelet membrane has been proposed to be the primary site of thrombin formation during haemostasis due to the presence of putative coagulation factor receptors and the presence phosphatidylserine (PS) (discussed further in *Sections 1.1.3.1.* and *6.2.3.*) (Hoffman and Monroe, 2001). In contrast, the formation of a densely-packed platelet plug can limit clot development by inhibiting access to TF, preventing the accumulation of fibrin, and restricting the diffusion of coagulation factors (Fogelson and Neeves, 2015). Additionally, platelets store and secrete various fibrinolytic regulators, including plasminogen, PAI-1, α_2-antiplasmin, and TAFI (Mosnier et al., 2003; Schadinger et al., 2010; Kim, 2015).

1.1.3. Coagulation cascade

The exposure of perivascular TF following damage to the endothelial lining initiates the extrinsic pathway of coagulation by binding activated factor (F) VII (FVIIa), which is constitutively present in the circulation at trace amounts (Wildgoose et al., 1992; Butenas and Mann, 1996; Hoffman and Monroe, 2001). Coagulation proceeds through a series of calcium-dependent proteolytic activation events (Hoffman and Monroe, 2001). The FVIIa-TF complex generates additional FVIIa while also converting FX and FIX to their active forms – FXa and FIXa, respectively. In turn, FIXa activates additional FXa, and FXa contributes to coagulation in two principal ways; first by further activating FVIIa, and second, by converting the inactive prothrombin zymogen

Figure 1.1. Platelet activation and aggregation.

Exposure of collagen initiates platelet adhesion, which is mediated by the platelet receptors GPVI, $\alpha_2\beta_1$, and the GPIb-IX-V complex. GPVI and $\alpha_2\beta_1$ direct collagen receptors, while GPIbα of the GPIb-IX-V complex binds to collagen via vWF. Adhesion triggers platelet activation through intracellular signaling mechanisms. Activation causes platelet shape change, de novo synthesis of TXA_2 and secretion of ADP from alpha platelet granules. TXA_2 and ADP causes further platelet activation through membrane-bound G protein-coupled receptors, and promote recruitment of other platelets. Thrombin, generated through the coagulation cascade is a potent platelet activator that cleaves PAR1 and PAR4 receptors on the surface of human platelets and can also bind GPIb. Inside-out signaling converts αIIbβ3 from a low-affinity to a high-affinity binding state, enabling cross-linking with adjacent platelets via fibrinogen and other molecules. This strongly promotes platelet aggregation and also induces further activation through intracellular signaling.


Thromboxane receptor
TXA2
ADP
P2Y1/P2Y12
Fibrinogen
PAR1/PAR4
αIIbβ3
Thrombin
Activation
vWF
GPIb-IX-V
GPVI
α2β1
Collagen

to the active serine protease, thrombin (Butenas and Mann, 1996; Hoffman and Monroe, 2001). In a final step, thrombin converts fibrinogen to fibrin, by cleaving N-terminal residues on the Aα and Bβ chains, which unmasks fibrin polymerization sites (Lane et al., 2005; Mosesson, 2005). Cross-linking of fibrin molecules produces an insoluble fibrin mesh.

Importantly, thrombin can promote further thrombin formation through a positive feedback loop in a so-called "amplification" stage, whereby activation of FXI leads to additional FIXa formation, and activation of the cofactors FVIII and FV serves to enhance the enzymatic activity of FIXa and FXa, respectively by several orders of magnitude (Hoffman and Monroe, 2001). This promotes a burst of thrombin generation via a "propagation" stage required for normal haemostasis. This is evidenced by the severe bleeding phenotype that occurs in FVIII or FIX deficiency (Bolton-Maggs and Pasi, 2003). Thrombin also activates TAFI and converts FXIII to FXIIIa, which stabilizes the fibrin mesh by cross-linking adjacent fibrin strands and by cross-linking α_2-antiplasmin with fibrin(ogen) (Schroder and Kohler, 2000; Hoppe, 2014).

Thrombin formation can also occur through a secondary, zinc-regulated pathway termed the 'contact pathway' which can be triggered when blood is exposed to artificial polyanionic surfaces, such as kaolin or silica, or certain physiological activators, such as nucleic acids or polyphosphates (Wu, 2015). Briefly, contact of FXII with these surfaces can trigger FXII autoactivation, leading to proteolytic activation of prekallikrein (PK) to kallikrein and FXI to FXIa, which colocalize with FXIIa by binding to high molecular weight kininogen (HK). FXIIa and kallikrein, in turn, can further promote FXII

activation. Inhibitors of this pathway include C1 inhibitor and histidine-rich glycoprotein (Jones et al., 2005; Wagenaar-Bos and Hack, 2006). The physiological relevance of the contact pathway is uncertain, and unlike the extrinsic pathway, the activation of the contact system is thought to have little or no role in haemostasis, as evidenced by the minor bleeding phenotype observed in the absence of FXI, and also by the lack of bleeding diatheses in individuals with FXII, HK or PK deficiencies (Renne et al., 2012).

Antithetically, thrombin formation is negatively regulated by various inhibitors which function at several nodes along the cascade, including antithrombin III (AT), tissue factor pathway inhibitor (TFPI) and activated protein C (APC). AT is a serine protease inhibitor that targets multiple enzymes within this cascade; most notably thrombin and FXa. Briefly, AT forms an irreversible covalent bond with these enzymes by interrupting protease-mediated cleavage of the AT reactive center loop at an intermediate reaction step (Gettins, 2002; Huntington, 2011). AT function is enhanced by glycosaminoglycans, including physiological cofactors such as heparan sulfate (Weitz, 2003), or by pharmacological heparin, which enhance AT's affinity towards its targets through a conformational change induced via binding of a specific pentasaccharide sequence (Jin et al., 1997; Chuang et al., 2001). In addition, efficient glycosaminoglycan-facilitated thrombin inhibition by AT requires the formation of a ternary complex in which AT and thrombin are bridged by a heparin sulfate/heparin oligosaccharide that is at minimum 18 monosaccharide units in length (Lane et al., 1984; Danielsson et al., 1986).

TFPI simultaneously binds and inactivates the FXa-FVIIa-TF complex, and may also function as an inhibitor of the FVa-FXa (prothrombinase) complex during the

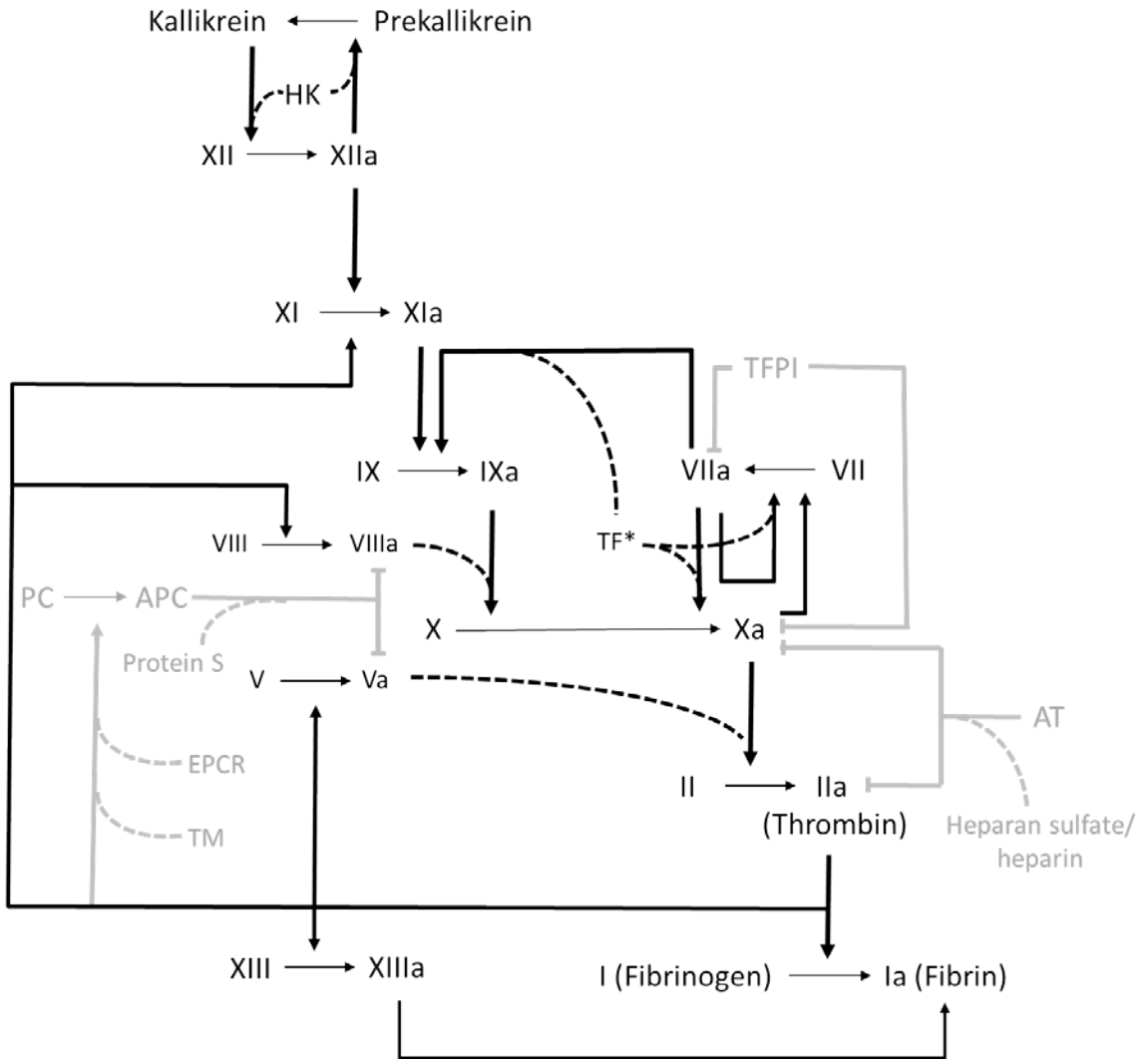
initiation stage of coagulation (Wood et al., 2014). The inactive protein C zymogen is converted to APC by thrombomodulin (TM)-bound thrombin in a reaction that is enhanced when protein C is bound to the endothelial protein C receptor (EPCR). Binding of TM to thrombin induces a conformational change in thrombin that enhances its proteolytic activity towards protein C. Once formed, APC downregulates the coagulation cascade by inactivating FVIIIa and FVa in a reaction that is enhanced by the presence of the cofactor, protein S (Esmon, 1989; Esmon, 2003). Minor regulators, such as heparin cofactor II and α-2 macroglobulin serve to modulate thrombin activity as well (Gettins, 2002). A simplified overview of coagulation, describing key elements is summarized in Figure 1.2.

1.1.3.1. Phospholipid membranes

Vitamin K-dependent proteases of the clotting cascade bind lipids via their N-terminal γ-carboxyglutamic (Gla) domains in a calcium-dependent manner. Briefly, calcium ions interact with carboxylic acid groups within the core of the Gla domain, resulting in a conformational change that exposes an N-terminal hydrophobic loop (Sunnerhagen et al., 1995; Freedman et al., 1996; Ohkubo and Tajkhorshid, 2008). The exposure of these hydrophobic residues allows the Gla domain to penetrate into the hydrophobic regions of the lipid bilayer. Additionally, complexes formed via calcium chelation by Gla residues and PS head groups, as well as calcium-dependent electrostatic bridging mechanisms between Gla residues and negatively charged phospholipids may also contribute to protein-membrane affinity (Zwaal et al., 1998; Ohkubo and Tajkhorshid, 2008), although these interactions only contribute

Figure 1.2. Regulation of coagulation.

Exposure of perivasular TF (*) to the vessel lumen initiates the extrinsic pathway of coagulation. FVIIa binds TF and actives FIX and FX. Activated FX (FXa) converts prothrombin to thrombin, which cleaves fibrinogen and amplifies the system through positive feedback by activating FV, FVIII, and FXI. Thrombin also activates the transglutaminase, FXIII, which promotes the cross-linking of adjacent fibrin strands. The contact pathway can be triggered by polyanionic surfaces, which cause FXII autoactivation, leading to the formation of kallikrein and FXIa. Inhibition (indicated in grey) of the coagulation cascade is mediated principally by AT, TFPI and APC. Activation of APC is mediated by TM-bound thrombin. Cofactor activity is indicated by dashed lines.


Kallikrein
Prekallikrein
HK
XII
XIIa
XI
XIa
TFPI
IX
IXa
VIIa
VII
VIII
VIIIa
TF*
PC
APC
Protein S
X
Xa
V
Va
AT
EPCR
TM
II
IIa
(Thrombin)
Heparan sulfate/
heparin
XIII
XIIIa
I (Fibrinogen)
Ia (Fibrin)

minimally to protein-membrane associations (Resnick and Nelsestuen, 1980; Atkins and Ganz, 1992). The homologous cofactors, FVa and FVIIIa bind to the cell membrane in a calcium-independent manner, via hydrophobic and electrostatic interactions between the C domains of their light chains and the cell membrane (Zwaal et al., 1998).

Membrane binding enhances the rates of enzymatic reactions by several orders of magnitude and localizes reactions to the site of injury. Firstly, high affinity binding promotes enzyme-cofactor assembly, allowing reactions to occur at physiological factor concentrations only on the activated cell membrane (Zwaal et al., 1998). Second, membrane binding restricts movement of enzymes, cofactors and substrates to a limited number of orientations along a two-dimensional plane (Kung et al., 1994), further increasing the likelihood of interactions that favour enzyme activity. Finally, the cell membrane can direct the activity of clotting factors by inducing conformational changes that promote specific interactions with their substrates or binding partners (Zwaal et al., 1998).

The composition of phospholipids in the cell membrane can influence the activation of clotting factors. Negatively charged phospholipids – including PS, in particular – allow for maximal activity of most clotting factors (Pei et al., 1993; Zwaal et al., 1998; Shaw et al., 2007). However, PS-containing membranes demonstrate reduced autoactivation of FVII in comparison to membranes with neutral charge (Neuenschwander et al., 1993). Furthermore, while the cofactor activity of most

phospholipids reaches a plateau with increasing concentrations, PS reaches peak activity and decreases at higher levels (Zwaal et al., 1998).

Phosphatidylethanolamine (PEth) has been shown to enhance protein C activation by the thrombin-TM complex (Horie et al., 1994). In the presence of phosphatidylcholine (PC) and PS, it can also promote the activity of coagulation complexes, and enhance the binding of FVa and FVIIIa to the membrane (Zwaal et al., 1998). Stereochemistry can also influence activation of clotting factors, as phosphatidyl-L-serine is a more efficient promoter of prothrombinase and intrinsic tenase activity than is phosphatidyl-D-serine. Thus, coagulation can be regulated by controlling the exposure of procoagulant lipid surfaces.

Most quiescent cells that are exposed to circulating blood limit thrombin generation by sequestering aminophospholipids, such as PS and PEth on the inner leaflet of their plasma membranes, while sphingophospholipids, such as PC and sphingomyelin are isolated to the outer leaflet (Manno et al., 2002; Lhermusier et al., 2011). This asymmetry is maintained through the concerted actions of adenosine triphosphate (ATP)-dependent phospholipid translocases known as flippases and floppases. Flippases selectively translocate PS and PEth to the inner leaflet, while floppase indiscriminately shuttles lipids to the outer layer.

Activation of cells causes the loss of membrane asymmetry and exposure of a procoagulant surface. Briefly, in response to influxes of intracellular calcium, flippases are inhibited, while a third class of membrane protein known as scramblase is activated and facilitates the unrestricted and passive exchange of lipids between the two leaflets,

ultimately resulting in the externalization of PS (Zwaal et al., 2005). Scramblase function requires the activity of the calcium-activated cation channel, transmembrane 16F (TMEM16F) (Suzuki et al., 2010; Yang et al., 2012).

1.1.3.2. Tissue factor

TF is a transmembrane glycoprotein and cofactor of FVIIa. It consists of a 219-residue, N-terminal extracellular domain, a 23-residue transmembrane domain and a 21-residue intracellular domain (Spicer et al., 1987). It is variably expressed in extravascular tissues, with high levels found in the brain, lung, heart, uterus, placenta, and testis, and low levels in skeletal muscles and joints. It is present in the subendothelial vessel wall where it forms a 'haemostatic envelope' that surrounds vascular tissue (Mackman, 2009). TF expression within the circulation is low, with leukocytes and leukocyte-derived microparticles (MPs) thought to be the major source in healthy individuals (Osterud, 2012). TF expression on cancer cells and on cancer cell-derived MPs and exosomes may occur in some malignant states. Finally, an alternatively spliced form of TF lacking the transmembrane domain exists as a soluble protein that lacks procoagulant activity (Yu and Rak, 2004).

It is generally accepted that TF activity is regulated by mechanisms that alter its functional state between encrypted (inactive) and decrypted (active) forms. Physiological mechanisms of encryption remain unresolved, but may involve the lack of an activated membrane, sequestration of TF in caveoli, homodimerization (Giesen and Nemerson, 2000; Morrissey, 2004), or the regulation of a disulfide bond between cys186 and cys209 (Chen et al., 2006). Cellular expression of PS and formation of disulfide bonds by protein

disulfide isomerase (PDI) have been proposed to contribute to TF decryption (Versteeg and Ruf, 2007).

1.1.3.3. Thrombin

Thrombin is the active 35.5 kDa serine protease derived from the inactive precursor, prothrombin, and is a central component in the regulation of haemostasis (Davie and Kulman, 2006). The inactive prothrombin molecule is released from hepatocytes and circulates at concentrations of around 1.4 μM (Kalafatis et al., 1994; Davie and Kulman, 2006). Proteolytic activation by prothrombinase removes the N-terminal Gla domain and two kringle domains of prothrombin, yielding a 36-residue light chain and 259-residue heavy chain that remain held together through an interchain disulfide bridge (Bode et al., 1989; Davie and Kulman, 2006). Enzymatic activity is mediated through an active site cleft that features a central catalytic triad, and surrounding specificity loops and hydrophobic residues that restrict access to the active site and only permit cleavage of specific targets. Additional sites are necessary for allosterically regulating and directing thrombin activity towards physiological substrates (Lane et al., 2005). These include anion binding exosites I and II, which bind to physiological substrates and regulators, as well as a Na^+-binding-site.

1.1.4. Other contributors to haemostasis

1.1.4.1. Endothelial cells

The endothelium is the epithelial lining of the blood vessels and lymphatics and plays an important role as both a promoter and attenuator of the haemostatic system. In the absence of vascular damage, endothelial cells help to ensure that blood is kept in a

fluid state by 1) forming a physical barrier between the blood and thrombogenic components of the subendothelial layers, such as collagen and TF, and 2) expressing factors that inhibit platelet activation and fibrin formation, and maintain vessel dilation.

Quiescent endothelial cells constitutively release platelet inhibitors such as PGI_2 and NO (Moncada et al., 1976; Radomski et al., 1987), which also function as vasodilators (de Nucci et al., 1988; Palmer et al., 1988). The endothelial membrane-bound cluster of differentiation (CD) 39 and CD73 variably regulate platelet activation via hydrolysis of adenine nucleotides (Marcus et al., 1997; Enjyoji et al., 1999; Koszalka et al., 2004), which can also be released from endothelial cells (LeRoy et al., 1984). The endothelium also expresses anticoagulant mediators such as TM, EPCR, TFPI and heparan sulfate and the plasminogen activator tPA (Aird, 2015; Kazmi et al., 2015). These factors maintain an anticoagulant environment in the absence of vascular injury, and ensure that in the event of an injury, the haemostatic response is appropriately regulated and spatially confined.

Endothelial cells also display procoagulant features following injury or activation. They expose procoagulant phospholipid surfaces onto which coagulation factors can bind, and can also express and release procoagulant mediators, including vWF, FVIII, TF, PAI-1 and PDI (Aird, 2015; Kazmi et al., 2015). Many of these pro- and anti-thrombotic mediators are variably expressed across different vascular beds, resulting in heterogeneity in endothelial cell phenotypes. Thus, the precise role of endothelial cells in maintaining haemostasis varies according to the phenotypic characteristics of endothelial cells that are present at the location of a haemostatic challenge (Kwaan and Samama, 2010).

1.1.4.2. Vascular smooth muscle cells and pericytes

Large veins and all vessels of the arterial system are surrounded by a medial layer of VSMCs that regulate vascular tone, while capillaries are supported by pericytes. Both cell types express mediators of coagulation which can influence the haemostatic response upon vascular injury (Rodgers, 1988; Bouchard et al., 1997; Caplice et al., 1998; Wang et al., 2009). Importantly, these cells express TF, which contributes towards the formation of the haemostatic envelope.

Vessel constriction is caused by the contraction of VSMCs, which is controlled through signaling pathways that modulate activity of contractile proteins in these cells (Hilgers and Webb, 2005). Inhibition of these pathways by effectors including NO and PGI_2 help control basal levels of vessel dilation, allowing for regulated blood flow (de Nucci et al., 1988; Palmer et al., 1988). When the vessel wall is breached, localized vasoconstriction restricts blood flow and limits hemorrhaging while a platelet plug and fibrin clot form to seal off the injury site (Zucker, 1947; Chen and Tsai, 1948). Thrombin and platelet-released factors, such as ADP may aid in this process by activating receptors on VSMCs (Ku and Zaleski, 1993; Wihlborg et al., 2004). Shear-induced mechanoregulation of endothelial cells can in turn modulate endothelial cell-mediated vasoregulation (Davies, 1995).

1.1.4.3. Erythrocytes

Red blood cells (RBCs) are anucleate cells and the most abundant cellular fraction in the circulation. Their primary function is in oxygen transport, but there is evidence that RBCs can also contribute to haemostasis. Transfusion of RBCs can cause reductions in

bleeding time (Hellema et al., 1961; Blajchman et al., 1994). Mechanisms for this are unclear, but may include altering viscosity of blood and promoting interactions between other components of the haemostatic system and the vessel wall, promoting platelet activation through the release of chemical messengers, such as ADP, and supporting the coagulation cascade through exposure of PS (Andrews and Low, 1999). *In vitro*, RBCs promote thrombin generation (Peyrou et al., 1999; Horne et al., 2006; Whelihan et al., 2012), enhance clot quality determined by thromboelastography (Roeloffzen et al., 2010) and increase clot resistance to fibrinolysis (Wohner et al., 2011). RBCs may also indirectly influence the haemostatic system, for example, through clearance and metabolism of the platelet inhibitors adenosine and inosine (Heptinstall et al., 2005).

1.1.4.4. Leukocytes

Leukocytes have a primary role as mediators of the immune system, but also happen to be the major source of blood-borne TF (Osterud, 2012). They are also involved in purine metabolism by converting ATP and ADP to adenosine through CD39 (Heptinstall et al., 2005) and binding adenosine deaminase through CD26 on lymphocytes (Kameoka et al., 1993). Proteases released from neutrophil granules can activate and degrade various components involved in coagulation and ECM proteins (Owen and Campbell, 1995; Perrin et al., 2010; Massberg et al., 2010). Cathepsin G, a serine protease released from neutrophil azurophilic granules, can have opposing actions in human platelet activation through cleavage of different PARs, and its absence is associated with prolonged tail bleeding times in mice (Faraday et al., 2013).

In addition, monocytes release TF-expressing MPs (described further in *Section 1.2.2.*), which have been proposed to have roles in coagulation. Localization of TF-expressing MPs to developing clots promotes fibrin deposition in a laser injury model, while P-selectin-stimulated MP shedding, and infused TF-positive MPs can reduce bleeding times in FVIII-deficient and wild type mice, respectively (Hrachovinova et al., 2003; Thomas et al., 2009; Thomas et al., 2015). In contrast, leukocytes express the uPA receptor, which can reduce fibrin clot formation by localizing uPA (Bai et al., 2009).

Finally, as part of a defense mechanism against pathogens, neutrophils undergo a type of programmed cell death termed NETosis, during which they release neutrophil extracellular traps (NETs). These complexes comprise of chromatin and granular enzymes, which concertedly entangle and kill invading pathogens (Brinkmann et al., 2004; Fuchs et al., 2007; Geddings and Mackman, 2014). NETs can promote clot formation through the activation of the contact pathway, inhibition of TFPI, and by enhancing the accumulation of RBCs and activated platelets (Geddings and Mackman, 2014). However, a role for NETs in haemostasis has not been established, and the deletion of 'protein arginine deaminase 4,' which is important in NET formation, does not alter bleeding time compared to wild type mice (Martinod et al., 2013).

1.2. Part 2: Hemorrhagic disorders and thrombosis

Haemostasis is maintained by a dynamic balance between pro- and anti-thrombotic mediators. Perturbation of this balance can result in a net tendency towards thrombosis or bleeding. The inability of the haemostatic system to adequately respond to

vascular damage results in bleeding, whereas dysregulation of the haemostatic system can lead to thrombosis.

Defects and factors that prevent a full response from platelets or the coagulation system under normal initiating conditions often cause a bleeding diathesis (George et al., 1990; Al Dieri et al., 2002). The tendency and severity of bleeding depend on the type of risk factor, the severity and location of vascular damage, and other confounding variables such as levels of prothrombotic factors and platelet activation, which may ameliorate or potentiate bleeding (George et al., 1990; Mulder and Llinas, 2004; van Bladel et al., 2011; Rendo et al., 2013).

Thrombosis in the arterial system occurs most frequently due to rupture of atherosclerotic plaques, which promotes a burst of platelet aggregation and thrombin generation, resulting in the formation of platelet-rich clots (Mackman, 2008). Arterial thromboembolism can also be triggered in the heart, such as in atrial fibrillation, which promotes stasis of blood, or as a result of mechanical heart valves, which induce clotting through contact activation (Previtali et al., 2011; Lyaker et al., 2013). In the venous system, the formation of clots is influenced by risk factors that cause hypercoagulability, endothelial damage, or venous stasis (Mackman, 2008). The presence of platelets in these thrombi is less pronounced, and clots appear red in colour owing to this and to the high incorporation of erythrocytes. As with bleeding, the propensity to clot is multifaceted and depends on the sum of the effects of the individual risk factors (Previtali et al., 2011).

The most common site of major thrombotic events in the venous system are the deep veins of the legs, where stagnant blood flow and hypoxia can occur in the valve

pockets, leading to activation of endothelial cells, and the accumulation and activation of other prothrombotic mediators (Esmon, 2009; Bovill and van der Vliet, 2011). Alternatively, venous thrombosis can occur at other sites, often in association with particular disease states and risk factors (Joffe and Goldhaber, 2002). The following section will discuss 1) haemophilia, a relatively common class of inherited bleeding disorders characterized by deficiencies of particular clotting factors, and 2) venous thromboembolism (VTE), which accounts for the vast majority of major thrombotic diseases occurring in the venous circulation.

1.2.1 Haemophilia

Haemophilia is a category of bleeding disorders that is characterized by a deficiency in FVIII (haemophilia A), FIX (haemophilia B), or FXI (haemophilia C). While FXI deficiency is usually associated with a mild phenotype, the absence of functional FVIII and FIX can result in severe bleeding, which may necessitate replacement factor therapy. Haemophilia A and B are two of the most common severe bleeding disorders, owing to their hemizygous inheritance in males, which is in contrast to other coagulation factor gene defects that are inherited in an autosomal recessive manner. Haemophilia A is the most common severe bleeding disorder, occurring in the population at a rate of 1 in every 5,000 male births, while haemophilia B occurs with an incidence of 1 in 25,000 male births.

Joints are the most common sites of spontaneous bleeding, and recurrence of such bleeds can eventually lead to haemophilic arthropathy (Mulder and Llinas, 2004). Bleeding at other sites, including muscle and intracranial bleeds can also occur (Bolton-

Maggs and Pasi, 2003). To limit spontaneous bleeds and the resulting long-term health effects, prophylactic administration of the missing factor or bypass agents can be given. However, regular treatment, which can total 2-3 self-infusions per week, is costly (Globe et al., 2004). Also, it is difficult to individually tailor dosing schedules, as the pharmacokinetics of these replacement factors vary within and between patients (Bjorkman et al., 2007; Ar et al., 2014) and frequent monitoring of circulating factor levels is generally not possible. Improper dosing is associated with a risk of thrombosis or bleeding, while unnecessary dosing carries a cost burden. Management of haemophilia in developing countries is further complicated by the fact that the majority of persons with haemophilia remain undiagnosed (Bolton-Maggs, 2006).

The severity of bleeding, to a large extent, depends on the levels of inducible FVIIIa or FIXa activity in circulation, which is in turn determined by the nature of the gene mutation (Lakich et al., 1993). For example, severe haemophilia A is characterized by < 1% FVIIIa activity, and imparts a high risk of bleeding in those affected. In contrast, moderate (1-5% FVIIIa activity) and mild (5-49% FVIIIa activity) haemophilia are associated with lesser risks of bleeding (Bolton-Maggs and Pasi, 2003). However, 25% of individuals with moderate haemophilia A experience frequent spontaneous bleeds and arthropathy, while up to 10% of those with severe haemophilia rarely experience bleeding and do not suffer from joint bleeds (van den Berg et al., 2007; den Uijl et al., 2014), illustrating the multifaceted nature of haemostasis.

1.2.1.1. Factor VIII

The Factor VIII molecule is a 2332-amino acid protein, derived from the F8 gene, located near the end of the long arm of the X chromosome (Xq28) (Vehar et al., 1984; Gitschier et al., 1984; Wood et al., 1984; Toole et al., 1984). It is produced in and released from endothelial cells (Everett et al., 2014). It possesses six domains, which identified from the N- to the C-terminus are A1, A2, B, A3, C1, and C2 (Ngo et al., 2008). Prior to secretion into circulation, a portion of the B domain is removed, yielding a heterodimeric procofactor consisting of an N-terminal heavy chain (A1, A2, B domains) and C-terminal light chain (A3, C1, C2 domains), which are held together by a metal ion-dependent bridge (Fay, 2004).

The vast majority of FVIII (>94%) circulates in the blood associated with vWF through non-covalent interactions that are mediated by the C2 and A3 domains (Lenting et al., 2007). Upon proteolytic cleavage by thrombin at residue 740, the truncated B domain is removed. Two additional cleavages at 372 and 1689 result in the liberation of FVIII from vWF and its conversion to an active, heterotrimeric cofactor (Fay, 2004). Disassociation from vWF allows FVIIIa to bind to activated cell membranes, where it can perform its cofactor function bound to FIXa as part of the intrinsic tenase complex. This cofactor activity increases the catalytic efficiency of FIXa-mediated FXa generation by 10^4-10^6-fold.

FVIIIa is unstable due to weak electrostatic interactions between the A2 domain and the rest of the molecule (Fay et al., 1991). This, coupled with proteolytic degradation by enzymes such as APC, results in its rapid inactivation (Fay, 2004).

1.2.1.2. Factor IX

The mature FIX zymogen is a 415-amino acid glycoprotein synthesized in and released from hepatocytes. Like the gene for FVIII, the F9 gene is located on the long arm of the X chromosome (Xq27.1) (Kurachi et al., 1993). The N-terminal region contains the Gla domain, which is followed by two epidermal growth factor (EGF)-like domains and a C-terminal protease domain (Thompson, 1986; Schmidt and Bajaj, 2003). Between the EGF2 domain and the protease domain is a 35-residue activation peptide spanning from Ala 146 to Arg 180. Cleavage after Arg 145 and Arg 180 by FVIIa, FIXa or FXIa releases the activation peptide, resulting in the formation of the active serine protease. The resulting N-terminal light chain and C-terminal heavy chain remain held together by a disulfide bond.

1.2.2 Venous thromboembolism

VTE is characterized by the development of clots in the deep veins of the legs (deep vein thrombosis (DVT)), or the embolization of these clots into the pulmonary circulation (pulmonary embolism (PE)), which can result in death. VTE is a major cause of morbidity and mortality with an estimated 35,000 to 70,000 new cases occurring in Canada per year, and 300,000 to 600,000 new annual cases in the United States (White, 2003; Beckman et al., 2010). Clinical risk factors are those which augment any aspect of Virchow's triad – namely, factors that contribute to increased hypercoagulability, venous stasis, or endothelial damage (Anderson, Jr. and Spencer, 2003).

Low molecular weight heparin (LMWH), warfarin or direct oral anticoagulants, such as dabigatran, rivaroxaban and apixaban are standard therapies to suppress and

prevent thrombosis in individuals with VTE or at high risk of developing VTE (Kearon et al., 2016). Treatment is maintained for 3 to 6 months following a first thromboembolic event, but may be continued indefinitely if the risk of recurrent VTE is high. Identifying patients that would benefit from extended therapy is complicated by the fact that anticoagulant treatment is associated with a small risk of life-threatening bleeds (Scarvelis et al., 2010).

1.2.2.1. Unprovoked VTE

Between 25 – 50% of patients with VTE present without any identifiable risk factors (unprovoked VTE) (White, 2003). Patients with an unprovoked proximal DVT or PE have a higher risk of recurrence than those with reversible provoking factors (Rodger et al., 2010); presumably due to the greater likelihood that the underlying causes of the initial thrombotic event persist after stopping anticoagulant therapy. Guidelines recommend extended therapy in patients with unprovoked VTE, but place a high value on patient preference (Kearon et al., 2012).

Potential biomarkers, such as D-dimer, thrombin generation, and soluble P-selectin have been and continue to be tested as markers for stratifying patients with unprovoked VTE into high and low risk groups, thereby directing intervention strategies. D-dimer is a fibrin degradation product, which has been shown to be a risk factor for recurrence in patients with unprovoked VTE (Palareti et al., 2006; Kearon et al., 2015). In the PROLONG trial, individuals with abnormal D-dimer were randomized to continue or to stop extended treatment, while those with normal levels remained off therapy. In DODS, patients who had completed anticoagulant therapy following an unprovoked VTE

were tested for D-dimer at enrolment and again 1 month after enrolment. Patients with positive D-dimer results restarted anticoagulation treatment, while those with negative results did not. PROLONG demonstrated a reduced risk of VTE in patients with negative D-dimer, but both studies still found a 6-7% risk of recurrence in this group, illustrating a continued need for additional risk markers.

Thrombin generation (discussed in *Section 1.3.1*) has been found by several investigators to be an independent predictor of recurrence in unprovoked VTE (Hron et al., 2006; Eichinger et al., 2008; Tripodi et al., 2008; Besser et al., 2008; Eichinger et al., 2010). However, the capacity of thrombin generation to predict recurrence may depend on analytical conditions (van Hylckama et al., 2007; van Hylckama et al., 2015). Finally, P-selectin (discussed in the next section) is a transmembrane glycoprotein expressed by activated platelets and endothelial cells. Elevated levels of soluble P-selectin were found to predict recurrence in a group of patients with a first unprovoked VTE (Kyrle et al., 2007).

1.2.2.2. P-selectin

P-selectin is a transmembrane glycoprotein expressed in the α-granules of platelets (Berman et al., 1986; Cummings and Smith, 1992) and Weibel-Palade bodies of endothelial cells (McEver et al., 1989; Bonfanti et al., 1989). Upon cell activation, it is mobilized to the plasma membrane (Hsu-Lin et al., 1984; Berman et al., 1986), where it has roles in inflammation and clot formation by tethering ligands on leukocytes and leukocyte-derived MPs, which may carry TF (Yang et al., 1999; Falati et al., 2003). The extracellular region comprises of an N-terminal, calcium-dependent (C-type) lectin

domain, followed by an EGF domain and six sushi domains (Cummings and Smith, 1992). Calcium-induced conformational changes to the lectin domain enable binding to specific moieties on carbohydrates and glycoconjugates (Geng et al., 1991; Cummings and Smith, 1992).

P-selectin glycoprotein ligand-1 (PSGL-1), which is a homodimeric mucin-like glycoprotein expressed on leukocytes and endothelial cells, is a well-documented native ligand of P-selectin (Moore et al., 1994; Yang et al., 1999). Binding to P-selectin requires the presence of specific fucosylated and sialylated O-linked glycans on PSGL-1, as well as sulfated tyrosine residues (Cummings and Smith, 1992; McEver and Zhu, 2010). These post-translational modifications allow for the formation of shear-dependent catch-bonds with the lectin domain of P-selectin (McEver and Zhu, 2010). P-selectin can also bind other glycoconjugates, such as CD24 and heparan sulfate, as well as GPIb (Aigner et al., 1998; Koenig et al., 1998; Romo et al., 1999).

1.2.2.3. Microparticles

MPs are 0.1 – 1 μm vesicles that are shed from membrane surfaces of most cells, and are thought to have a host of physiological roles (Freyssinet, 2003; Mause and Weber, 2010). MP formation is incompletely understood, but is a regulated process that can be governed through multiple mechanisms. In general, vesiculation involves localized alterations in cell membrane surface tension, which is achieved through remodeling of the cytoskeleton in concert with additional changes that influence membrane curvature. Briefly, these additional factors can involve localized forces exerted by nearby proteins, conformational characteristics of membrane constituents, or modification of membrane

lipid composition due to loss of asymmetry between the inner and outer leaflets, as described in *Section 1.1.3.1* (Muralidharan-Chari et al., 2010). The process of MP formation has been previously reviewed (Muralidharan-Chari et al., 2010; Morel et al., 2011).

MP formation that is driven by or results in the loss of membrane asymmetry (outlined in Figure 1.3) is of particular importance to the coagulation process because it results in the exposure of PS, providing a catalytic surface for coagulation enzymes. These procoagulant characteristics are enhanced in some MPs, which also carry TF (Biro et al., 2003; Del Conde et al., 2005). Due to their procoagulant nature, MPs have been proposed to participate in haemostasis (Freyssinet, 2003; Owens and Mackman, 2011). Further, elevated MP levels found in several prothrombotic conditions suggests that they may also contribute towards thrombogenesis (Warkentin et al., 1994; Dignat-George et al., 2004; Chirinos et al., 2005; Morel et al., 2005). Indeed, endogenously derived, human pericardial MPs induce thrombosis in rats (Biro et al., 2003).

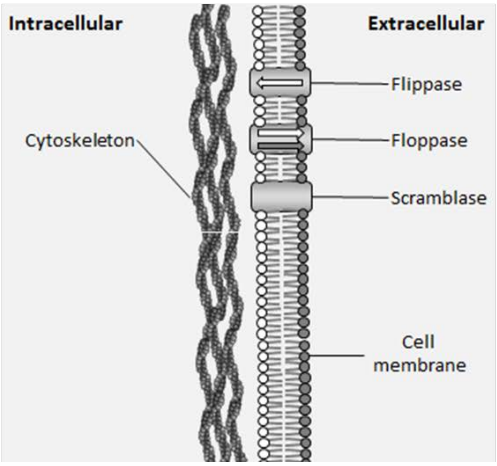
1.2.2.4. P-selectin-binding, procoagulant MPs in thrombosis and VTE

P-selectin has been implicated as a regulator of MP-mediated thrombus formation. P-selectin stimulation induces shedding of MPs expressing PSGL-1 and TF from monocytes (Hrachovinova et al., 2003; Bernimoulin et al., 2009). Infusion of recombinant P-selectin-Fc chimeric protein into mice stimulates PSGL-1-dependent release of monocyte-derived MPs that accumulate at thrombus sites and promote clot formation in haemophilia A mice (Hrachovinova et al., 2003), and increased circulating, soluble

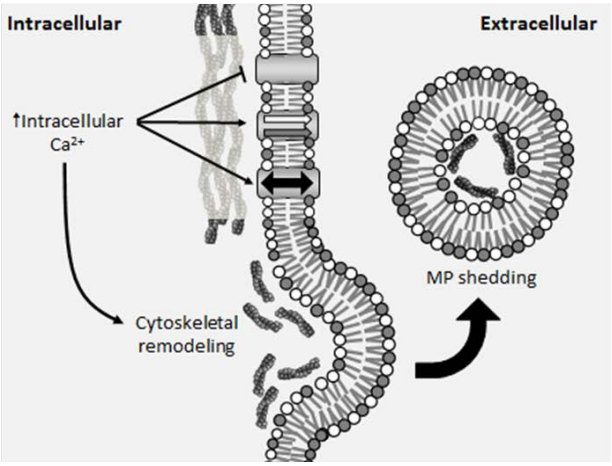
Figure 1.3. Loss of membrane asymmetry and MP formation.

A) Quiescent cells maintain an asymmetric distribution of phospholipids on their cell membrane through phospholipid translocase activity. Flippase selectively transfers aminophospholipids, such as PS and PEth to the inner leaflet, while floppase transfers phospholipids to the out leaflet. Flippase activity dominates, resulting in asymmetric phospholipid distribution. B) Stimuli resulting in sufficiently high levels of cytosolic calcium trigger pathways that cause cytoskeletal remodeling, flippase inhibition, further floppase activation and promote the activity of scramblase, which causes bidirectional translocation of phospholipids. The resulting change in surface tension cause membrane blebbing, leading to MP shedding.

A


Intracellular
Extracellular
Flippase
Floppase
Scramblase
Cytoskeleton
Cell membrane

B


Intracellular
Extracellular
↑Intracellular Ca2+
MP shedding
Cytoskeletal remodeling

P-selectin was found to be predictive of VTE in patients with cancer (Ay et al., 2008; Ay et al., 2010).

Interactions between P-selectin and PSGL-1 are required for binding between monocyte MPs and platelets, and for the transfer of surface proteins (Del Conde et al., 2005). Fusion of leukocyte MPs to platelets, which is mediated by the PSGL-1-associated carbohydrate, CD15, supports transfer and activation of TF (Rauch et al., 2000).

Haematopoietic and cancer cell-derived, TF-expressing MPs are capable of promoting coagulation in arterial laser injury and inferior vena cava (IVC) models of thrombosis, respectively (Chou et al., 2004; Thomas et al., 2015). The capacity of these MPs to initiate or contribute towards thrombosis is thought to depend on their ability to dock onto sites of thrombus formation by binding to cell adhesion proteins on endothelial cells or platelets (Owens and Mackman, 2011). The propagation of arteriolar thrombi by monocyte-derived MPs, which express PSGL-1 and TF, is inhibited in P-selectin-deficient mice (Falati et al., 2003). Also, TF-expressing MPs derived from PSGL-1-expressing tumour cells induce venous thrombosis in mice, and this can be abrogated by blocking P-selectin binding (Thomas et al., 2009). Importantly, this study also showed that the ability to induce thrombosis in tumour-bearing mice resulted from endogenous tumour-derived MPs rather than from tumour cells. However, cancer cell-derived MPs have also been shown to promote venous thrombosis through a P-selectin-independent mechanism (Thomas et al., 2015). Lastly, Basavaraj and Hansen found that levels of circulating, PSGL-1-expressing MPs are increased in patients with unprovoked VTE (Basavaraj and Hansen, 2012).

1.2.2.5. Procoagulant MPs as predictive biomarkers of VTE

TF-expressing MPs are elevated in patients with VTE (Tesselaar et al., 2009; Zwicker et al., 2009; Campello et al., 2011; van Doormaal et al., 2012; Rautou and Mackman, 2013) and other thrombotic diseases. This correlation is most strongly defined in individuals with cancer. TF-bearing MPs appear to be predictive of VTE in patients with pancreatic and potentially other forms of cancer (Khorana et al., 2008; Zwicker et al., 2009; van Doormaal et al., 2012; Thaler et al., 2012; Zwicker et al., 2013). Recently, it was shown that patients randomized to LMWH on the basis of having high levels of TF-positive MPs had a 5.6% cumulative incidence of VTE at two months, while those who did not receive thromboprophylaxis had an incidence of 27.3%. In addition, the incidence of VTE was 7.8% in patients with cancer and low levels of TF-positive MPs (Zwicker et al., 2013).

1.3. Part 3: Laboratory methods for investigating thrombosis and haemostasis

Investigation of thrombosis and haemostasis can be performed using a litany of tools and methodologies. This section will be limited to discussing techniques related to those used in the following chapters; namely, thrombin generation assays (TGAs), MP detection methods, and murine bleeding models.

1.3.1. Thrombin generation assay

The TGA is a kinetic assay that uses a chromogenic or fluorogenic substrate to monitor *in vitro* thrombin generation over time (Hemker and Beguin, 1995; Baglin, 2005). Thrombin has several roles in haemostasis, and its activity is a function of the activity of

several pro- and anti-coagulant mediators (van Veen et al., 2008b; Brummel-Ziedins, 2013). Thus, the plasma or whole blood TGA is considered a global measure of coagulation, and may be a useful surrogate to monitor the condition of the haemostatic system.

Small fluorogenic substrates, such as those used in the calibrated automated thrombogram and Technothrombin assay, enable continuous measurement of thrombin generation in plasma samples, and generate signals that are not affected by the turbidity of plasma. Variable quenching of the fluorescent signal in whole blood however, limits the use of existing commercially available fluorescent thrombin substrates to plasma (Tappenden et al., 2007; Al Dieri and Hemker, 2008). Another caveat of these small synthetic substrates is that their cleavage by thrombin is unaffected by α2-macroglobulin-mediated inhibition, which does not affect the access of small compounds to the thrombin catalytic site (Baglin, 2005). This can be corrected with the use of an algorithm that estimates and subtracts α2-macroglobulin-inhibited thrombin. In addition, at high levels of fluorescence, there is a loss of linear association between signal and fluorescent marker; a phenomenon termed the "inner-filter effect." Thus, at high thrombin concentrations, the levels of active thrombin may be underestimated unless this is taken into account using a standard curve consisting of known thrombin concentrations (Hemker et al., 2003).

The kinetics of thrombin generation can be assessed using a thrombin generation curve, which represents the rate of thrombin formation over time. This is obtained by determining the first derivative of the total amount of thrombin formed over time. The

curve follows the initiation, amplification, propagation and termination of thrombin generation. Several parameters of the thrombin generation curve, such as the endogenous thrombin potential (ETP), peak thrombin, time to peak (TTP), and lag time are often reported, and can be used to compare different curves (Figure 1.4).

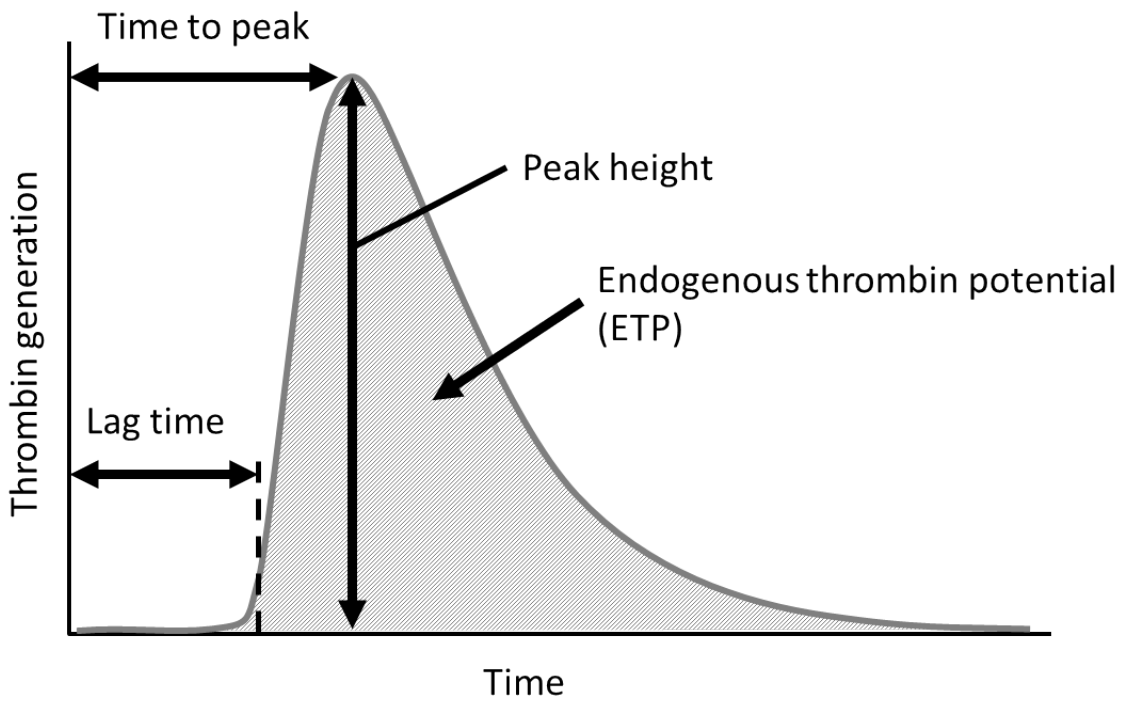
1.3.1.1. Thrombin generation as a clinical and research tool

Traditional clotting assays, such as the prothrombin time (PT) and activated partial thromboplastin time (aPTT) reach their endpoint when fibrinogen levels are exhausted, but 95% of the total thrombin has yet to be formed (Brummel et al., 2002). Therefore, in contrast to TGAs, these assays cannot inform about the different stages of thrombin formation or discriminate between samples having normal variations in clotting factor and inhibitor levels.

Studies have demonstrated that thrombin generation can be used to quantify thrombosis or bleeding risk. It was found to be an independent marker of first VTE in healthy controls and recurrent VTE in patients with a variety of risk factors, including thrombophilia, cancer, D-dimer and oral contraceptive use (Tans et al., 2003; van Hylckama et al., 2007; Eichinger et al., 2008; Tripodi et al., 2008; Lutsey et al., 2009; Ay et al., 2011). It may also estimate the risk of thrombosis in patients with lupus anticoagulant (Boeer et al., 2013). Thrombin generation parameters can predict bleeding in patients with haemophilia A or B (Dargaud et al., 2005; Pike et al., 2015) and are reduced in warfarinized patients with low FIX, who have increased risk of bleeding despite having an international normalized ratio (INR) in the target range of 2-3 (Dargaud et al., 2013).

Figure 1.4. Thrombin generation curve.

Thrombin generation assays are analyzed by plotting the rate of thrombin generation versus time. Four common parameters obtained from this curve are depicted.


Time to peak
Peak height
Endogenous thrombin potential
(ETP)
Lag time
Thrombin generation
Time

Further, since thrombin is a common downstream product of both the extrinsic and contact pathways of coagulation, and its formation is attenuated by all of the coagulation inhibitors, abnormalities at any node of the coagulation system can potentially manifest themselves in a TGA. Importantly, these abnormalities can be differentiated according to how they modify the thrombin generation curve.

For example, TFPI prolongs the lag time when TF is used to induce thrombin generation, while AT and prothrombin levels influence peak height and ETP (van't Veer and Mann, 1997; Hockin et al., 2002; Allen et al., 2004). With respect to various thrombophilias, FV Leiden can be excluded when the lag time of a thrombin generation curve that is initiated using 0.5 pM TF is prolonged by more than 1.5 minutes with the addition of APC. The prothrombin G20210A mutation can be excluded when peak thrombin concentration is less than 426 nM, and protein S deficiency can be excluded when ETP is reduced by less than 37% after addition of APC (Hezard et al., 2006).

Thrombin generation assays can also be modified to be highly sensitive to different components of the coagulation cascade. Initiation with APTT reagent or > 5 pM TF can be used to measure the contact or extrinsic pathways, respectively (Al Dieri et al., 2002; van Veen et al., 2008b). Initiation with FIXa has been used to measure differences in FVIII levels below 1% (Ninivaggi et al., 2011). These observations suggests that not only can thrombin generation be used to inform about global clotting tendency, but also to identify specific abnormalities, thereby providing a more detailed and comprehensive coagulation profile.

However, thrombin generation assays are labour-intensive, inter-laboratory practices lack standardization, and preanalytical steps in the assay can be a major source of variation (Baglin, 2005; van Veen et al., 2008b; Dargaud et al., 2010). Importantly, a range for thrombin generation parameters that can be used to distinguish healthy individuals from those at risk for developing coagulopathies remains to be defined (Brummel-Ziedins et al., 2005a; Danforth et al., 2012). Thus, despite the potential clinical value of the thrombin generation assay, the scope of its use has been limited to research.

1.3.1.2. Assessment of thrombin generation in whole blood

As described in *Section 1.1*, thrombin generation is regulated by several physiological effectors. Many of these components, including platelets, RBCs, and leukocytes are often removed by centrifugation prior to a TGA, and thus, their influence is usually not considered. However, these components are known to variably affect TGA parameters. For example, platelets derived from different donors demonstrate varying effects on thrombin generation (Oliver et al., 1999; Allen et al., 2004) and TGAs using platelet-rich plasma (PRP) better differentiate bleeders from non-bleeders among individuals with FXI deficiency (Pike et al., 2015). In addition, haematocrit is also correlated with TGA parameters (Horne et al., 2006), while neutophil-derived proteases variably influence thrombin generation under different conditions (Goel and Diamond, 2003; Perrin et al., 2010). Moreover, isolation, storage and handling of plasma introduce preanalytical variability and make point-of-care assessment using plasma impractical due to the length of time required for preparation.

A handful of techniques have been used to measure continuous thrombin generation in whole blood. Thuerlemann et al. developed an amperometric biosensor that measures thrombin generation in whole blood through cleavage of an amperogenic thrombin substrate (Thuerlemann et al., 2009). Ninivaggi et al. measured thrombin generation using a rhodamine-110-labeled substrate and a porous matrix on which to prepare a thin layer of diluted whole blood (Ninivaggi et al., 2012). More recently, our lab has developed a substrate that can be used to detect thrombin activity in whole blood by monitoring the change in the efficiency of Förster resonance energy transfer (FRET) after thrombin-mediated cleavage (Qiao et al., 2016). The substrate consists of two fluorophores in close approximation, separated by a structured region harbouring a thrombin cleavage site. The change in the ratio of fluorescence of these fluorophores due to cleavage by thrombin is used to construct a thrombin generation curve.

1.3.2 MP Quantification

1.3.2.1. Flow cytometry

Flow cytometry has been the most prevalent method used for assessing MPs. It permits high-throughput sample analysis, and can detect cells and particles directly in plasma (Jy et al., 2004). In conventional flow cytometers, suspended cells and particles are individually passed along a narrow stream of fluid by hydrodynamic forces and detected as they intersect the path of a laser beam (van der Pol et al., 2010; Lacroix et al., 2010; van der Pol et al., 2014). The size of a particle, which is proportional to the amount of light it scatters as it interacts with the beam, is determined by comparing the particle's small-angle light scatter (forward scatter (FSC)) with that of standardized sizing beads.

MPs can be differentiated from cells and platelets by selectively counting particles that fall within a <1 μm detection gate. The intensity of FSC light, however, is affected by the refractive indexes of cells and MPs, which are heterogeneous and different than that of polystyrene sizing beads. Thus, sizing estimates of MPs may not be accurate.

Particles that are smaller than the incident light mainly scatter light at larger angles. Therefore, cells can be differentiated based on the amount of their granular contents by measuring the intensity of light scattered perpendicular to the incident light (side scatter (SSC)). The amount of SSC light from MPs, which have a diameter on the same order of magnitude as the incident light, may therefore mask the SSC light scattered by their granular contents. Although this makes it difficult to discriminate between MPs based on granularity, it makes it possible to delineate MPs by size according to their SSC properties (van der Pol et al., 2014).

Markers on cells and MPs can be detected using fluorescently-conjugated antibodies and ligands (Jy et al., 2004; Lacroix et al., 2010). In addition, cytoplasmic or membrane dyes may be used (Boilard et al., 2014). The small surface area of MPs, however, offers limited binding sites for detection antibodies, potentially resulting in weak signals that may not be distinguishable from background noise (Lacroix et al., 2010). Moreover, antibodies may also bind Fc receptors present on the MP surface. Aggregation of fluorescent markers may also interfere with MP detection by appearing within the MP detection gate.

The detection limit of conventional flow cytometers for measuring small vesicles is around 300 nm, which is larger than the size of most MPs, making them indiscernible

from background noise based on size (van der Pol et al., 2010; van der Pol et al., 2014). In addition, MPs may not be individually distributed by hydrodynamic forces as cells are, causing multiple MPs to be detected as a single event. Thus, the capacity of these cytometers to discriminate between small particles and to perform multi-labeling studies on MPs is limited. Therefore, conventional flow cytometers are poorly-suited for detecting MPs, and alternative devices with higher sensitivity to submicron-sized particles, such as next-generation flow cytometers and impedance-based flow cytometers are increasingly used for this purpose (Janes and Rommel, 2011; Zwicker et al., 2013; Headland et al., 2014). These machines have reduced background-to-noise ratios and improved control of fluidics, which enables detection of smaller particles. Instead of detecting light scatter, impedance-based flow cytometers determine particle size by monitoring the change in impedance as a particle passes between two electrodes.

1.3.2.2 Enzyme-linked immunosorbent assay

An enzyme-linked immunosorbent assay (ELISA) has also been used as a method to evaluate MP samples in plasma (Osumi et al., 2001; Nomura et al., 2003). Briefly, diluted plasma samples are incubated at 200 rpm overnight at room temperature in 96-well microtiter plates containing immobilized antibodies. Wells are subsequently washed and MPs are labeled by the addition of biotinylated antibodies against a selected MP marker. MPs are detected using a peroxidase-conjugated avidin with a peroxidase substrate. This method has been used to detect platelet MPs using antibodies against GPIX and GPIb for capture and detection of MPs, respectively. Pooled platelet samples from healthy controls were used as standards. Measuring peroxidase activity via substrate

cleavage permits signal amplification, thereby enabling detection of antigens expressed at low surface concentrations. Unlike flow cytometry, this method does not enumerate individual MPs, and may include detection of exosomes. Further, overnight storage of MPs may contribute to loss of signal (Shah et al., 2008).

1.3.2.3. Activity/functional assays

MPs can also be quantified using functional assays that measure the procoagulant activity of a MP sample. One strategy uses a FXa generation assay to measure TF activity of MPs isolated from plasma (Khorana et al., 2008; Tesselaar et al., 2009; Lee et al., 2012). Briefly, MPs are isolated from diluted platelet-free plasma by serial centrifugation and washing steps using a standardized protocol of 20,000 x g for 15 minutes at 4°C. A small volume of supernatant is left after each centrifugation step so as to not disturb the MP pellet. A FXa generation assay performed in the presence and absence of an anti-TF antibody is used to determine MP TF activity, which is compared to known concentrations of relipidated TF standards. This method has been noted for its sensitivity compared to other types of techniques used to measure TF-expressing MPs (Lee et al., 2012). It has been frequently used to correlate MP TF activity in patient plasma samples with mortality or VTE (Tesselaar et al., 2009; Ay et al., 2011; van Doormaal et al., 2012; Thaler et al., 2012). It was found that MP TF activity may be predictive of VTE in patients with pancreatic cancer (Khorana et al., 2008).

In addition, MP procoagulant activity can be measured using a clotting assay which monitors change in turbidity of plasma containing MPs (Vaezzadeh and Gross, 2011; Campello et al., 2014). Finally, a TGA can be used to measure MP levels in plasma

after concentrating MPs by centrifugation from 1 mL of plasma. Using a TGA, MPs were found to be elevated in patients with VTE (Bidot et al., 2008). Similar to detection by ELISA, these methods only measure MP levels in bulk, rather than at the individual level. Also, serial centrifugation steps can result in the loss of MPs (Shah et al., 2008). Further, since these assays only measure MPs with procoagulant activity, MPs not expressing TF or PS are excluded from analysis. Finally, these assays measure all procoagulant MPs within a sample, and on their own, cannot be used to detect a specific subset of procoagulant MPs.

1.3.2.4. Functional solid-phase capture assays

Functional solid-phase capture assays are used for quantifying a specific subset of procoagulant MPs (Aupeix et al., 1997; Bal et al., 2010). This method couples the concept of an ELISA-based assay with that of a functional MP assay. Briefly, MPs are captured onto the surface of a 96-well microtiter plate using an immobilized antibody or ligand, and their procoagulant activity is subsequently measured using a prothrombinase assay or FXa generation assay. Commercially available capture assays include the Zymuphen MP-activity assay and Zymuphen MP-TF assay from Aniara (West Chester, USA), which use immobilized annexin V and TF antibodies to capture PS and TF-expressing MPs, respectively.

Like ELISA and functional assays, this method does not enable detection of individual MPs. This method does not require centrifugation of MPs, and sample incubation to isolate MPs has been performed for as little as 30 minutes. However, unlike the MP TF activity assay, in which MPs are isolated by centrifugation, the Zymuphen TF

activity assay was unable to detect elevated MP levels in plasma derived from lipopolysaccharide (LPS)-treated blood (Lee et al., 2012).

1.3.2.5. Other methods of MP quantification

Other methods of MP measurement include nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), Raman spectroscopy, resistive pulse sensing (RPS), transmission electron microscopy (TEM), atomic force microscopy (AFM), and analysis of MP protein content. Briefly, NTA and DLS use laser light to monitor the Brownian motion of particles within a solution (van der Pol et al., 2010; Yuana et al., 2011; van der Pol et al., 2014). NTA tracks individual particles, while DLS monitors the interference patterns of refracted light over time to calculate a sizing distribution. Raman spectroscopy detects inelastic light-scattering of macromolecules, with the amount of signal detected used as an indicator of MP volume. RPS measures the change in current as MPs pass through a pore, and estimates the size of MP based on the change in resistance (van der Pol et al., 2014). TEM analyzes fixed samples using electrons, which can provide high-resolution images of MPs. To measure MPs, AFM uses a <100Å probe positioned at the end of a cantilever to scan over an atomically flat surface containing MPs bound to immobilized antibodies (van der Pol et al., 2010; Yuana et al., 2011). Deflection of the probe and cantilever caused by intermolecular forces between the probe and MPs are detected using a laser. AFM generates a three-dimensional image of the surface with subnanometer resolution. Finally, protein concentration can be used as a surrogate for MP concentration and proteomic analysis can be performed to evaluate MP protein

composition (Thomas et al., 2009; Bernimoulin et al., 2009; Yuana et al., 2011). The different MP detection methods are compared in Table 1-1.

1.3.2.6. Pre-analytical variables affecting MP measurement

MPs are sensitive to handling and storage (Yuana et al., 2011). Blood collection in citrate, ethylenediaminetetraacetic acid (EDTA), citrate-theophylline-adenosine-dipyridamole (CTAD), acid-citrate-dextrose (ACD) or heparin can differentially affect MP count and PS exposure. High shear stress during blood collection may activate platelets and lyse RBCs, affecting the final MP sample. Centrifugation steps for plasma isolation can variably affect levels of MPs and contaminating platelets, while centrifugation steps to isolate MPs can result in the loss of MPs. Finally, storage of MPs and plasma can affect MP count and characteristics. Freeze-thawed platelet-poor plasma results in increased levels of PS-expressing platelet MPs, while storage of MPs under different conditions resulted in variable loss of MPs (Shah et al., 2008; Yuana et al., 2011).

1.3.3. Modeling haemostasis in vivo

In vitro investigation of thrombosis and haemostasis cannot fully replicate or account for the conditions of a physiological system. Animal models offer a physiological system in which to perform controlled studies. *In vivo* investigation of haemostasis is achieved through the use of animal bleeding models, which attempt to induce hemorrhaging in living tissue using controlled and replicable methods. Mice are important tools in biomedical research because they can be genetically manipulated to model human disease or to investigate the molecular mechanisms regulating health and

Table 1-1. MP detection methods

Adapted from (van der Pol et al., 2010; Lacroix et al., 2010; van der Pol et al., 2014).

	MP Size detection limit	Quantification	MP markers	Procoagulant activity
Flow cytometry (conventional)	> 300 nm	Individual particles + Bulk	Yes	No
Flow cytometry (Impedance and next gen)	> 150 nm	Individual particles + Bulk	Yes	No
ELISA	N/A	Bulk	Yes	No
Functional assay	N/A	Bulk	No	Yes
Functional capture assay	N/A	Bulk	Yes	Yes
NTA	> 50 nm	Individual particles	Limited by photobleaching	No
DLS	> 1 nm	Individual/Bulk	No	No
Raman spectroscopy	?	Individual/Bulk	No/measures distinct chemical composition	No
RPS	> 50 nm	Individual particles	No	No
TEM	~ 1 nm	Individual particles	Yes	No
AFM	< 1 nm	Individual particles	Yes	No
Protein content	N/A	Bulk	No/yes	No

disease. A plethora of transgenic and knockout models relevant to thrombosis and haemostasis research already exist, which simplifies investigation of a large variety of haemostatic mediators.

1.3.3.1. Tail tip transection models

Hemorrhagic injury to the tail is often induced in order to evaluate bleeding in mice and rats. In one type of model, the tail is transected near the tip at a fixed diameter or distance (Greene et al., 2010; Rand et al., 2012; Liu et al., 2013; Kahr et al., 2013). This severs the ventral artery, two lateral tail veins, and the smaller dorsal vein. Bleeding time can be measured by blotting the tail tip with filter paper until bleeding ceases, or can be determined by submerging the tail in saline and recording the time at which the resulting stream of blood from the tail disappears. In the event of rebleeding, the total combined duration of bleeding events is sometimes recorded. Alternatively, the volume of blood loss can be quantified by collecting blood into saline and measuring haemoglobin levels using spectrophotometry. The volume of blood in samples is determined by comparing to a standard curve. Blood loss may also be estimated by measuring the change in body weight before and after bleeding, as this was shown to correlate with blood loss measured using haemoglobin (Liu et al., 2013). Mortality may also be used as an endpoint in this model (Bi et al., 1995).

Increased bleeding is observed in a variety of hemorrhagic conditions including in mice with coagulation factor deficiencies (Bi et al., 1995; Wang et al., 1997) and in mice with platelet adhesion, activation, aggregation, or secretion defects (Novak et al., 1988; Sambrano et al., 2001; Iwatsuki et al., 2004; Strassel et al., 2007). Prolonged bleeding is

also observed in response to various anticoagulant and antiplatelet drugs. Moreover, differential bleeding responses allow comparison of different antithrombotics and their doses (Lambourne et al., 2012; Liu et al., 2013).

Results of tail tip transection bleeding models can vary according to the methodology that is employed. In one study, bleeding volume discriminated between 0.5 and 1.5 mg/kg daily doses of prasugrel, while bleeding time did not (Liu et al., 2013). Also, bleeding in PAR3-deficient mice was prolonged when the tail was amputated 5 mm from the tip, but not when it was transected 0.5 - 1 mm from the tip (Kahn et al., 1998; Weiss et al., 2002). In rats, the positioning of the tail (vertical vs. horizontal), immersion of the tail in saline or exposure to air, and the temperature of saline bath (room temperature vs. 37°C) all influenced bleeding times (Dejana et al., 1979). The type of anesthetic used has also been postulated to be a potential source of variation, as different anesthetics can differentially influence blood pressure. Strain, sex, age and sharpness of the blade used to amputate the tail tip are also potential sources of preanalytical variability and should be kept constant (Greene et al. JTH 2010). In addition, bleeding results can vary widely, which often necessitates large sample sizes to demonstrate differences between groups.

1.3.3.2. Lateral tail vein bleeding models

Injury to a single lateral tail vein is a less severe model of tail bleeding than tail tip transection. In contrast to tail tip transection, puncturing the tail vein enabled discrimination between bleeding phenotypes in mice that are doubly deficient in fibrinogen and vWF, and their fibronectin-depleted littermates (Wang et al., 2014). In a

variation, a transverse incision is made to lacerate the lateral tail vein (Broze, Jr. et al., 2001). Initial bleeding time is measured by monitoring bleeding in prewarmed saline, while rebleeding events are noted by monitoring mice over several hours and into the next day. Prolonged bleeding times are observed in mice after intraperitoneal (IP) administeration of drugs such as aspirin (50 mg/kg IP) and heparin (100 IU/kg IP) and in mice with platelet and coagulation defects. FVIII- and FIX-deficient mice show normal bleeding times, but exhibit rebleeding (Broze, Jr. et al., 2001).

1.3.3.3. Saphenous vein bleeding models

The saphenous vein bleeding models are similar to the lateral tail vein bleeding model in that injury is induced in a single vein, which is less severe than tail tip transection. In one model, the saphenous vein is exposed by removing the skin on the ventral side of the hind limb, and punctured using a 23 G needle (Pastoft et al., 2012). Blood is wicked away without disrupting the injury site until bleeding ceases. The injury is extended by making an approximately 1 mm longitudinal distal incision. Blood is again continuously wicked away and the bleeding time is recorded. The clot can be repeatedly disrupted using a blunted needle in order to achieve multiple re-bleeds and record several bleeding times per mouse. Data can be reported as average bleeding time and number of clots formed within a defined length of time. This model has primarily been used to assess bleeding in mice with compromised coagulation pathways, including FVIII- and FIX-deficient mice and mice receiving dabigatran, in which it has been reported to more closely reflect clinical dosing in humans compared with tail bleeding models (Pastoft et al., 2012; Pastoft et al., 2013; Monroe and Hoffman, 2014).

In a different model of saphenous vein bleeding, a laser is used to induce a hemorrhagic injury, approximately 50 µm in diameter, to the exposed vein (Getz et al., 2015). Injuries are repeated at 5-minute intervals. The vessel is observed by intravital microscopy, and the bleeding time after each injury is recorded. Injection of fluorescently-conjugated GPIX or fibrin antibodies permits labeling and measurement of accumulated platelets and fibrin, respectively. Transgenic and knockout models of mice deficient in talin and GPIb were found to have more severe bleeding in this model than mice with coagulation defects.

1.3.3.5. Other murine bleeding models

Cuticle bleeding in mice has been performed by amputating the fifth digit below the nail bed (Suh et al., 1995). Bleeding is monitored by dabbing with Whatman paper and the time to cessation is recorded. Another model measures renal bleeding after making an incision to the surface of the kidney using a template device (Bird et al., 2012). The kidney is maintained under warm Ringer's solution. The average bleeding time of two incisions to the same kidney is recorded.

In haemophilia mice, joint bleeding can be induced by blunt trauma to the knee using a spring-loaded device for controlled and replicable injuries (Valentino et al., 2004; Valentino et al., 2009). Alternatively, bleeding can be initiated by inserting a 30 G needle into the joint cavity through the infra-patellar ligament (Ovlisen et al., 2008). In the joint bleeding models, one knee is injured while the other serves as a control. Mice can be evaluated over the course of several days, during which repeated injuries can be performed. After sacrifice, bleeding is measured by assigning a visual score based on

histological analysis. In addition, other measurements, such as joint diameter before and after injury as well as other histological markers, such as synovial hyperplasia, vessel hyperplasia, hemosiderin staining, villus formation, and cartilage erosion can be assessed and used as part of a haemarthrosis scoring system. Few comparative studies have been performed between different bleeding models, but there is evidence that they may not always correlate well (Lavelle and MacIomhair, 1998).

Chapter 2: Objectives

2.1. Overall objective

Haemostatic dysregulation is a leading cause of morbidity and mortality. Characterizing and understanding mechanisms that drive thrombosis and haemostasis can help guide intervention strategies. New clinical and investigative methods that complement existing ones will be essential in this endeavour. We identified several areas of research that would benefit from the development of novel research methodologies. These included 1) *in vivo* bleeding models 2) detection of circulating, procoagulant plasma MPs, and 3) whole blood TGAs. The overall objective of the studies described in this thesis was to establish important clinical and investigative tools for facilitating research or clinical assessment in these particular areas. The specific objective and rationale for each study is described in detail:

Objective 1: To develop and characterize a novel murine arterial bleeding model and to compare haemostasis in different bleeding models

Several murine bleeding models, targeting different tissues and vessels, exist (Suh et al., 1995; Broze, Jr. et al., 2001; Angelillo-Scherrer et al., 2005; Buyue et al., 2008). However, it is unclear how the choice of bleeding model can influence the results of a study – although, there is evidence that it can (Lavelle and MacIomhair, 1998). Further, although venous and arterial beds differ in terms of shear, expression of coagulation mediators, and regulation of vasomotor tone – all of which can influence haemostasis – there is no model for evaluating bleeding in an isolated mouse artery. Thus, we

hypothesized that platelets or coagulation variably influence haemostasis in arterial and venous bleeding models. To test this, we developed a novel saphenous artery bleeding model that would be analogous to an existing saphenous vein bleeding model. We characterized and compared haemostasis in these models along with a tail tip transection model, which causes both venous and arterial bleeding.

Objective 2: To develop and characterize a method for evaluating circulating procoagulant, P-selectin-binding MPs, and to assess these MPs and total MP TF activity as risk markers of recurrence in patients with prior unprovoked VTE

P-selectin-binding MPs are proposed mediators of thrombosis in some animal models (Falati et al., 2003; Thomas et al., 2009), and could therefore be predictors of thrombotic disease. However, limitations in MP detection methods hinder assessment of these MPs in patients. Thus, to address the role of procoagulant, P-selectin-binding MPs in patients, new dedicated and practicable methods are needed. We therefore developed a capture-based activity assay to measure procoagulant, P-selectin-binding MPs in plasma and tested whether they are predictive of recurrence in patients with unprovoked VTE. Since MP TF activity is associated with development of VTE in certain cancer patients, but has not been assessed as a predictor of recurrence in unprovoked VTE, it was also measured as part of our study.

Objective 3: To develop and characterize a proof of concept for a chip that performs whole blood TGAs under multiple experimental conditions at the point of care.

The TGA is a global coagulation test that may potentially have far-reaching implications in the clinical assessment of haemostatic and thrombotic disorders (van Veen et al., 2008b). For this to be realized, point of care testing and a more comprehensive characterization of thrombin generation profiles in the general population are two areas that need to be addressed. Whole blood TGAs can potentially help to address these issues, but would benefit from more practicable implementation methods. Whole blood test strips and chips could provide novel approaches for facilitating point of care TGAs. Scaled down tests would also support performance of multiple TGAs using small volumes of sample. This would allow for more comprehensive testing by evaluating thrombin generation under different initiating conditions.

Chapter 3: Comparison of the effect of coagulation and platelet function impairments on various mouse bleeding models

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Forward: In Chapter 3, we generated a novel murine saphenous artery bleeding model and compared it with saphenous vein and tail tip transection models. We showed that all bleeding models were similarly sensitive to unfractionated heparin, whereas saphenous vein bleeding was less sensitive to $\alpha_{IIb}\beta_3$ inhibition than were artery and tail bleeding models. Inhibition of ADP-induced platelet aggregation *ex vivo* required higher levels of $\alpha_{IIb}\beta_3$ inhibitor than arterial and tail vein bleeding models. The results suggest that models that incorporate arterial bleeding may be more sensitive to platelet inhibition than venous bleeding models. The study also provides a new tool for evaluating haemostasis in arteries.

Running title: Coagulation and platelets in mouse bleeding models

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3.1. Summary

Background: Haemostatic impairments are studied *in vivo* using one of several murine bleeding models. However it is not known whether these models are equally appropriate for assessing coagulation or platelet function defects.

Objective: To assess the performance of arterial, venous and combined arterial and venous murine bleeding models towards impaired coagulation or platelet function.

Methods: Unfractionated heparin (UFH) or $\alpha_{IIb}\beta_3$ inhibitory antibody (Leo.H4) were administered to mice, and their effects on bleeding in saphenous vein, artery, and tail tip transection models were quantified and correlated with their effects on plasma clotting and ADP-induced platelet aggregation, respectively.

Results: All models exhibited similar sensitivity with UFH (EC_{50} dose = 0.19, 0.13 and 0.07 U/g, respectively) (95% CI = 0.14 - 0.27, 0.08 - 0.20, and 0.03 - 0.16 U/g respectively). Maximal inhibition of *ex vivo* plasma clotting could be achieved with UFH doses as low as 0.03 U/g.

In contrast, the saphenous vein bleeding model was less sensitive to $\alpha_{IIb}\beta_3$ inhibition (EC_{50} = 6.9 μg/mL) than tail transection or saphenous artery bleeding models (EC_{50} = 0.12 and 0.37 μg/mL, respectively) (95% CI = 2.4 - 20, 0.05 - 0.33, and 0.06 - 2.2 μg/mL, respectively). The EC_{50} of Leo.H4 for ADP-induced platelet aggregation *in vitro* (8.0 μg/mL) was at least twenty-fold higher than that of the tail and arterial, but not the venous bleeding model.

Conclusion: Venous, arterial and tail bleeding models are similarly affected by impaired coagulation, while platelet function defects have a greater influence in models incorporating arterial injury.

3.2. Introduction

Haemostasis involves 1) the activation and aggregation of platelets to form a primary platelet plug and 2) the conversion of soluble fibrinogen to an insoluble fibrin mesh through the activation of clotting factors. If any of these components is impaired due to either gene mutations or administration of antithrombotic drugs, the haemostatic process may be compromised and excessive bleeding may occur. Murine bleeding models are routinely used to assess these impairments (Andre et al., 2003; Petrich et al., 2007; Cho et al., 2008; Sheffield et al., 2009; White et al., 2010; Stolla et al., 2011; Rand et al., 2012). Several bleeding models are currently in use (Table 3-1). However, different models can generate different and disagreeing results (Matsuno et al., 2002; Schumacher et al., 2007; Bird et al., 2012). Unfortunately, it is not known which models most accurately reflect the effect of different haemostatic impairments on bleeding.

Some of these models exclusively assess bleeding from veins or microvasculature, while others measure bleeding from arteries, veins and microvasculature. Thus, different models measure bleeding under varying haemodynamic forces. This in turn, influences the contribution of platelets and clotting factors. Platelet adhesion and aggregation occur through different mechanisms under different shear rates (Savage et al., 1998; Ruggeri et al., 2006; Maxwell et al., 2007; Nesbitt et al., 2009). Similarly, the activation of clotting

Table 3-1. Vessel injuries made in different murine bleeding models.

Bleeding model	Arterial injury	Venous injury	Microvasculature injury	References
Tail tip transection	Yes	Yes	Yes	(Andre et al., 2003; Cho et al., 2008; Sheffield et al., 2009; White et al., 2010; Stolla et al., 2011; Rand et al., 2012)
Cuticle bleeding	Yes	Yes	Yes	(Suh et al., 1995; White et al., 2010)
Lateral tail vein transection	No	Yes	Yes	(Broze, Jr. et al., 2001)
Saphenous vein	No	Yes	No	(Buyue et al., 2008; Pastoft et al., 2012)
Renal bleeding	No	No	Yes	(Bird et al., 2012)
Template bleeding time	No	No	Yes	(Matsuno et al., 2002)
Saphenous artery	Yes	No	No	

factors, the deposition of fibrin, and the structure of fibrin clots are all influenced by haemodynamic forces (Repke et al., 1990; Campbell et al., 2010; Neeves et al., 2010).

Importantly, platelets and clotting factors can differ in the extents to which they contribute towards haemostasis. The relative contribution of each can depend on the type of vessel that is injured. This is illustrated by the different bleeding symptoms of patients with impaired platelet function compared to those with coagulation impairments. Patients with platelet disorders tend to experience mucocutaneous bleeding and can exhibit prolonged template bleeding times (Duke, 1910; George et al., 1990; George, 2000) and individuals receiving platelet $\alpha_{IIb}\beta_3$ inhibitors report increased bleeding resulting from cuts to the skin and may experience petechia (Simpfendorfer et al., 1997; Curtis et al., 2002). This bleeding diathesis is distinct from that of patients with coagulation disorders or those receiving heparin, who commonly experience joint bleeds and who do not have prolonged template bleeding times (Borchgrevink and Waaler, 1958; Borchgrevink, 1961; Kaneshiro et al., 1969; Peyvandi and Mannucci, 1999; George, 2000).

Thus, although the common purpose of all murine bleeding models is to assess the effect of some variable on haemostasis, due to the differences in vessel properties, they may not be equally appropriate for assessing platelet and coagulation impairments. Unfortunately, it is not clear how the differences between bleeding models influence bleeding caused by different haemostatic impairments. Nor is it evident which murine bleeding models are best suited for assessing defects of coagulation and which are more apt for assessing impairments of platelet function.

To address this issue, we used unfractionated heparin (UFH) to model impairments of coagulation, and a monoclonal inhibitory antibody directed against murine $\alpha_{IIb}\beta_3$ (Leo.H4) to model impairments of platelet aggregation. We assessed how changes to these parameters affect haemostasis in the artery, vein and tail by using saphenous artery, saphenous vein and tail tip transection bleeding models, respectively. In addition, we correlated the effect of these drugs on bleeding with their effects on *ex vivo* plasma clotting and ADP-induced platelet aggregation, respectively. We show here that UFH affects tail, saphenous vein and artery bleeding to a similar extent, suggesting that arterial, venous and tail bleeding models can be equally sensitive at detecting coagulation defects. In contrast, Leo.H4 increases saphenous artery and tail bleeding at lower doses than those required to increase saphenous vein bleeding, suggesting that bleeding models that incorporate arterial injury are more sensitive to platelet aggregation defects than venous bleeding models.

3.3. Materials and methods

3.3.1. Tail tip transection model

Weight-matched, male C57Bl/6 mice were anaesthetized with a 10% ketamine, 5% xylazine, 5% atropine cocktail. UFH (LEO Pharma, Ballerup, Denmark), Leo.H4 (Emfret Analytics, Wurzburg, Germany), or saline was infused through a catheter in the left jugular vein. Tail bleeding was assessed as described previously (Rand et al., 2012). Briefly, five minutes after infusion, the tail tip was transected at a point that measured 1 mm in diameter. The tail was subsequently immersed into pre-warmed saline from which

1 mL samples were collected at various time points over 30 min. 70 µl of ZAP-OGLOBIN II lytic reagent (Beckman Coulter, Mississauga, Canada) was added directly to the samples to lyse red blood cells. Blood loss was determined by lysing red blood cells from these samples, measuring the optical density at 405 nm, and converting this value into volume of blood using a standard curve derived from samples with defined volumes of blood.

3.3.2. Saphenous vein bleeding model

Saphenous vein bleeding was assessed as previously reported (Buyue et al., 2008; Pastoft et al., 2012), but modified so that a single bleeding time was recorded instead of inducing and assessing multiple re-bleeds. Briefly, mice were anaesthetized as described above. The right saphenous vein was exposed and connective tissue at the surface was removed in order to limit pooling of blood within tissues. UFH, Leo.H4 or saline was infused through the jugular vein. Five minutes after infusion, the saphenous vein was transected using a 25 gauge needle. Extravasated blood was removed using Kim wipes until blood no longer accumulated outside of the vessel. The time required for this to occur was noted, but was not used as the primary measure of saphenous vein bleeding. A ~1 to 2 mm longitudinal incision was then made along the medial wall of the vessel, starting from the point at which the vein was transected and towards the distal end of the vein. The time at which blood no longer accumulated outside of the vessel following this incision was used as the saphenous vein bleeding time. The maximum bleeding time was arbitrarily set as 900 s.

3.3.3. Saphenous artery bleeding model

In this novel method to assess bleeding, mice were anaesthetized, infused with UFH, Leo.H4 or saline, and the superficial vessels at the medial surface of the leg were exposed as described above. The saphenous artery was transected near the femur using a 27 gauge needle. Blood was gently removed using Kim wipes, and time to cessation of bleeding was recorded. Maximum bleeding time was arbitrarily set as 900 s.

3.3.4. Ex vivo clotting assay

900 µl of blood was withdrawn from the right carotid artery into 100 µl of 3.2% sodium citrate 15 to 20 min after intravenous infusion of UFH or saline. To obtain platelet poor plasma, blood was centrifuged at 6,000 x g for 5 min, and the isolated plasma was then centrifuged again at 6,000 x g for 5 additional min. 40 µl of platelet poor plasma was then incubated with 30 µl of HemosIL APTT reagent (Instrumental Laboratory, Bedford, USA) at 37°C for 5 min and subsequently recalcified with 20 mM $CaCl_2$. Clotting was measured by monitoring sample turbidity (Kim et al., 2007). This was achieved by measuring absorbance at 350 nm for the duration of 2 h and quantifying the area under the curve (AUC).

3.3.5. Platelet aggregation

Leo.H4 was incubated at various concentrations with citrated, whole blood for 10 min. This was then diluted 1:3 in saline containing 1.5 mM $CaCl_2$, and incubated at 37°C for 3 min. Platelets were activated with the addition of 0.1 mM ADP. Aggregation was measured by impedance aggregometry for 10 min using a Multiplate Analyzer (DiaPharma, West Chester, USA) and the area under the curve (AUC) was determined.

3.3.6. Statistical analysis and dose response curves

Results are depicted as mean ± standard error of the mean (SEM). The significance of differences was determined by ANOVA with the Bonferroni correction used for multiple comparisons. $P < 0.05$ was considered statistically significant. Sigmoidal dose response curves were fitted to the data and the dose required to achieve 50% of the maximal response (EC_{50} values) and their 95% confidence intervals (CI) were determined using GraphPad Prism 4. Constraints were set as follows: the "bottom" of each dose curve for bleeding experiments was set to be equivalent to the mean of the controls. For saphenous vein and artery bleeding experiments, the "top" of the curve was set as 900 s. For platelet aggregometry experiments, the "top" was defined by the mean of the saline control, and the "bottom" was set as zero.

3.4. Results

3.4.1. Dose response with UFH is similar in tail, venous and arterial bleeding models

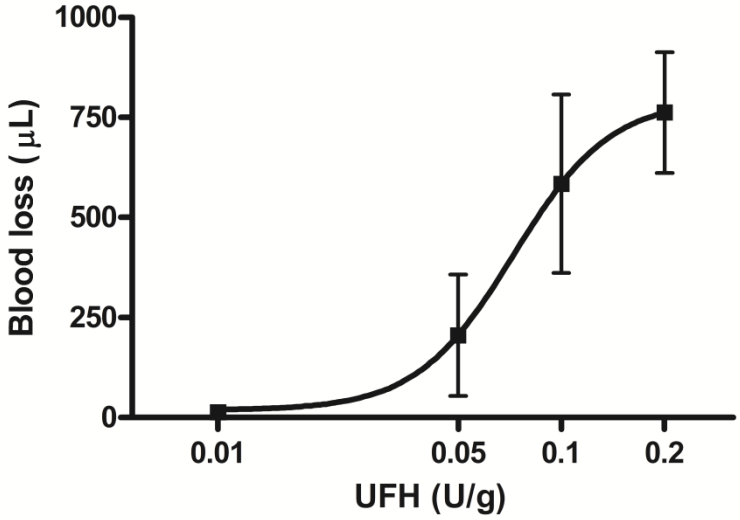
UFH produced a similar dose-dependent increase in bleeding in all three models. The EC_{50} doses for the tail tip transection, saphenous vein and saphenous artery models were 0.07 U/g (of mouse weight) (95% CI = 0.03 - 0.16 U/g), 0.19 U/g (95% CI = 0.14 - 0.27 U/g) and 0.13 U/g (95% CI = 0.08 - 0.20), respectively (Figure 3.1 and Table 3-2). Therefore, coagulation impairments similarly influence bleeding in all three models.

In addition, the amount of bleeding that was caused by only transecting the saphenous vein – that is, without generating a subsequent longitudinal incision – was also observed. Doses of UFH between 0.01 and 0.3 U/g did not increase bleeding after this

Figure 3.1. Dose response curves of bleeding models in response to intravenous infusion of UFH.

Fitted sigmoid log dose response curves for (A) tail transection and (B) saphenous vein (▲) or artery (■) bleeding models were generated to obtain EC_{50} values. Mean ± SEM of 3 to 5 mice are shown. Tail bleeding responses represent blood loss over 30 min, while saphenous vein and arterial bleeding models depict bleeding time. Maximum bleeding time was arbitrarily set at 900s.

A


1000
750
500
250
0
Blood loss (μL)
0.01
0.05
0.1
0.2
UFH (U/g)

B

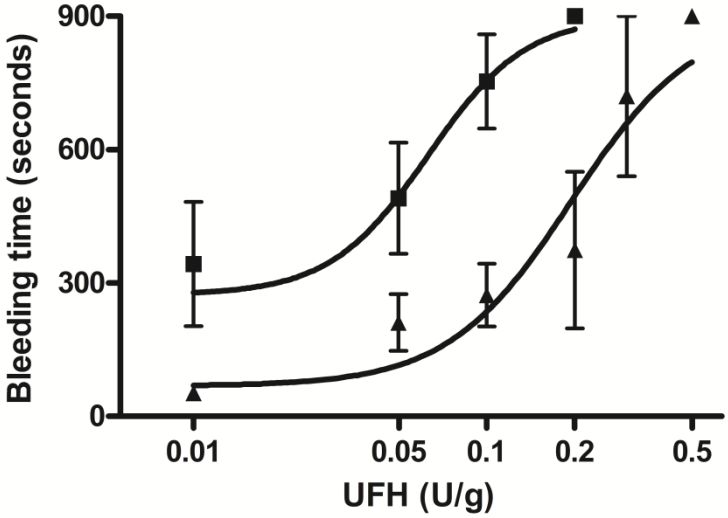

900
600
300
0
Bleeding time (seconds)
0.01
0.05
0.1
0.2
0.5
UFH (U/g)

Table 3-2. Summary of bleeding data.

EC_{50} values of tail tip transection, saphenous vein and saphenous artery bleeding, along with their 95% CI are shown.

* 95% CI of EC_{50} for Leo.H4 in venous bleeding model lies outside the CI range of tail and arterial bleeding data.

	UFH		Leo.H4	
	EC_{50} (U/g)	**95% CI**	**EC_{50} (µg/mL)**	**95% CI**
Tail transection	0.07	0.03 – 0.16	0.12	0.05 – 0.33
Saphenous vein	0.19	0.14 – 0.27	*6.9*	*2.4 – 20**
Saphenous artery	0.13	0.08 – 0.20	0.37	0.06 – 2.2

transection, nor did they demonstrate a trend towards increased bleeding. However, at a dose of 0.5 U/g, UFH did cause prolongation of bleeding after transection of the saphenous vein ($P < 0.05$) (data not shown).

3.4.2. UFH inhibits ex vivo clotting at lower doses than those required to increase bleeding

We characterized the effects of various UFH doses on fibrin clot formation using a plasma clotting assay (Figure 3.2). Clot formation was not inhibited by 0.01 U/g UFH. It was however, significantly inhibited with 0.03 U/g UFH ($P < 0.05$). Higher doses did not cause any further inhibition. Therefore, lower doses of UFH are required to achieve the maximum effect on *ex vivo* clotting than to elicit the maximum bleeding response *in vivo.*

3.4.3. Tail tip transection and saphenous artery bleeding models are more sensitive to $\alpha_{IIb}\beta_3$ inhibition than saphenous vein bleeding

Although Leo.H4 produced a dose-dependent increase in bleeding in all three models, the EC_{50} values varied. In the tail tip transection and arterial bleeding models the EC_{50} values were 0.12 µg/mL and 0.37 µg/mL, respectively. In contrast, the EC_{50} value in the saphenous vein bleeding model was 6.9 µg/mL (Figure 3.3 and Table 3-2). The 95% confidence intervals for tail tip transection and arterial bleeding models did not overlap with that of the saphenous vein bleeding model (Table 3-2).

Therefore, the tail tip transection and saphenous artery bleeding models were more sensitive to Leo.H4 than the saphenous vein bleeding model. In addition, we noted the bleeding times after transecting the saphenous vein with a 27 gauge needle. As seen

Figure 3.2. Effects of UFH infusion on *in vitro* plasma clotting time.

Plasma clotting assays were performed from platelet poor plasma of mice intravenously infused with different doses of UFH. Blood was withdrawn 15 to 20 min following UFH infusion. Optical density of the samples was measured over 2 h, and results were recorded as AUC. Mean ± SEM of 3 to 7 mice are shown. ***** represents $P < 0.05$.

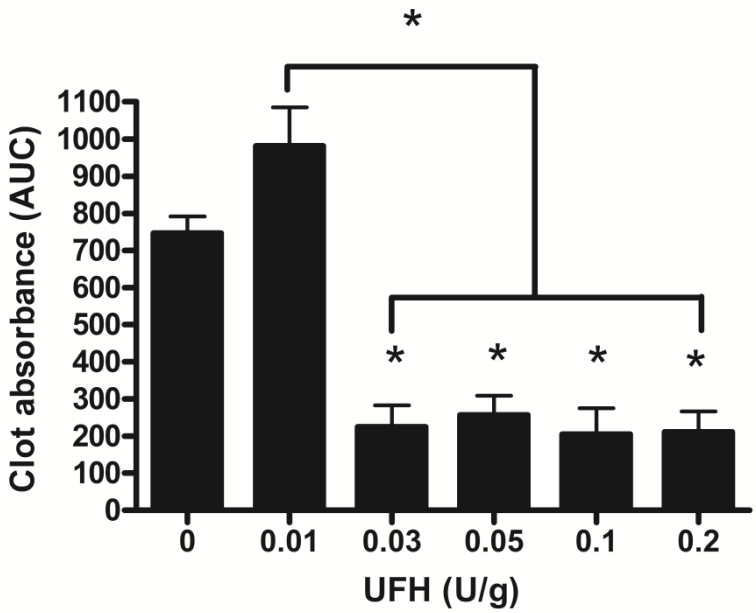
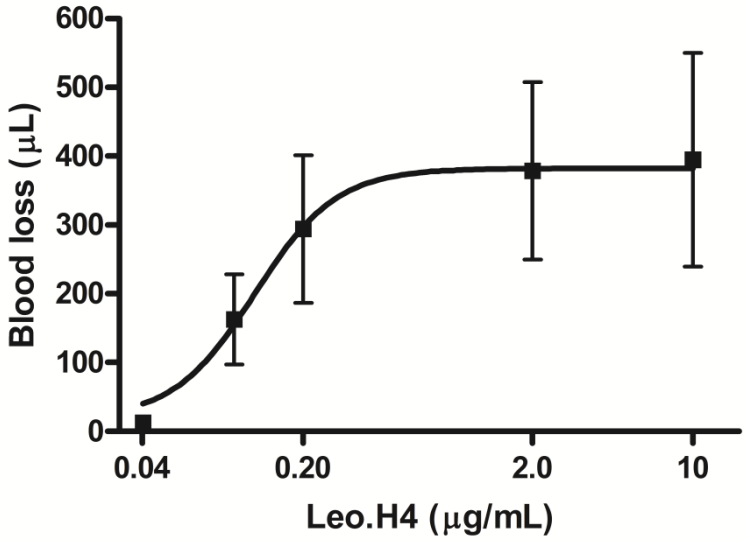

*
1100
1000
900
800
700
600
500
400
300
200
100
0
Clot absorbance (AUC)
* * * *
0
0.01
0.03
0.05
0.1
0.2
UFH (U/g)

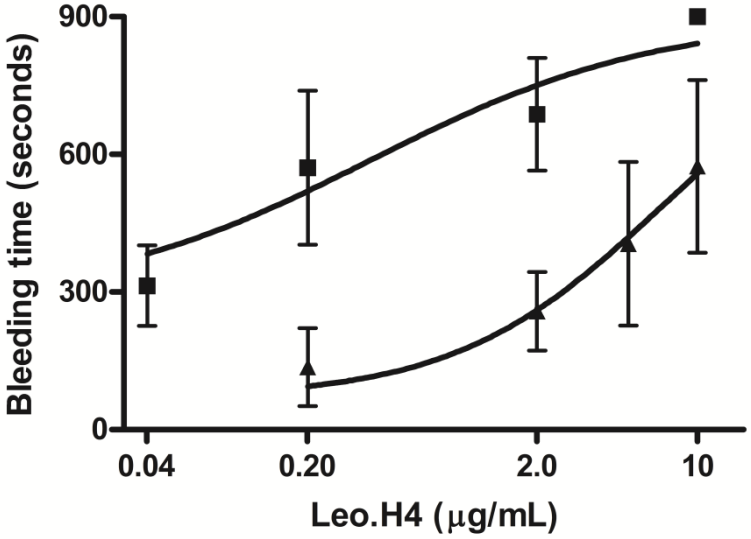
Figure 3.3. Dose response curves of bleeding models in response to intravenous infusion of Leo.H4.

Fitted sigmoid log dose response curves for (A) tail transection and (B) saphenous vein (▲) or artery (■) bleeding models were generated to obtain EC_{50} values. Mean ± SEM of 3 to 10 mice are shown. Doses of Leo.H4 have been converted from mg per gram body weight to μg per ml of circulating blood using 72 μl/g as the relation between volume of blood in circulation and body weight (Gregersen and Rawson, 1959). Tail bleeding responses represent blood loss over 30 min, while saphenous vein and arterial bleeding models depict bleeding time. Maximum bleeding time was set at 900 s.

A


600
500
400
300
200
100
0
Blood loss (μL)
0.04
0.20
2.0
10
Leo.H4 (μg/mL)

B


900
600
300
0
Bleeding time (seconds)
0.04
0.20
2.0
10
Leo.H4 (μg/mL)

with UFH, only the highest dose of Leo.H4 used (10 µg/mL) increased bleeding ($P < 0.05$) (data not shown).

3.4.4. Tail transection and saphenous artery models, but not the saphenous vein bleeding model, are more sensitive to Leo.H4 than in vitro ADP-induced platelet aggregation

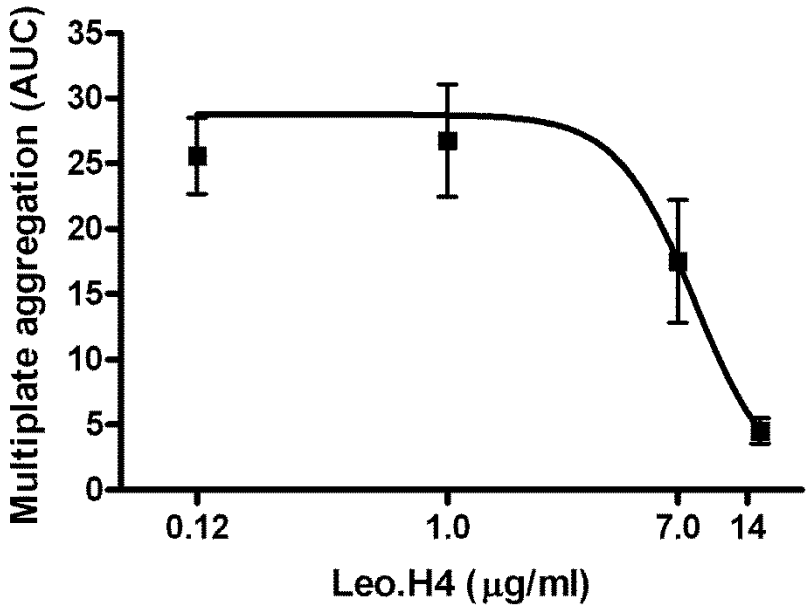
Impedance-based platelet aggregometry was used to characterize the effects of $\alpha_{IIb}\beta_3$ inhibition on platelet aggregation *in vitro*. The EC_{50} value of Leo.H4 was 8.0 µg/mL (95% CI = 5.9 - 11 µg/mL), which is over fifty-fold higher than the EC_{50} values for tail and arterial bleeding models, but comparable to the value for saphenous vein bleeding (Figure 3.4).

3.5. Discussion

The tail tip transection model has long been employed as a standard method to assay a broad range of hemorrhagic phenotypes in mice. Alternative models however, have also been developed and utilized in various studies. Since differences between arteries and veins can influence platelets and coagulation factors, and since different bleeding models can generate varying results, it is necessary to address how arterial and venous injuries differentially affect bleeding caused by platelet function and coagulation impairments. Here, we have characterized and compared the capacity of different bleeding models—which incorporate different combinations of venous and arterial bleeding—to detect defects in coagulation and platelet aggregation in order to explore the conditions under which different models would be most useful. We chose to use UFH and

Figure 3.4. Inhibition of *in vitro* ADP-induced platelet aggregation by Leo.H4.

Impedance-based platelet aggregometry was performed on citrated mouse blood incubated with Leo.H4. Aggregation was initiated using 0.1 mM ADP. Mean ± SEM of 3 to 7 samples and the fitted sigmoid log dose response curve are shown.


Multiplate aggregation (AUC)
35
30
25
20
15
10
5
0
0.12
1.0
7.0
14
Leo.H4 (μg/ml)

the $\alpha_{IIb}\beta_3$ inhibitory antibody, Leo.H4, to model coagulation impairment and platelet impairment, respectively. We have shown that UFH potentiates bleeding in tail transection, saphenous vein and saphenous artery models to a similar extent, while inhibition of $\alpha_{IIb}\beta_3$ has a greater influence on bleeding from the tail tip and saphenous artery than from the saphenous vein.

The tail transection model yielded an EC_{50} of 0.07 U/g in response to intravenous UFH infusion. This is in agreement with previous tail bleeding studies which have shown substantial bleeding responses at similar and higher doses, but not at lower doses (Lavelle and MacIomhair, 1998; Wang and Xu, 2005). We found a comparable effect after UFH infusion using a saphenous vein bleeding model (EC_{50} = 0.19 U/g). This response to UFH is in line with the prolongation of saphenous vein bleeding observed in mouse models of haemophilia A or B, which also have compromised coagulation (Buyue et al., 2008; Pastoft et al., 2012). Likewise, UFH was equally effective at increasing bleeding from the saphenous artery (EC_{50} = 0.13 U/g). These findings suggest that bleeding responses in venous, arterial and tail transection models are similarly influenced by impairment of coagulation.

We found that a UFH dose lower than these EC_{50} values was still capable of maximally inhibiting the ability of plasma to clot *ex vivo*, demonstrating that bleeding responses are less sensitive to circulating levels of UFH than *ex vivo* clotting assays. Correlations between *ex vivo* clotting assays and bleeding models following anticoagulation however, may be dependent on the type of anticoagulant administered (Lambourne et al., 2012).

In contrast to what was observed with UFH, the dose response to Leo.H4 was not similar in all models. The saphenous vein bleeding model yielded an EC_{50} dose that was several-fold higher than that in both the tail tip transection and saphenous artery bleeding models. Transecting the tail tip severs and exposes the two lateral tail veins, the dorsal vein and the ventral artery. Thus both tail transection and saphenous artery models exhibit arterial bleeding.

While there are examples to the contrary (Li and Cooley, 1995; Hupkens and Cooley, 1996), platelets are generally thought to be more important in the pathogenesis of arterial thrombosis than venous thrombosis, owing partly to the higher shear in arteries (Baumgartner, 1973; Yamamoto et al., 1998; Aird, 2007). The same is believed to be true in injury-induced thrombosis models (Yamamoto et al., 1998). Indeed, studies using perfusion chambers have shown that platelet adhesion is correlated with increasing wall shear (Baumgartner, 1973; Turitto and Baumgartner, 1979; Turitto et al., 1980). Interestingly, in PAR3 -/- mice, tail bleeding is prolonged when the tail is transected 5 mm from the tip, but not when it is severed 0.5 - 1 mm from the tip (Kahn et al., 1998; Weiss et al., 2002). When cut 1 mm from the tip, bleeding is thought to be primarily venous, while a 5 mm transection induces both venous and arterial bleeding (Maroney et al., 2012). These findings, taken together with our results, suggest that models that incorporate arterial bleeding may be more sensitive at assessing platelet defects than methods that rely entirely on venous bleeding. It is noteworthy that prolongation of saphenous vein bleeding was observed with higher doses of Leo.H4, suggesting that severe defects in platelet aggregation can affect bleeding in the saphenous vein model.

β3-integrin -/- mice exhibit prolonged bleeding following laceration of the lateral tail vein (Broze, Jr. et al., 2001).

We also noted that bleeding after saphenous vein transection (as opposed to saphenous vein incision) is only prolonged when high doses of UFH are used. This is consistent with findings that haemophilia A and B mice do not display prolonged bleeding following saphenous vein transection (Buyue et al., 2008; Pastoft et al., 2012). Bleeding is also not prolonged in mice with coagulation defects immediately following laceration of the lateral tail vein (Broze, Jr. et al., 2001). Instead, these mice exhibit delayed bleeding following this injury. In contrast, bleeding times are prolonged in FVIII-deficient mice following repeated induced bleeding events after incision of the saphenous vein (Pastoft et al., 2012). These observations, coupled with our findings regarding UFH dose-dependent prolongation of bleeding times after saphenous vein incision, may suggest that certain coagulation impairments only cause delayed bleeding in venous models that involve a small injury, but are more likely to cause immediate prolongation of bleeding in a model with more extensive vein injury such as a longitudinal incision of the lateral tail vein.

Lastly, we found that the EC_{50} of Leo.H4 required to inhibit platelet aggregation *in vitro* was several-fold higher than the EC_{50} in tail and arterial bleeding models. A daily dose of 0.015 mg/kg of the platelet inhibitor, prasugrel, prolongs bleeding time and increases blood loss in mice (Liu et al., 2013). However, 500-fold more prasugrel is required to inhibit ADP-induced platelet aggregation (Hashimoto et al., 2007). Thus, bleeding can be increased in mouse models by doses of antiplatelet drugs that have little

or no effect on *in vitro* platelet aggregation. Similarly, the tail bleeding model might over emphasize the effect of platelet function impairments.

In summary, our findings suggest that coagulation is equally required for haemostasis in isolated venous and arterial bleeding models, as well as in a tail tip transection model. In contrast, inhibition of platelet aggregation has a greater influence on bleeding in arterial and tail transection models than in a venous model. One implication is that venous bleeding models might offer a more suitable method than tail tip transections for assessing the effects of antithrombotics – such as thrombin inhibitors – on coagulation in isolation of their collateral effects on platelets. The findings also emphasize the importance of utilizing models that incorporate arterial injury when assessing the effects of platelet function impairments on haemostasis. In addition, *ex vivo* coagulation assays are more sensitive to heparin inhibition than *in vivo* bleeding models, whereas arterial and tail bleeding models are more sensitive to $\alpha_{IIb}\beta_3$ inhibition than *in vitro* impedance-based platelet aggregometry. These findings help to highlight the advantages and limitations of several bleeding models and will assist investigators in choosing an appropriate model when evaluating the effects of inhibitors or defects of coagulation or platelet function.

Chapter 4: Measuring microparticle tissue factor activity and procoagulant, P-selectin-binding microparticles prior to recurrence in unprovoked venous thromboembolism

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[4]Department of Infectious diseases and Immunity, Université Laval Centre de Recherche du CHU de Québec, Québec City, Québec, Canada

Forward: In Chapter 4, we developed and characterized a functional capture assay to measure procoagulant, P-selectin-binding MPs in plasma. We evaluated whether these MPs or MP TF activity predicted risk of recurrence in patients with previous unprovoked VTE. The capture assay was sensitive to changes in plasma MP levels resulting from treatment of blood with LPS. Neither MP TF activity, nor procoagulant, P-selectin-binding MPs were associated with recurrent VTE in patients with prior unprovoked VTE. This study introduces new methods for evaluating MPs based on receptor-ligand interactions, which could be important for studying MPs in health and disease. Specifically, it provides a tool for evaluating plasma levels of P-selectin-binding MPs,which are proposed mediators of thrombosis.The results also add to existing literature regarding the role of MPs in unprovoked VTE by addressing the association between MPs and future recurrent events

Running title: Procoagulant MPs and recurrent unprovoked VTE

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Athourship contributions: N. Vaezzadeh, E. Boilard, Z. Lysov and R. Ni performed experiments. N. Vaezzadeh and P.L. Gross wrote and edited the paper. N. Vaezzadeh, C. Kearon and V. Bhagirath contributed to the interpretation of results. C. Kearon and V. Bhagirath and P.C. Liaw provided patient samples. N. Vaezzadeh, P.L. Gross and C. Kearon designed the study.

This manuscript is in preparation.

4.1. Summary

Background: Unprovoked venous thromboembolism (VTE) is associated with a higher risk of recurrence than provoked VTE. Recruitment of procoagulant microparticles (MPs) via P-selectin-binding membrane glycoproteins or other unknown mechanisms is thought to contribute towards thrombosis.

Objectives: To develop and characterize a functional capture assay capable of measuring procoagulant, P-selectin-binding MPs in plasma, and to evaluate whether these MPs or total MP TF activity is predictive of recurrence following unprovoked VTE.

Methods: Plasma samples from patients enrolled in the DODS trial, who had completed anticoagulant therapy after presenting with unprovoked VTE, were used to assess the relationship between MPs and recurrent VTE during 2.2-year follow-up. A functional capture assay was developed, which used immobilized P-selectin to capture P-selectin-binding plasma MPs. MP TF activity and procoagulant, P-selectin binding MPs were evaluated in patient plasma samples.

Results: Using 1:5 diluted plasma and high orbital shaking speeds, procoagulant, P-selectin-binding THP-1 MPs could be detected at levels as low as $1.0 \times 10^4 – 2.0 \times 10^4$/µl. Elevations in procoagulant, P-selectin-binding MPs were detected in LPS-treated blood. However, total MP TF activity and procoagulant, P-selectin binding MPs were not elevated in patients with recurrent VTE compared to those without recurrence.

Conclusions: We have developed a functional assay to detect P-selectin-binding, procoagulant MPs in plasma. Neither the total MP TF activity nor procoagulant, P-selectin binding MPs were predictive of recurrence following unprovoked VTE.

4.2. Introduction

Venous thromboembolism (VTE) is a major cause of morbidity and mortality worldwide that manifests as deep vein thrombosis (DVT) or pulmonary embolism (PE) (White, 2003; Beckman et al., 2010). Between 25-50% of patients with VTE present without any identifiable risk factors (White, 2003), and face a higher likelihood of experiencing recurrent VTE once anticoagulation is discontinued (Rodger et al., 2010). In the absence of bleeding risks, guidelines recommend extended anticoagulation in patients with unprovoked VTE, but place a high value on patient preference (Kearon et al., 2012). Improved methods are needed to stratify patients with unprovoked VTE into groups with high and low risk of recurrence after withdrawal of anticoagulants in order to help determine which patients can safely stop receiving oral anticoagulant therapy and which ones should continue anticoagulation indefinitely. Biomarkers such as D-dimer are being evaluated (Palareti et al., 2006; Kearon et al., 2015), but this remains an unmet need.

Microparticles (MPs) are 0.1 – 1 μm vesicles that are shed from the surfaces of cells (Freyssinet, 2003). A large proportion of MPs display procoagulant features, such as high levels of exposed phosphatidylserine and procoagulant membrane proteins, such as tissue factor (TF) (Owens et al., 2012). Elevated levels of these MPs in circulation

may be indicative of a hypercoagulable state as evidenced by observations that high MP levels correlate with numerous thrombotic conditions, while low levels can accompany certain bleeding diatheses (Sims et al., 1989; Warkentin et al., 1994; Castaman et al., 1996; Dignat-George et al., 2004; Chirinos et al., 2005; Morel et al., 2005).

TF-bearing MPs and MP TF activity are predictive of VTE in some cancer patients (Khorana et al., 2008; Zwicker et al., 2009; van Doormaal et al., 2012; Zwicker et al., 2013). In rats, MPs derived from the pericardial blood of cardiac surgery patients induce thrombus formation in a TF-dependent manner in a venous stasis model (Biro et al., 2003). These findings suggest that TF-bearing MPs may play an important role in the pathogenesis of venous thrombosis, and that they might be useful as predictive biomarkers to identify patients who are at risk for developing VTE. TF-bearing MPs derived from tumour cells or hematopoietic cells, such as monocytes, potentiate thrombosis by localizing to sites of thrombus formation through adhesion molecule interactions mediated by MP P-selectin glycoprotein ligand-1 (PSGL-1) and platelet or endothelial P-selectin; thus not all TF-bearing MPs are necessarily involved in thrombus propagation (Rauch et al., 2000; Falati et al., 2003; Chou et al., 2004; Del Conde et al., 2005; Thomas et al., 2009).

However, although one study reported that PSGL-1 expressing MPs were elevated in patients with unprovoked VTE (Basavaraj and Hansen, 2012), it is uncertain if procoagulant, P-selectin-binding MP-mediated thrombus formation is relevant in clinical settings. Also, there are limitations to conventional quantitation methods, such as flow cytometry, in reliably enumerating MPs expressing particular surface antigens or multiple

antigens (van der Pol et al., 2010; Lacroix et al., 2010; van der Pol et al., 2014) and there is a lack of a widely available, alternative method for detecting levels of PSGL-1-expressing, procoagulant MPs. Furthermore, while TF-bearing MPs appear to precede the development of VTE in cancer patients, it is not known whether these or P-selectin-binding, procoagulant MPs predict thrombosis in patients without malignancy. To address the question of whether procoagulant MPs can predict recurrence in patients with unprovoked VTE, we first developed a capture-based activity assay to evaluate levels of procoagulant, P-selectin-binding MPs in plasma. Using both this assay, as well as a previously validated assay that measures total circulating MP TF activity, we assessed plasma samples, collected as part of the DODS trial (Kearon et al., 2015). In this study patients with unprovoked proximal DVT or PE after 3 to 7 months of treatment, stopped anticoagulation because they had normal D-dimer levels. Specifically, we evaluated whether MP levels, sampled one month after stopping anticoagulation, predicted VTE recurrence during follow-up.

4.3. Materials and methods

4.3.1. Materials

THP-1 cells were obtained from American Type Culture Collection (Manassas, VA, USA). RPMI-1640, Hanks' Buffered Salt Solution containing calcium (HBSS), calcium ionophore A23187, and CellTracker Green CMFDA were all purchased from Life Technologies (Burlington, ON, Canada). Recombinant P-selectin-Fc was purchased from R&D Systems (Minneapolis, MN, USA), and the human monoclonal TF antibody,

HTF-1 was from American Diagnostica (Stanford, CT, USA). Protein A-coated 8-well strips and Superblock blocking buffer were obtained from Thermo Fisher Scientific (Waltham, MA, USA). RecombiPlasTin was purchased from Instrumentation Laboratory (Bedford, MA, USA). Recombinant human factor VIIa was obtained from Haematologic Technologies Inc. (Essex Junction, VT, USA), recombinant human factor X was from Enzyme Research Laboratories (South Bend, IN, USA), and the factor Xa chromogenic substrate BIOPHEN CS-11(65) was purchased from Aniara (West Chester, OH, USA).

4.3.2. Study population

We assayed stored plasma samples from patients enrolled in the DODS trial. The study design of DODS has been described in detail (Kearon et al., 2015). Briefly, patients were between 18 and 75 years of age, had a previously reported first incident of unprovoked proximal DVT or PE and had completed 3 to 7 months of warfarin therapy. Patients with negative D-dimer levels were allowed to stop anticoagulation. D-dimer levels were measured again 1 month after warfarin cessation to determine whether or not to restart anticoagulant therapy. Two hundred sixty-three patients with negative D-dimer levels, who remained off anticoagulation after this 1 month follow-up were included in the study of MP TF activity, and 283 were included in the study of procoagulant, P-selectin-binding MPs.

4.3.3. Plasma collection

Blood was collected into citrated vacutainer tubes. Plasma was obtained by successive centrifugation at 1,700 x g for 15 and 5 minutes and stored at -80 °C.

4.3.4. THP-1-derived MP generation

THP-1 cells were cultured in RPMI-1640 supplemented with 10% (v/v) FBS and 1% penicillin-streptomycin. Confluent cells were washed with and resuspended in HBSS prior to labeling or induction of MP shedding. For high-sensitivity flow cytometry studies, cells were fluorescently labeled using CellTracker Green CMFDA (20 μM) and subsequently washed prior to MP generation. To prepare high MP yields, MPs were generated using a combination of calcium ionophore-induced shedding, as well as mechanically-stimulated MP release. Briefly, cells were first incubated with calcium ionophore A23187 (10 μM) for 20 minutes at room temperature and subsequently pelleted by centrifugation at 2,200 x g for 5 min. The supernatant was aspirated, and the resulting cell pellet was resuspended in 1 mL of HBSS. The cells were then removed by centrifugation at 600 x g for 5 min and the top half of the MP-rich supernatant was collected. MPs were either used immediately, or serially diluted in a 1:1 solution of citrated pooled plasma and HBSS with 0.32% sodium citrate and stored at -80 °C for later use.

4.3.5. High-sensitivity flow cytometry

CMFDA-labeled MPs were stored at -80 °C until enumerated using high-sensitivity flow cytometry using a FACSCanto II SORP as previously described (Boilard et al., 2014).

4.3.6. LPS-induced MP generation in blood

MPs were generated from blood cells using LPS as previously described (Lee et al., 2012), and plasma was isolated and stored according to protocols detailed in DODS

(Kearon et al., 2015). Briefly, blood was collected from healthy donors into 3.2% sodium citrate. MP formation was induced by incubating blood with 10 µg/mL LPS for 5 hours at 37 °C with gentle rocking. Plasma was isolated by centrifuging blood at 1,700 x g for 15 and 5 minutes. Samples were aliquoted and stored at -80 °C until later use.

4.3.7. MP TF activity assay

MP TF activity was measured using methods similar to those previously described (Khorana et al., 2008; Thaler et al., 2012; Lee et al., 2012). Briefly, MPs from 200 µl of plasma samples were washed twice in HBSA (137 mM NaCl, 5.38 mM KCl, 5.55 mM glucose, 10 mM HEPES, and 0.1% BSA, pH 7.5) by centrifugation at 20,000 x g for 15 min at 4 °C. Duplicate samples of washed MPs were incubated with 4 µg/mL HTF-1 or control IgG. Samples were then incubated in sealed wells at 37 °C for 2 hours in the presence of 2.5 nM FVIIa, 75 nM FX and 5 mM $CaCl_2$. The reaction was stopped using 5 mM EDTA, and FXa activity was measured with a chromogenic substrate. The change in absorbance at 405 nm was recorded over 15 minutes at 37 °C, and converted into pg/mL TF using a standard curve prepared from serial dilutions of RecombiPlasTin (0 - 20 pg/mL).

4.3.8. Capture of P-selectin-binding MPs

Recombinant P-selectin or a control antibody was immobilized onto protein A-coated wells according to the manufacturer's instructions with some minor modifications. Briefly, wells were rinsed with TBS, containing 0.05 % Tween-20. One hundred microlitres of Superblock buffer containing 9.4 nM of rP-selectin-Fc or a control IgG antibody was incubated in each well for 30-60 minutes. Wells were washed twice with

TBS and then once more using HBSS. Following this, 100 µl of MP samples were incubated in sealed wells for 1 hour under continuous, vigorous shaking. For patient samples, plasma samples were mixed in a 1:5 ratio with HBSS containing 0.32% sodium citrate prior to incubation in duplicate wells at 360 rpm. Afterwards, wells were gently washed once with HBSS, taking care not to disturb captured MPs.

4.3.9. Measurement of FXa generation by P-selectin-bound MPs

Wells containing captured MPs were incubated at 37 °C for 1 hour with 300 µl of a TBS solution containing 5 nM FVIIa and 150 nM FX in the presence of 5mM $CaCl_2$. To measure Factor Xa generation, 225 µl of the reaction mixture was removed from the MP-coated wells and added to a 96 well-plate containing a FXa chromogenic substrate. Absorbance at 405 nm was measured over time at 37 °C.

4.3.10. Statistical analysis

Statistical significance between experimental and control wells was determined using a student's t-test, while the effects of LPS treatment in coated wells was analyzed using a two-way analysis of variance (ANOVA) with a Bonferroni post-test to compare the effects of well-coating in different groups. The effect of LPS on samples from donors was also assessed using a paired t-test. A one-way ANOVA was used to evaluate the effects of parameters with variable conditions.

4.4. Results

4.4.1. MP TF activity and risk of recurrent VTE.

We measured MP TF activity in the plasma of patients with prior unprovoked VTE. Median MP TF activity was 0.22 pg/mL in patients with recurrence (25th - 75th percentile: 0.15 – 0.29 pg/mL) and 0.24 pg/mL in patients without recurrence (25th - 75h percentile: 0.15 – 0.33 pg/mL). Thus, MP TF activity did not predict recurrent VTE in this patient population (Figure 4.1).

4.4.2. A novel capture-based activity assay to detect procoagulant, P-selectin-binding MPs in citrated platelet-poor plasma.

The effect of procoagulant MPs on thrombus formation may be dependent on the expression of P-selectin-binding ligands. In order to quantify procoagulant activity from MPs expressing adhesion proteins that mediate P-selectin binding, we developed and characterized a novel capture assay to measure FXa generation from isolated MPs bound onto immobilized P-selectin in a 96-well plate. MPs derived from THP-1 cells, which coexpress PSGL-1 and TF, were captured onto P-selectin- or IgG-coated wells. MP-mediated FXa generation was significantly higher in wells containing P-selectin than in control wells (Figure 4.2A and B). This was reversed with EDTA (Figure 4.2A). Enhanced detection of P-selectin-binding MPs was achieved by increasing the orbital shaking speed during incubation of MPs in wells (Figure 4.2C).

Despite slightly reduced FXa generation, the assay permitted detection of THP-1-derived MPs from samples containing 0.32% sodium citrate (Figure 4.2B). In the

Figure 4.1. MP TF activity and VTE recurrence.

Total TF activity from isolated MPs was measured from plasma samples of DODS patients with (n = 32) or without (n = 231) recurrent VTE. MPs were isolated and washed by serial centrifugation prior to measuring TF-dependent FXa generation.

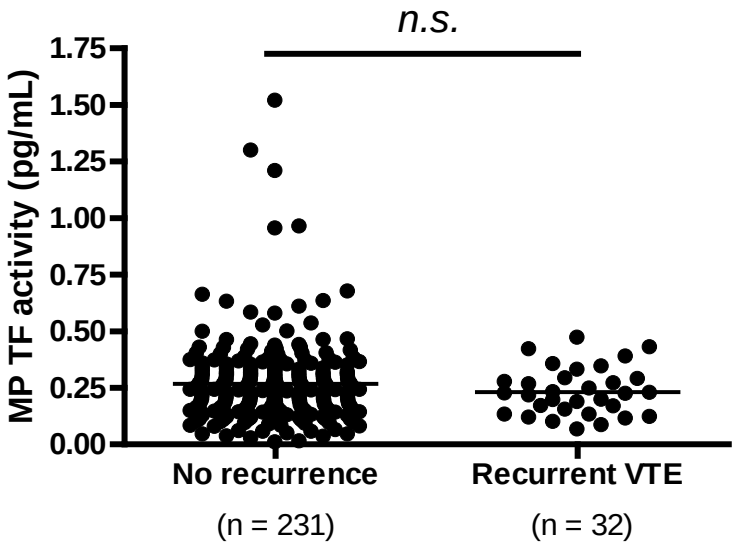
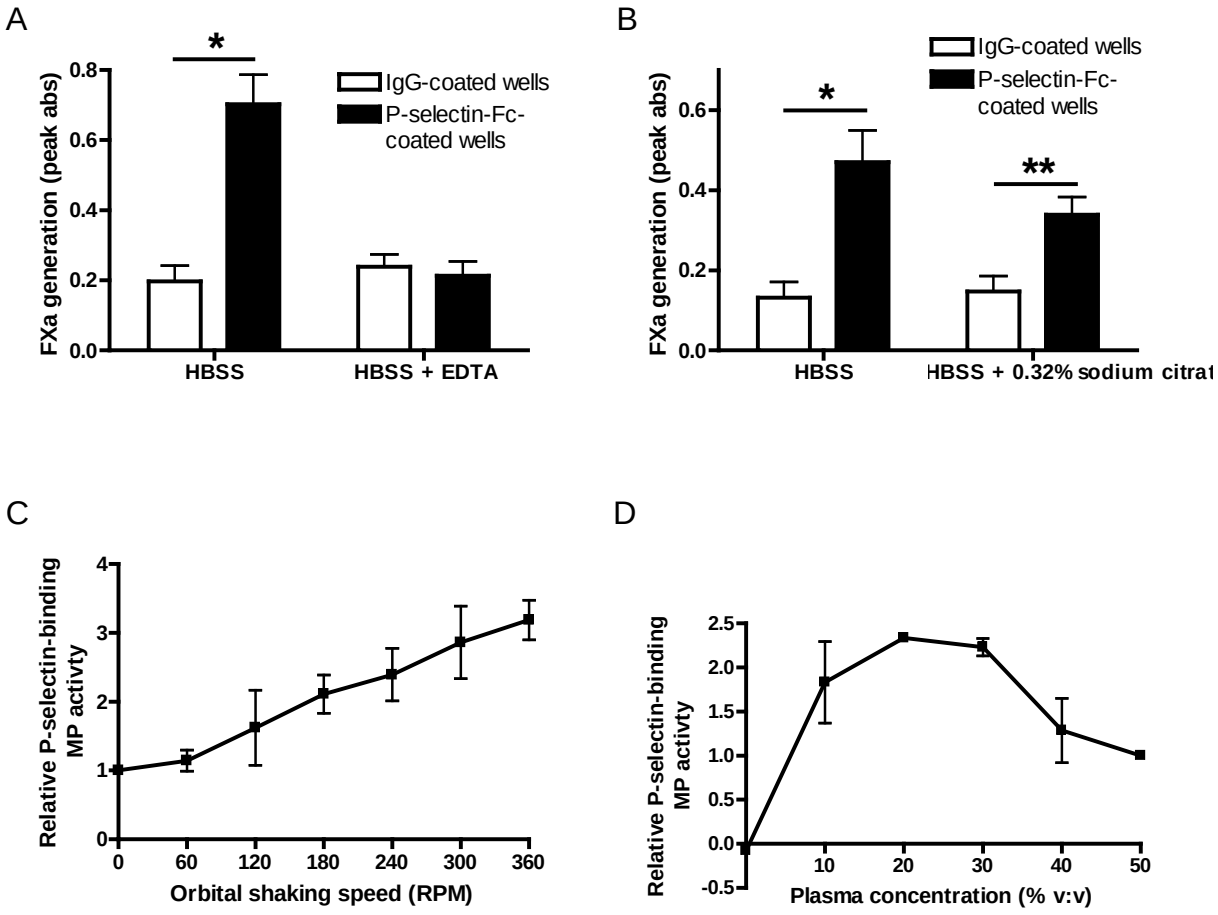

n.s.
MP TF activity (pg/mL)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
No recurrence
(n = 231)
Recurrent VTE
(n = 32)

Figure 4.2. A novel capture-based activity assay to detect procoagulant, P-selectin binding MPs.

THP-1 MP adherence to P-selectin- or IgG-coated wells in the presence or absence of (A) 10 mM EDTA and (B) 0.32% sodium citrate. Levels of bound MPs were measured by performing FXa generation assays after washing wells. Relative levels of FXa activity, determined by subtracting activity of MPs in control wells from that of MPs in P-selectin coated wells, show the effects of (C) increasing orbital shaking speeds and (D) plasma MP sample dilution during the MP capture step of the assay. Mean ± SEM; N = 3-7; * $P < 0.05$, ** $P < 0.01$.


A
*
IgG-coated wells
P-selectin-Fc-coated wells
FXa generation (peak abs)
0.0
0.2
0.4
0.6
0.8
HBSS
HBSS + EDTA
B
IgG-coated wells
P-selectin-Fc-coated wells
*
**
FXa generation (peak abs)
0.0
0.2
0.4
0.6
HBSS
HBSS + 0.32% sodium citrat
C
Relative P-selectin-binding MP activty
0
1
2
3
4
0
60
120
180
240
300
360
Orbital shaking speed (RPM)
D
Relative P-selectin-binding MP activty
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
10
20
30
40
50
Plasma concentration (% v:v)

presence of citrated plasma, the highest signal was obtained when the sample was diluted to contain 20% v/v plasma in citrated buffer (Figure 4.2D).

To evaluate assay sensitivity and reproducibility, serial dilutions of THP-1 MPs were stored in plasma and tested across different days, correlating results of the assay with MP levels determined by high-sensitivity flow cytometry. We found that there was high inter-assay variation, which could be corrected by dividing the value obtained for each sample by the mean of all samples on the same plate or by a known reference sample (not shown). Assessing the ratio in procoagulant activity between P-selectin-coated wells and control wells both enabled concentration-dependent detection of THP-1 MPs ranging in levels from between 1.1 x 10^7 to at least 8.7 x 10^8/mL (Figure 4.3), as well as the difference between procoagulant activity from P-selectin-coated wells and control wells (not shown) both enabled concentration-dependent detection of THP-1 MPs ranging in levels from between 1.1 x 10^7 to at least 8.7 x 10^8/mL. In addition, normalized results measuring the difference in activity between P-selectin and control wells showed a linear relationship at MP concentrations above 1.1 x 10^7/mL (r^2 = 0.9994) (not shown).

4.4.3. LPS-induced increase in procoagulant plasma MPs is reflected in the MP capture assay.

In order to determine if the assay is sensitive to increases in levels of endogenous plasma MPs, LPS was used to induce MP formation in blood from healthy donors. As has been shown before (Lee et al., 2012), LPS caused an increase in total MP TF activity, which ranged from 0 - 0.21 pg/mL prior to LPS treatment to 8.0 - 19.6 pg/mL after (Figure 4.4A). Similarly, using the P-selectin-binding MP capture assay, there was

Figure 4.3. P-selectin-binding MP capture assay sensitivity using THP-1 MPs.

Normalized FXa activity represented as the ratio of 'P-selectin-bound MPs'-to-'non-specifically-bound MPs' are plotted against MP concentration, as determined by high-sensitivity flow cytometry. Assays were performed on THP-1 MPs previously titrated with 50% v/v plasma in 10% sodium citrate and stored at -80°C. Data were normalized by dividing results of individual samples by the averaged results of all samples from the same plate. Mean ± SEM; N = 3.

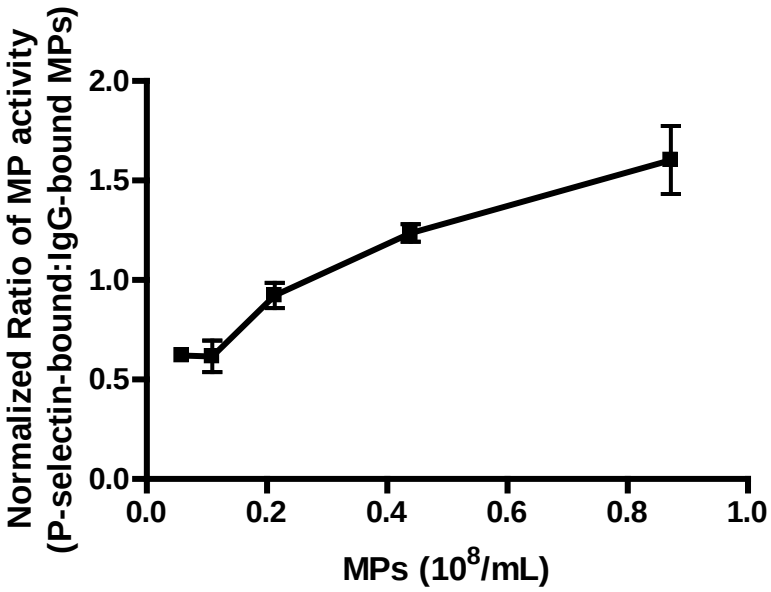
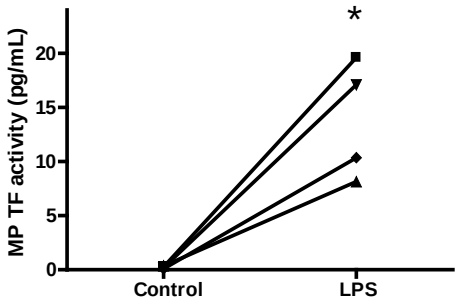

Normalized Ratio of MP activity
(P-selectin-bound:IgG-bound MPs)
2.0
1.5
1.0
0.5
0.0
0.0
0.2
0.4
0.6
0.8
1.0
MPs (10^8/mL)

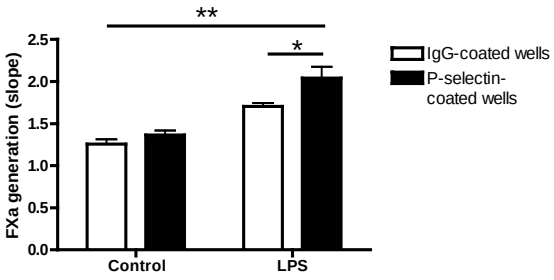
Figure 4.4. Sensitivity to LPS-induced increase in blood-derived MPs.

(A) Effect of LPS on MP TF activity in blood from four donors determined by MP TF activity assay. (B) FXa activity showing effect of LPS on P-selectin-bound MPs and non-specifically-captured MPs using the capture assay. (C) Increases in the ratio of 'P-selctin-bound MPs'-to-'non-specifically-captured' MPs following LPS treatment of blood from four donors. Bar graph shows mean ± SEM; * $P < 0.05$, ** $P < 0.0001$.

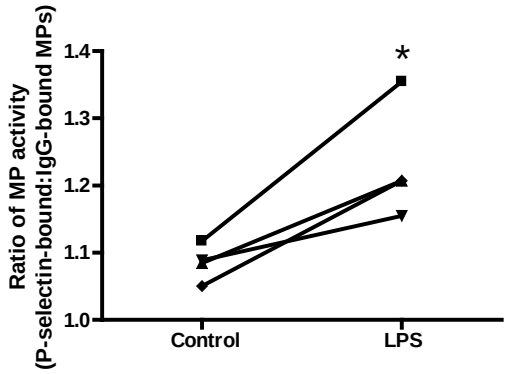
A


MP TF activity (pg/mL)
20
15
10
5
0
*
Control
LPS

B


FXa generation (slope)
2.5
2.0
1.5
1.0
0.5
0.0
**
*
Control
LPS
IgG-coated wells
P-selectin-coated wells

C


Ratio of MP activity (P-selectin-bound:IgG-bound MPs)
1.4
1.3
1.2
1.1
1.0
*
Control
LPS

a significant increase in FXa activity following LPS treatment in wells containing captured MPs. LPS caused a 1.4- and 1.5-fold increase in FXa activity from MPs captured in control wells and P-selectin-coated wells, respectively (Figure 4.4B). Additionally, LPS caused a statistically significant relative increase in FXa generation from MPs in P-selectin-coated wells compared with MPs in IgG coated wells (Figure 4.4B and C). The results demonstrate that increases in plasma MP levels are reflected in the P-selectin-binding MP capture assay.

4.4.4. The procoagulant, P-selectin-binding MP capture assay to assess MPs and risk of recurrent VTE.

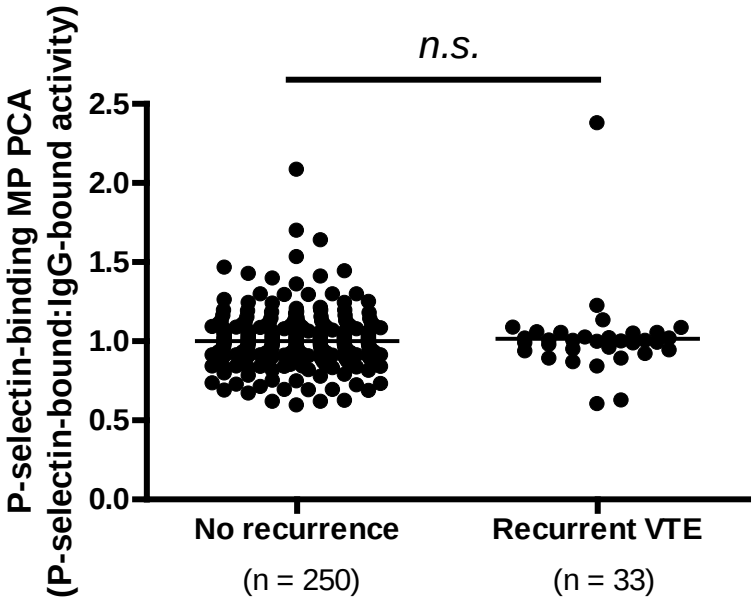
To determine whether procoagulant, P-selectin-binding MPs predict future recurrent thrombotic events in patients with unprovoked VTE, we also measured procoagulant, P-selectin-binding MPs in DODS patient plasma samples. The median normalized ratio of 'P-selectin-bound'-to-'IgG-bound' MP activity was 1.0 (25th - 75th percentile: 0.90 – 0.1 pg/mL) and 0.99 (25th - 75th percentile: 0.94 – 1.0 pg/mL) in patients with and without recurrence, respectively during the 2.2-year follow-up (Figure 4.5).

4.5. Discussion

Whether TF-expressing or procoagulant, P-selectin-binding MPs are involved in the pathogenesis of recurrent VTE or can predict recurrence in patients with unprovoked VTE is unknown. Here, we 1) examined whether total MP TF activity is an independent predictor of recurrence following unprovoked VTE, and 2) developed and characterized a

Figure 4.5. Procoagulant, P-selectin-binding MP capture assay to assess MPs and risk of recurrent VTE.

Ratio of captured MP FXa generation in P-selectin-coated wells relative to control wells assessed using plasma samples from DODS patients with (n = 33) and without (n = 250) recurrent VTE.


n.s.
P-selectin-binding MP PCA
(P-selectin-bound:IgG-bound activity)
2.5
2.0
1.5
1.0
0.5
0.0
No recurrence
(n = 250)
Recurrent VTE
(n = 33)

capture assay in order to determine whether P-selectin-dependent procoagulant MPs add further value in this clinical scenario. We found no association between either elevated MP TF activity, or procoagulant, P-selectin-binding MPs, and increased risk of developing recurrent VTE in patients with a prior unprovoked VTE event who stopped their anticoagulant.

Studies examining MP PS activity or TF activity have found no evidence of increased MP levels in patients with unprovoked VTE (Ay et al., 2009; Thaler et al., 2014). Using impedance-based flow cytometry, Zwicker *et al*. found that median levels of TF-expressing MPs in patients with idiopathic VTE was within the undetectable range (Zwicker et al., 2009).

The absence of MP TF activity following an unprovoked event is not sufficient to rule out the contribution of MPs in the pathogenesis of VTE in this disease state because MPs may be consumed by the clot if they contribute to its development (Ramacciotti et al., 2009), which may cause their circulating levels to decline during or subsequent to a thromboembolic event. This study partially addresses the unmet need to evaluate MP levels prior to recurrence in patients with unprovoked VTE, using samples from a large set of VTE patients enrolled in a well-defined prospective trial (DODS). Further, MPs were measured months after the initial thromboembolic event, and thus, would not be expected to be sequestered within a clot. However, MPs may be acute biomarkers of thrombosis. Khorana and colleagues showed that in a small group of patients with pancreatic cancer, MP TF activity levels increased in the months leading to thrombosis in the two individuals who developed VTE (Khorana et al., 2008). Therefore, future

prospective studies are needed to evaluate whether MP levels acutely increase prior to VTE, and to determine whether frequent testing is needed to identify this.

While levels of TF-bearing MPs can correlate with the development of thrombosis (Khorana et al., 2008; Zwicker et al., 2009; van Doormaal et al., 2012; Zwicker et al., 2013; Thomas et al., 2015), there are mechanisms in which only a subset of TF-expressing MPs – including, those that display P-selectin ligands such as PSGL-1 – contribute to thrombus formation (Falati et al., 2003; Thomas et al., 2009), and therefore, measuring total TF activity of all MPs in these situations could obfuscate results. Conventional flow cytometric detection of immunostained TF-expressing MPs is associated with technical limitations. Previous studies have reported the use of solid-phase capture assays using immobilized antibodies or ligands to detect procoagulant MPs expressing particular surface markers (Aupeix et al., 1997; Bal et al., 2010), but not P-selectin binding markers.

The interaction between P-selectin and PSGL-1 is calcium-dependent. EDTA abolished MP detection in our assay, demonstrating that THP-1 MPs in P-selectin-coated wells were captured in a calcium-dependent manner. However, in samples containing 0.32% sodium citrate, P-selectin-bound MPs could still be differentiated from non-specifically-bound MPs, since residual calcium remains available for P-selectin-PSGL-1 interactions (Bournazos et al., 2008). There was a positive correlation between FXa generation and THP-1 MPs in plasma samples at MP levels above 1.0×10^4/µl, although the detection limit using these MPs is likely to be between this value and 2.0×10^4 MP/µl.

This is the same order of magnitude as the reported detection limit of impedance-based flow cytometric measurement of TF-positive MPs (1.0×10^4/µl) (Zwicker et al., 2009).

The P-selectin-binding MP capture assay may be useful in other patient cohorts or in the assessment of other selectin- or mucin-binding MPs, which may be associated with VTE or involved in other diseases. Evaluation of P-selectin-binding MPs may be useful in patients with pancreatic or other forms of cancer, as MPs derived from the pancreatic cancer cell line, Panc02 have demonstrated P-selectin/PSGL-1-dependent thrombosis in animal models (Thomas et al., 2009) and selectin-mucin interactions have been proposed to be a key mechanism in the pathogenesis of Trousseau syndrome (Del Conde et al., 2007; Hauselmann and Borsig, 2014). In addition, the levels and characteristics of lectins or their ligands on tumour cells may indicate tumour progression and inform prognosis (Leathem and Brooks, 1987; Brooks and Leathem, 1991; Hauselmann and Borsig, 2014; Lange et al., 2014). Interestingly, in patients with haematologic malignancies who undergo allogeneic stem cell transplants, levels of PSGL-1-expressing MPs may be a biomarker of disease progression (Trummer et al., 2011).

There are several limitations of this study that must also be taken into consideration. Firstly, blood-borne factors such as GPIb and soluble P-selectin, by binding P-selectin binding partners on MPs, may act as inhibitors in our capture assay. Under these circumstances however, we suspect that their inhibitory effects in the assay would be reflective of similar inhibitory effects in circulation. Secondly, MP TF antigen levels were not measured in this assay. MP TF may be expressed in an encrypted state, and its activity may become enhanced only upon incorporation into the membranes of

activated cells where high PS exposure facilitates TF-FVIIa activity. However, unlike monocytes, PS levels and TF activity of MPs is not altered following freezing or ionomycin treatment suggesting that MP TF activity is not encrypted (Tesselaar et al., 2007; Johnson et al., 2009; Lee et al., 2012). Third, the results of the MP assays may have been affected by the long-term storage of plasma samples prior to evaluation of MP activity. However, others have noted that MP activity is not greatly affected by long-term storage (Lacroix et al., 2012; Willemze et al., 2014).

The possibility cannot be excluded that active TF-bearing MPs or procoagulant, P-selectin binding MPs may contribute towards thrombosis at levels that are below the detection limits of these activity assays. In order to induce P-selectin-mediated thrombus formation using an IVC ligation model in mice, Thomas et al. (2009) administered a dose of 18,000 MP/g/mouse. It can be presumed that this dosage results in a circulating concentration of ~2.5 x 10^2 MP/µl. However, this value may greatly underestimate the true number of infused MPs, as the authors showed that a 1000-fold reduction in concentration still resulted in significantly reduced vessel occlusion time. The study reported that MP concentration was determined by obtaining the corresponding MP protein concentration using a bicinchoninic acid assay. In contrast, MP counts in our study were determined directly by high-sensitivity flow cytometry using CMFDA-labeled MPs. It is unclear how these differences may affect quantification and whether data from animal studies can be extrapolated in this way. In addition, the capacity of the assay to discern increases in levels of human blood-borne MPs was shown by demonstrating increases in MP activity in blood treated with LPS. This is an important validation of

sensitivity, as not all existing MP assays are capable of detecting MPs formed this way (Lee et al., 2012). Lastly, TF-positive MPs in cancer-associated thrombosis have been shown to exist at concentrations as high as 1.0 x 10^7/μl, which is approximately three orders of magnitude greater than our estimated detection limit (Zwicker et al., 2009). Thus, although P-selectin-binding MPs likely comprise of only a fraction of TF-positive MPs, under pathological conditions, procoagulant, P-selectin binding MPs may exist at levels that would permit detection in the procoagulant, P-selectin-binding MP capture assay.

MPs can potentially support thrombus formation. However, there is a lack of evidence to support the notion that MPs are involved in the pathogenesis of non-cancer-associated VTE. This study investigated previously unaddressed questions surrounding the relationship between MPs and unprovoked VTE. Specifically, we measured both MP TF activity and procoagulant, P-selectin-binding MPs in a large cohort of patients with unprovoked VTE, and we contribute to the literature by demonstrating that MP activity was not elevated in patients who subsequently experienced a recurrent VTE event compared with those who did not. The development of a novel P-selectin-binding MP capture assay introduces a tool which can be used to further investigate the role of MPs in health and disease.

Chapter 5: A whole blood thrombin generation assay on a chip

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Forward: In Chapter 5, we designed and characterized a whole blood thrombin generation assay which is performed on a multichannel chip, using microlitre volumes of capillary blood. We showed that contact pathway-activated thrombin generation performed in this way can provide acceptable levels of intra- and inter-individual variation, and that multiple assays can be initiated under different conditions on the same chip. We also showed that pre-made chips can be stored for later use, that the results are sensitive to variations in prothrombin levels, and that an individual chip can differentiate between FVIII- and FIX-deficient plasma. The results demonstrate a proof of concept for a novel method of point of care thrombin generation testing, which could have important research and clinical implications.

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Authourship contributions: Nima Vaezzadeh performed the experiments. S. Qiao, R. Selvagnapathy and A. Mohammadzadeh provided technical assistance. N. Vaezzadeh and P.L. Gross designed the study and wrote the manuscript.

This manuscript is in preparation

5.1. Summary

Background: Whole blood thrombin generation assays (TGAs) permit point-of-care testing of thrombin generation. Comprehensive evaluation of coagulation using TGAs requires assessment under multiple experimental conditions, but standard laboratory approaches may be too limiting for such practices at the point of care. The objective of this study was to develop and characterize a point-of-care device for performing multiple thrombin generation assays under variable conditions using a drop of fingertip capillary blood.

Methods: A 20 × 25 mm chip, which uses capillary action to divert blood into four parallel channels containing predeposited reagents, was designed and constructed. Thrombin generation in each of the channels was initiated through contact activation and was measured by monitoring cleavage of a fluorogenic thrombin substrate made in-house.

Results: Four simultaneous thrombin generation curves could be derived using as little as 10 µl of non-anticoagulated fingertip capillary blood. The mean coefficients of variation (CVs) between channels were 12% (endogenous thrombin potential (ETP)), 13% (peak thrombin), 16% (time to peak (TTP)) and 12% (lag time). Mean intra-individual CVs for ETP, peak thrombin, TTP and lag time 13%, 9%, 18% and 19%, respectively. Mean inter-individual CVs were 10%, 15%, 28% and 29% for these parameters, respectively. These results showed inter-individual differences in the peak thrombin ($P < 0.01$), TTP ($P < 0.01$), and lag times ($P = 0.01$) of apparently healthy donors. The ETP ($P < 0.01$) and peak thrombin ($P < 0.05$) were increased in blood containing elevated levels of

prothrombin. All thrombin generation parameters correlated with the amount of predeposited contact pathway activator present in individual channels. Chips remained stable after three days of storage without any change in thrombin generation parameters. Lastly, chips containing recombinant factors VIII (FVIII) and IX (FIX) enabled differentiation between FVIII- and FIX-deficient plasma.

Conclusions: We have developed of a chip for evaluating thrombin generation under multiple experimental conditions using a drop of whole blood or plasma. This could have use as a low-cost, point-of-care method to characterize the coagulation cascade, including applications in haemophilia diagnostics.

5.2. Introduction

Thrombin is a central mediator of haemostasis that serves as a critical protease in both fibrin formation and platelet activation (Lane et al., 2005; Davie and Kulman, 2006). Thrombin generation assays (TGAs), which measure the extent to which a sample of blood or plasma supports thrombin formation and activity, therefore inform about the overall condition of the haemostatic system (Hemker and Beguin, 1995; Rand et al., 1996; Baglin, 2005; Hemker et al., 2006; van Veen et al., 2008b). The results can be correlated with various haematological conditions and clinical outcomes (Tans et al., 2003; Dargaud et al., 2005; Brummel-Ziedins et al., 2005b; Hron et al., 2006; Eichinger et al., 2008; Dargaud et al., 2013; Boeer et al., 2013) and are more sensitive measures of coagulation than clinical laboratory methods that are presently employed, such as

prothrombin time (PT) and activated partial thromboplastin time (aPTT) (van Veen et al., 2008b; Lance, 2015).

Recently, our lab has described a novel thrombin substrate that allows TGAs to be performed in minute volumes of non-anticoagulated whole blood drawn by finger prick, thereby enabling point of care testing. Substrate cleavage is measured by monitoring the change in Förster resonance energy transfer (FRET). Incorporating this substrate, along with predeposited initiators, onto a test strip or chip-based fluidic device can greatly facilitate point of care testing.

In addition, utilizing chips containing multiple branching channels can enable evaluation of thrombin generation under multiple assay conditions. Assessment of thrombin generation under multiple experimental conditions – for instance, in the presence of different initiators or inhibitors – provides more information about the coagulation cascade than can be obtained by testing a single set of parameters (Al Dieri et al., 2002; Hezard et al., 2006; Duckers et al., 2008; Machlus et al., 2009; Duckers et al., 2010; Castoldi and Rosing, 2011). A point of care method to evaluate thrombin generation under multiple experimental conditions therefore affords the opportunity to obtain a more complete and informative evaluation of the coagulation cascade, which would be useful in clinical research and practice.

As a proof of concept, we have developed and characterized a point-of-care device that performs multiple simultaneous TGAs under varying conditions through activation of the contact pathway of coagulation. Blood is passively drawn into multiple

parallel channels, where it reacts with predried reagents. The results are promising for new and potentially useful applications of the TGA.

5.3. Materials and methods

5.3.1.Materials

The thrombin substrate, "T13" was produced in-house (Qiao et al., 2016). APTT reagent was purchased from Instrumentation Laboratory (Bedford, MA, USA) and prothrombin was from Haematologic Technologies Inc. (Essex Junction, VT, USA). FVIII- and FIX-deficient plasmas were obtained from Affinity Biologicals Inc. (Ancaster, ON, Canada). Recombinant FVIII (Kogenate) and recombinant FIX (Benefix) were provided by the Hamilton General Hospital. The Cricut Explore digital craft cutter was purchased from Provo Craft & Novelty Inc. (South Jordan, UT, USA). Acetate plate sealers were purchased from Thermo Fischer Scientific (Waltham, MA, USA), and hydrophilic pressure-sensitive adhesive was generously provided by Adhesives Research (Glen Rock, PA, USA).

5.3.2. Chip fabrication

A four-channel device was designed using AutoCAD software. The different layers of the device were prepared and assembled using previously described methods (Yuen and Goral, 2010) with some minor alterations. Briefly, the channel and chip layers were made using a digital craft cutter (Cricut Explore). An acetate film was used to construct the floor and side walls of the channels. A structural support layer with low background fluorescence was adhered to the underside of the chip. Reagents were

deposited onto the chip and dried in a vacuum chamber. Lastly, a transparent film coated with a hydrophilic pressure-sensitive adhesive was used to fashion the top wall (Figure 5.1).

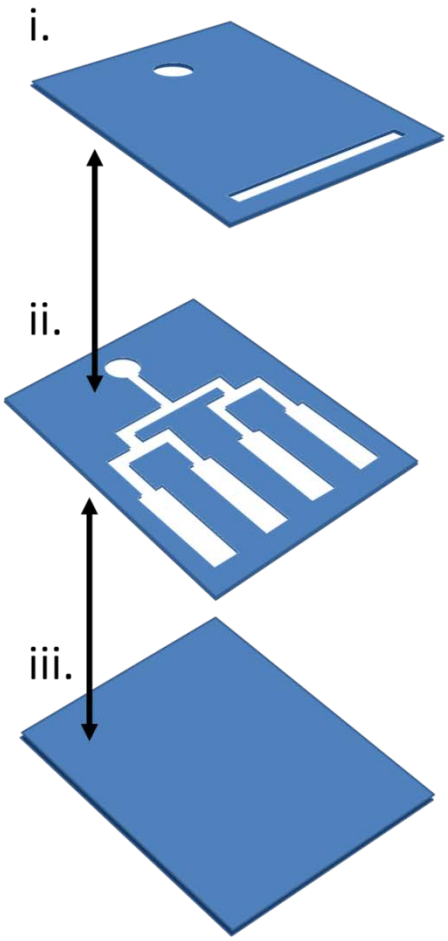
5.3.3. Whole blood thrombin generation assay

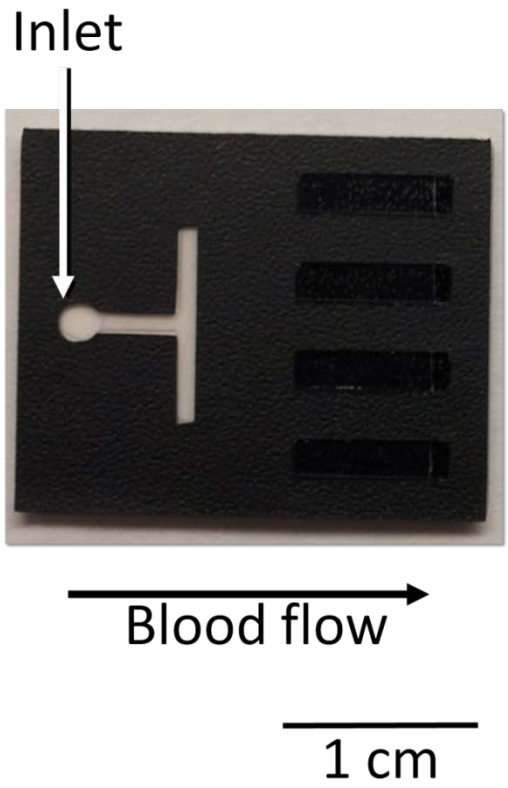
Blood was drawn by finger prick using a contact-activated lancet and the first drop was wiped away. In some instances, 10 µl of whole blood or plasma was deposited into the channel inlet using a pipette. In other instances, donors were instructed to place a drop of blood into the chip by contacting their finger to the channel inlet. The chip was mounted onto a 96-well plate template and aligned to allow for sample detection using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA). Thrombin generation was monitored by measuring the rate of change in the ratio of 581 to 526 nm fluorescence emissions within each channel.

5.3.4. Statistical analysis

Analysis of variance (ANOVA) was used to identify differences between experiments containing more than two groups. A Bonferroni post-test was used to compare between these individual groups. A student's t-test was performed to compare paired groups. Coefficients of variation (CV), reported as a percentage, were obtained according to the following formula: (standard deviation/mean) x 100.

Figure 5.1. Design of multichannel chip for performing thrombin generation assays. (A) Depiction of channel design showing (i) the top layer, comprising of a hydrophilic adhesive film that forms the top channel wall, (ii) a middle layer, which forms the side walls of the channel, and (iii) a bottom layer that functions as the channel floor and structural support. (B) A top view of a completed chip is shown containing an additional cover layer with low background fluorescence. The direction of blood flow by capillary action from the chip inlet to the four parallel channels is shown.


i.
ii.
iii.


Inlet
Blood flow
1 cm

5.4. Results

5.4.1. A TGA performed on a chip

Materials and methods for chip fabrication were selected on the basis of their ability to draw whole blood into the channels through capillary action, generate consistent thrombin generation curves, not induce contact pathway activation, and enable rapid prototyping of designs (data not shown). To determine whether our final construction method allows for acceptable reproducibility between channels and chips, we added recalcified, platelet-poor plasma, containing T13 and APTT reagent to the empty chip (Figure 5.2A, Table 5-1). Mean CVs for endogenous thrombin potential (ETP), thrombin peak, time to peak (TTP) and lag time between channels on the same chip (intra-chip CV) were 5, 8, 6, and 16%, respectively. Mean CVs for these parameters between different chips (inter-chip CV) were 4% (ETP), 5% (thrombin peak), 17% (TTP), and 28% (lag time). This data shows that thrombin generation can be measured on the chip, and that the design and construction of the device allow for acceptable levels of intra- and inter-chip variation for most thrombin generation parameters.

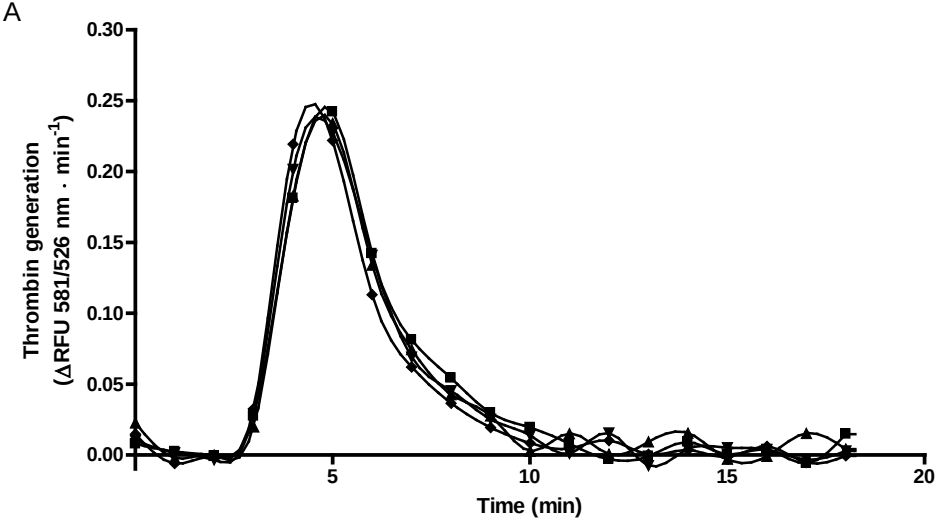
5.4.2. On-chip TGA performed on a drop of blood obtained via finger prick

TGA parameters differ between healthy individuals. To evaluate the performance of TGAs on chips containing predeposited reagents, we tested finger prick-derived whole blood from healthy donors. (Figure 5.2B and 5.3, Table 5-2). Mean intra-chip CVs for ETP, thrombin peak, TTP and lag time were 12, 13, 16, and 12%, respectively. Mean inter-chip CVs for these parameters were 13, 9, 18, and 19%, respectively. Finally, mean

Figure 5.2. Thrombin generation curves measured on a chip.

(A) A representative sample of a thrombin generation curve from APTT reagent-activated platelet-poor plasma in four channels of the same chip. (B) A representative figure of a thrombin generation curve from donor blood inside four channels of a single chip containing predeposited thrombin substrate and APTT reagent.

A


Thrombin generation
(ΔRFU 581/526 nm · min-1)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
5
10
15
20
Time (min)

B

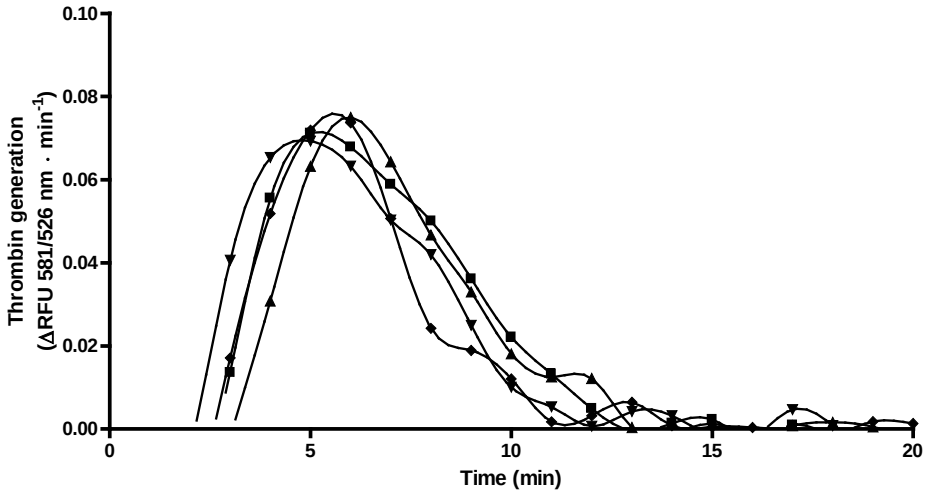

Thrombin generation
(ΔRFU 581/526 nm · min-1)
0.10
0.08
0.06
0.04
0.02
0.00
0
5
10
15
20
Time (min)

Table 5-1. Coefficients of variation in chips using platelet-poor plasma.

N = 8.

TGA Parameter	Intra-chip CV (%)	Inter-chip CV (%)
ETP	5	4
Peak thrombin	8	5
TTP	6	17
Lag time	16	28

Figure 5.3. Thrombin generation in whole blood from healthy donors.

A drop of fingertip capillary blood from four healthy donors was used to measure thrombin generation parameters in chips containing predeposited thrombin substrate and APTT reagent. (A) The ETP, (B) peak thrombin, (C) TTP, and (D) lag time are shown. Each point represents the average of four values from a single four-channel chip. * $P < 0.05$, ** $P < 0.01$.

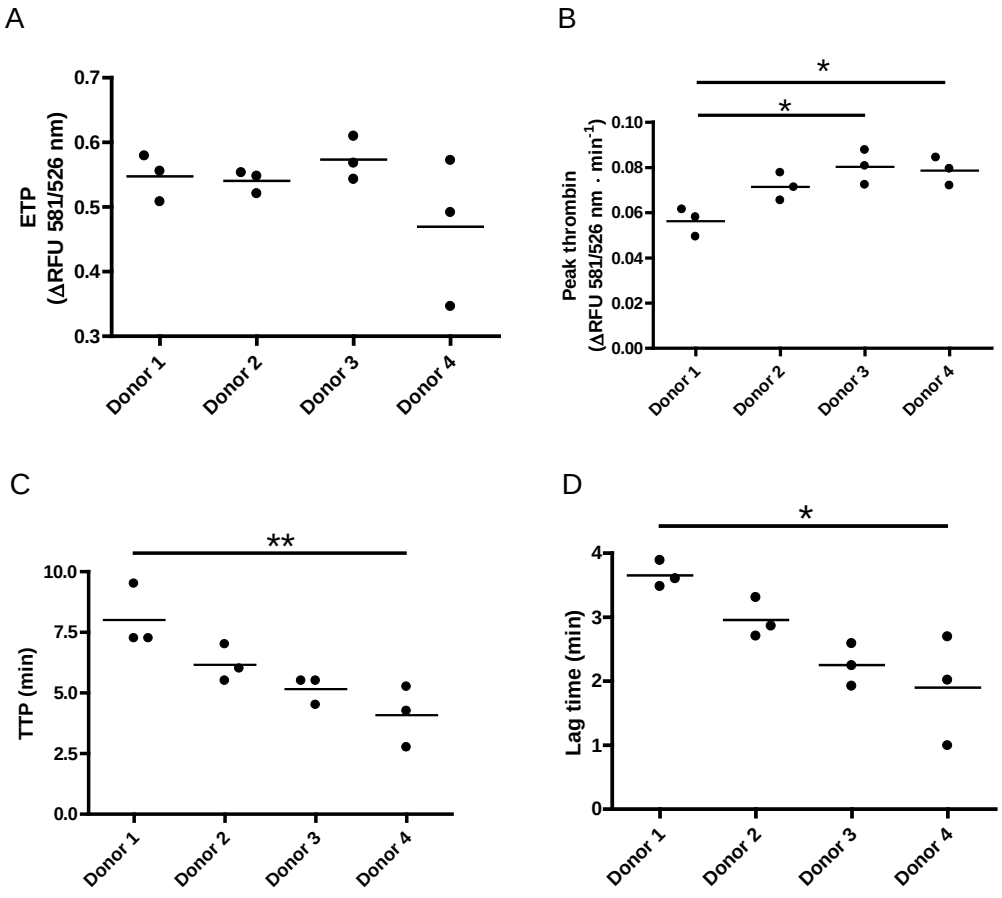

A
ETP
(ΔRFU 581/526 nm)
0.7
0.6
0.5
0.4
0.3
Donor 1
Donor 2
Donor 3
Donor 4
B
Peak thrombin
(ΔRFU 581/526 nm . min-1)
0.10
0.08
0.06
0.04
0.02
0.00
*
*
C
TTP (min)
10.0
7.5
5.0
2.5
0.0
**
D
Lag time (min)
4
3
2
1
0
*

Table 5-2. Coefficients of variation in chips using whole blood from donors.

N = 12

	Intra-individual CV (%)		Inter-individual CV (%)
TGA Parameter	**Intra-chip**	**Inter-chip**	
ETP	12	13	10
Peak thrombin	13	9	15
TTP	16	18	28
Lag time	12	19	29

inter-individual CVs were 10, 15, 28, and 29% for ETP, TP height, TTP, and lag time, respectively. Among four healthy donors, we found a statistically significant difference based on donor thrombin peak ($P < 0.01$), TTP ($P < 0.01$), and lag time ($P = 0.01$), but not ETP. These results show that on-chip TGAs performed on non-anticoagulated whole blood can differentiate between thrombin generation parameters in apparently healthy individuals.

5.4.5. Different channels within a chip perform independent TGAs

To examine whether TGAs within channels of the same chip are isolated from the one another, different volumes of APTT reagent were dried in parallel channels (Figure 5.4). APTT reagent was positively correlated with ETP ($P < 0.01$) and thrombin peak ($P < 0.01$), and inversely correlated with TTP ($P < 0.01$) and lag time ($P < 0.05$). These results demonstrate that adjacent channels can be used to perform separate TGAs under different assay conditions.

5.4.6. Short-term storage of pre-made chips does not affect thrombin generation

We were interested in whether chips could be prepared ahead of time with predeposited reagents and stored for later use. We found that 3-day storage of chips at 4 °C in the presence of desiccant did not cause a significant change in any of the thrombin generation parameters (Figure 5.5). Therefore, chips do not have to be used immediately after preparation and can be stored for later use.

Figure 5.4. Independent thrombin generation within individual channels of the same chip.

Four different conditions were tested on the same chip by depositing different amounts of APTT reagent in different channels. (A) The resulting ETP, (B) peak thrombin, (C) TTP, and (D) lag time are shown. Mean ± SEM; N = 3. $P < 0.05$ for all values (ANOVA).

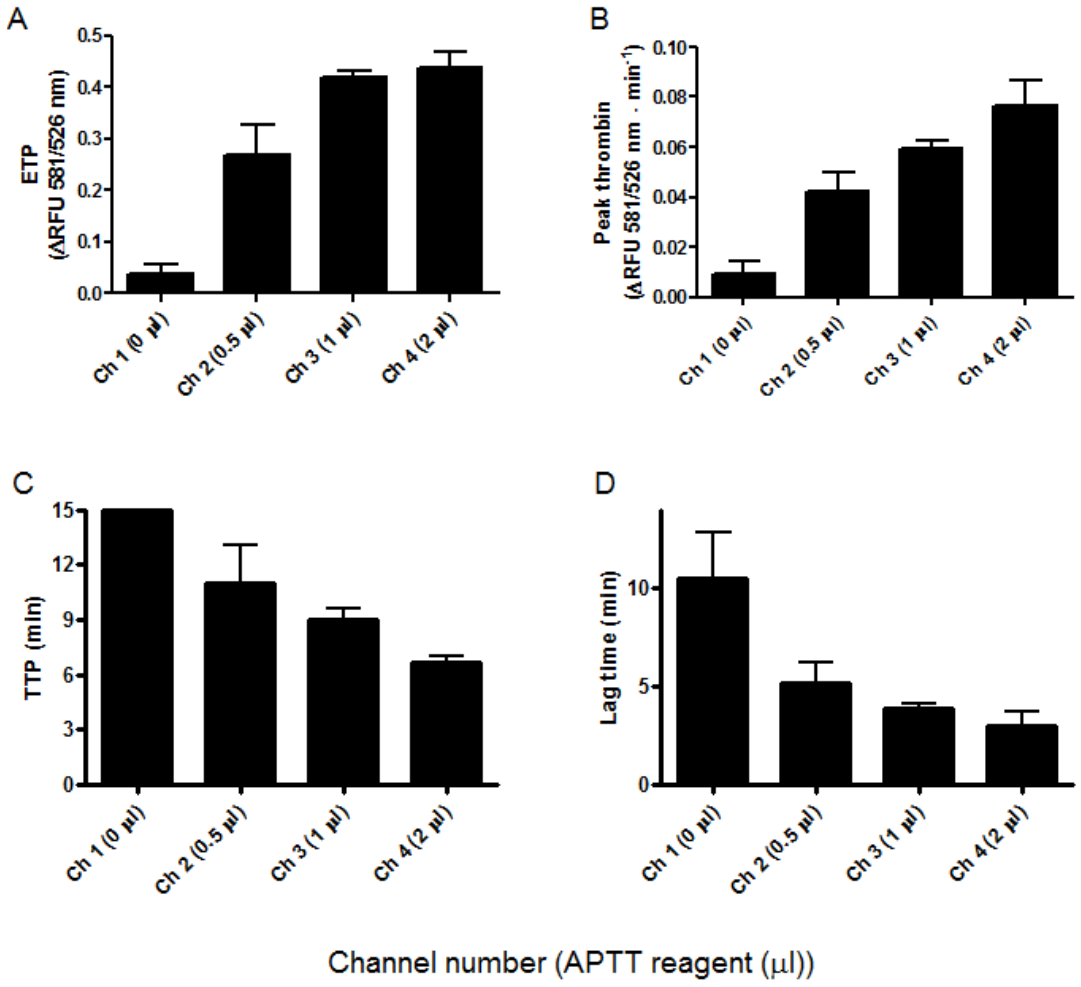
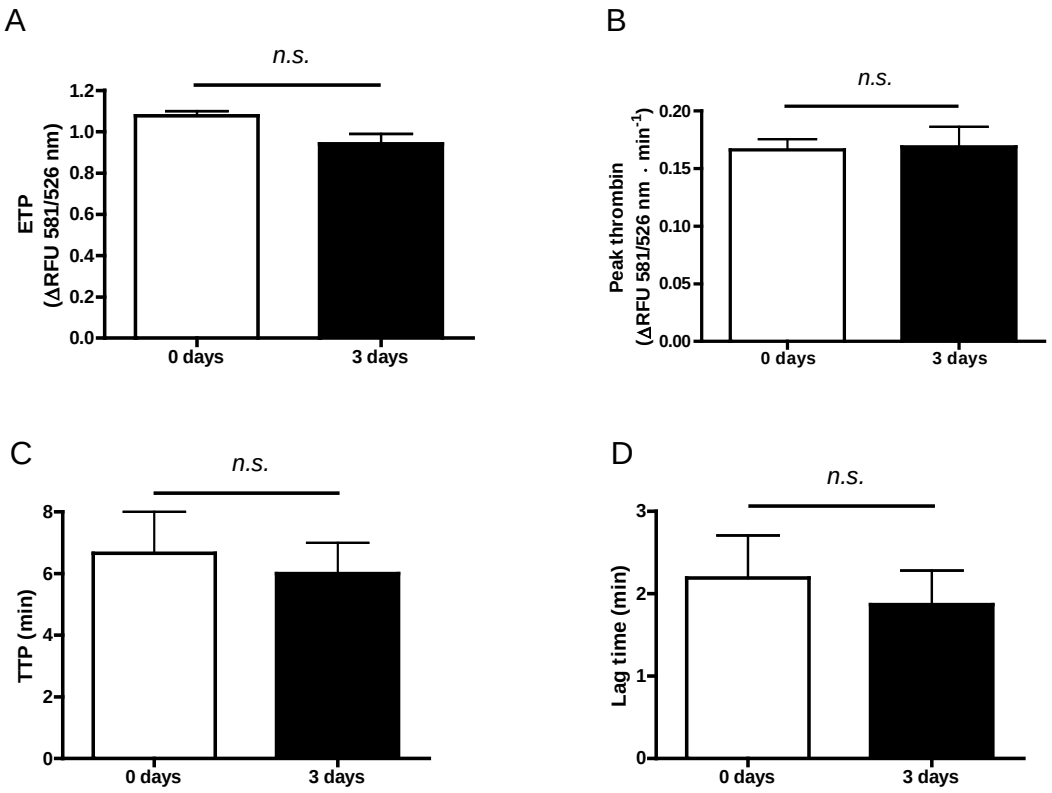

A
ETP
(ΔRFU 581/526 nm)
0.5
0.4
0.3
0.2
0.1
0.0
Ch 1 (0 µl)
Ch 2 (0.5 µl)
Ch 3 (1 µl)
Ch 4 (2 µl)
B
Peak thrombin
(ΔRFU 581/526 nm · min-1)
0.10
0.08
0.06
0.04
0.02
0.00
Ch 1 (0 µl)
Ch 2 (0.5 µl)
Ch 3 (1 µl)
Ch 4 (2 µl)
C
TTP (mIn)
15
12
9
6
3
0
Ch 1 (0 µl)
Ch 2 (0.5 µl)
Ch 3 (1 µl)
Ch 4 (2 µl)
D
Lag time (mIn)
10
5
0
Ch 1 (0 µl)
Ch 2 (0.5 µl)
Ch 3 (1 µl)
Ch 4 (2 µl)
Channel number (APTT reagent (µl))

Figure 5.5. Effect of 3-day storage on chip function.

The effect storing chips with predeposited reagents for 3 days at 4 °C on the (A) ETP, (B) peak thrombin, (C) TTP, and (D) lag time are shown. Mean ± SEM; N = 3-4.


A
n.s.
ETP
(ΔRFU 581/526 nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 days
3 days
B
n.s.
Peak thrombin
(ΔRFU 581/526 nm · min-1)
0.20
0.15
0.10
0.05
0.00
0 days
3 days
C
n.s.
TTP (min)
8
6
4
2
0
0 days
3 days
D
n.s.
Lag time (min)
3
2
1
0
0 days
3 days

5.4.7. Blood prothrombin levels correlate with ETP and thrombin peak using on-chip TGA

To investigate the effects of hypercoagulable blood on our chip-based TGA, we titrated prothrombin into blood samples prior to measuring thrombin generation (Figure 5.6). Increasing levels of prothrombin caused an increase in the ETP ($P < 0.01$) and thrombin peak ($P < 0.05$), but did not significantly change the TTP or lag time. Therefore, increased hypercoagulability caused by elevated prothrombin levels is reflected in TGAs performed on the chip.

5.4.8. Addition of rFVIII and rFIX to chips allows discrimination between FVIII- and FIX-deficient plasma

To demonstrate the utility of a multichannel TGA on a chip, we prepared channels with T13 and APTT reagent, along with rFVIII or rFIX, which were placed in different channels of the same chip. A third channel, prepared without any clotting factors, was used as a negative control. Thrombin was generated in recalcified, FVIII-deficient plasma only in channels containing rFVIII (Figure 5.7A, Table 5-3). Similarly, thrombin generation in FIX-deficient plasma could only be measured in channels containing rFIX (Figure 5.7B, Table 5-3). These results show that on-chip TGAs can be used to discriminate between FVIII- and FIX-deficient plasma, and illustrate how it may have practical, real-world applications.

Figure 5.6. Effect of prothrombin on thrombin generation parameters.

Prothrombin was added to whole blood from healthy donors. The effect of prothrombin on (A) ETP, (B) peak thrombin, (C) TTP, and (D) lag time is shown. Chips contained predeposited thrombin substrate and APTT reagent. Mean ± SEM; N = 3. * $P < 0.05$, ** $P < 0.01$.

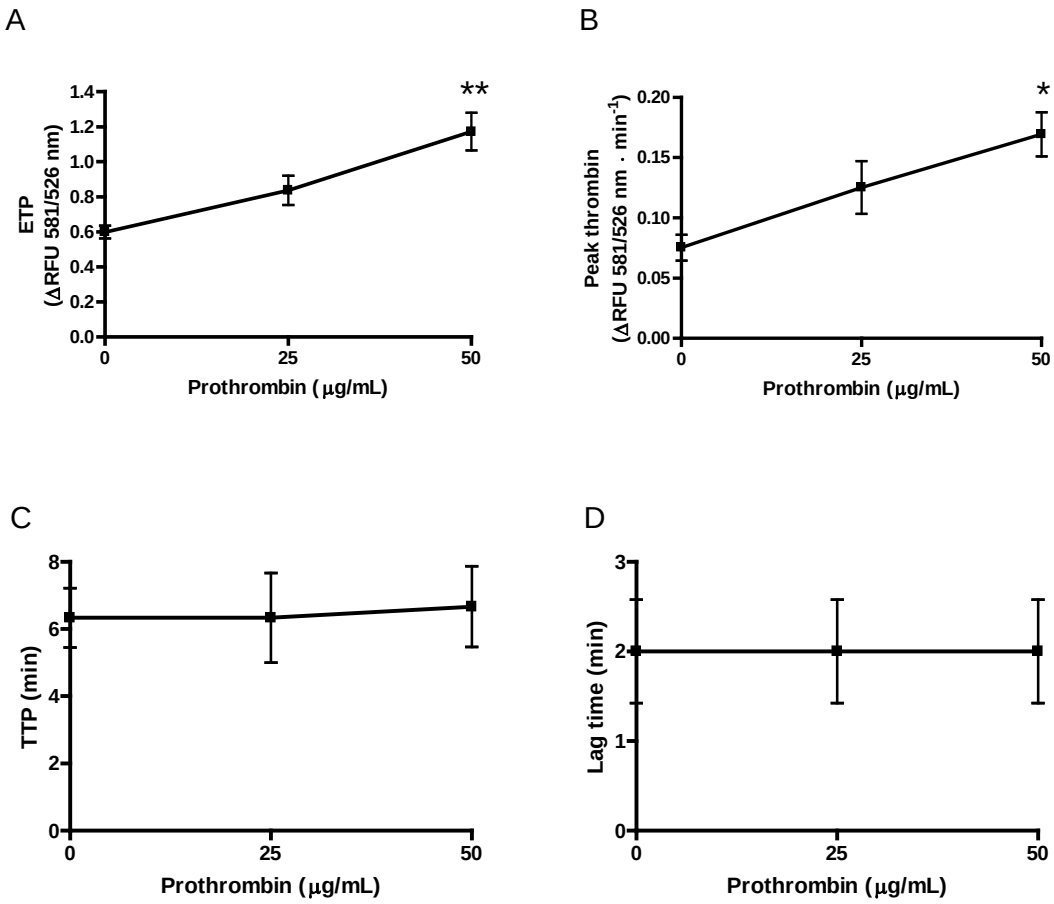
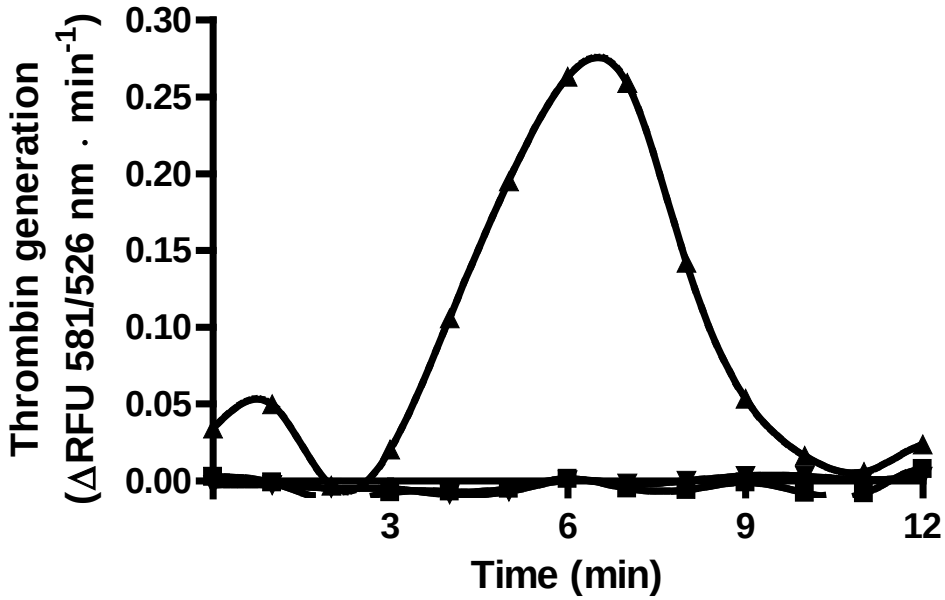

A
B
C
D
ETP
(ΔRFU 581/526 nm)
Peak thrombin
(ΔRFU 581/526 nm · min-1)
TTP (min)
Lag time (min)
Prothrombin (μg/mL)
**
*

Figure 5.7. Discrimination between FVIII- and FIX-deficient plasma.

Representative thrombin generation curves after addition of re-calcified (A) FVIII-deficient plasma or (B) FIX-deficient plasma to chips in which channels contained thrombin substrate along with (▲) rFVIII, (▼) rFIX, or (■) thrombin substrate, alone. N = 3.

A


Thrombin generation
(ΔRFU 581/526 nm · min-1)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
3
6
9
12
Time (min)

B

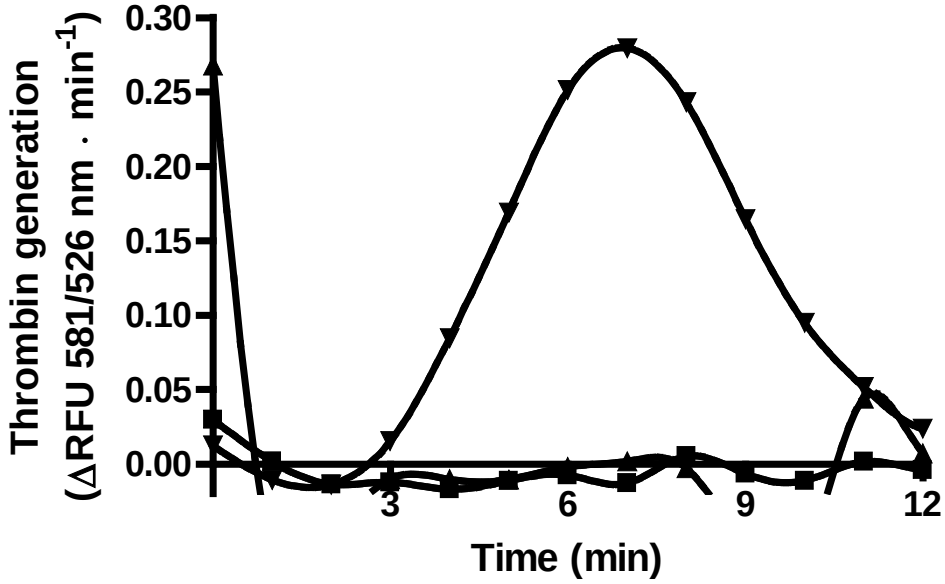

Thrombin generation
(ΔRFU 581/526 nm · min-1)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
3
6
9
12
Time (min)

Table 5-3. Thrombin generation assay parameters in channels containing predeposited rFVIII or rFIX.

Mean ± SEM; N = 3. * $P < 0.05$.

	FVIII-deficient plasma			FIX-deficient plasma		
TGA Parameter	**rFVIII**	**rFIX**	**Neg ctrl**	**rFVIII**	**rFIX**	**Neg ctrl**
ETP (Δ 581/526 nm)	1.2 ± 0.18 *	-	-	-	1.4 ± 0.01 *	-
Peak thrombin (Δ 581/526 nm · min^{-1})	0.28 ± 0.04 *	-	-	-	0.24 ± 0.02 *	-
TTP (min)	7.0 ± 0.58 *	> 12	> 12	> 12	8.3 ± 1.3 *	> 12
Lag time (min)	2.5 ± 1.2 *	> 12	> 12	> 12	2.8 ± 0.38 *	> 12

4.5. Discussion

A point-of-care device that performs multiple TGAs simultaneously and under varying conditions would be a valuable tool for achieving simple and comprehensive assessment of coagulation. Here, we report a proof of concept for a novel chip that permits the evaluation of multiple whole blood TGAs in parallel, independent channels containing predeposited reagents. The chip can be fabricated using low-cost materials and methods. Blood is drawn into the chip and passively diverted into four branching channels through capillary action – without the use of external pumps – which facilitates ease of use. To demonstrate the utility of performing multiple TGAs on this device, we have used it to differentiate between FVIII- and FIX-deficient plasma samples.

The ability to monitor thrombin generation in whole blood permits point-of-care testing and evaluation in a more physiological milieu than plasma. Thuerlemann et al. developed an amperometric biosensor in the form of an individual test strip, which can monitor thrombin generation in whole blood (Thuerlemann et al., 2009). Ninivaggi and colleagues also described a method for detecting thrombin generation in 50% diluted whole blood, in which samples were deposited atop a porous matrix and detected using a rhodamine-110-based thrombin substrate (Ninivaggi et al., 2012). One novel feature of our design is the ability to divert small volumes of undiluted blood into four or more assay channels, which permits evaluation of thrombin generation under multiple sets of conditions with only a single application of sample. The use of a large polypeptide as a fluorogenic substrate allows for an inexpensive method to achieve this and does not require correction for α_2-macroglobulin, which is necessary for TGAs that use small,

synthetic substrates (Baglin, 2005; van Veen et al., 2008b). The channel was specifically fabricated to be compatible with conventional microplate fluorometers, but the design can be modified for other instruments.

Assessing thrombin generation under a variety of experimental conditions can yield additional information about the coagulation cascade than can be achieved by using exogenous TF alone. For example, while TGAs initiated with TF are sensitive to variations in tissue factor pathway inhibitor (TFPI), antithrombin III (AT), and prothrombin levels (van't Veer and Mann, 1997; Hockin et al., 2002; Allen et al., 2004), certain hypercoaglable states are better differentiated when exogenous thrombomodulin (TM) or activated protein C (APC) are also present in the assay mixture (Hezard et al., 2006; Dargaud et al., 2006). TGAs can also be modified to be highly sensitive to different components of the coagulation cascade. Initiation with contact pathway activator or high concentrations of TF can be used to assess the contact or extrinsic pathways, respectively (Al Dieri et al., 2002; van Veen et al., 2008b; Thuerlemann et al., 2009). Initiation with activated FIX (FIXa) has been used to measure differences in FVIII levels below 1% (Ninivaggi et al., 2011).

The ability to perform TGAs in non-anticoagulated capillary blood eliminates the effects of divalent cation chelating anticoagulants and thus permits assessment under more physiologically relevant conditions. However, this likely also introduces added variability due to time-sensitive effects of extravascular TF, which may interfere with results if blood is not transferred from the fingertip to the chip quickly enough (unpublished data). The inter-assay CVs were slightly greater than those in other whole

blood thrombin generation assays, which used citrated blood or plasma – although, these other studies also used diluted samples (Thuerlemann et al., 2009; Ninivaggi et al., 2012). Regardless, we found that intra-individual CVs were still generally less than the observed inter-individual variations, and significant differences were nonetheless observed among our small group of healthy donors. In addition, donors were tested multiple times across several days, and the results show that there was limited variation despite potential day-to-day donor differences.

The effects of inter-assay variation may be mitigated by performing repeated measurements – which is simplified by the low invasiveness of the blood-drawing procedure and low cost of running each test – and averaging the results. The use of negative control channels may help identify and control for the effects of extravascular or blood-borne TF. In addition, all test results were included in this study, and median CVs were generally lower than the means (not shown). Thus, the intra-chip and intra-individual variation may be further reduced by excluding tests in which there was improper channel filling or unacceptable variation between replicate channels. Correlating channel filling and substrate dispersion with thrombin-mediated cleavage may reveal how channel filing contributes to variation. Finally, fabrication of channels using more precise methods than those described here would likely aid in reducing intra- and inter-chip variation.

Elevated prothrombin levels are a risk factor for VTE (Poort et al., 1996). We showed that this assay was sensitive to increases in prothrombin at levels which are within the normal range of prothrombin variation in healthy individuals (Butenas et al.,

1999). The resulting increase in the ETP and thrombin peak is characteristic of the effect of prothrombin on thrombin generation curves, in plasma, initiated by TF (Butenas et al., 1999; Allen et al., 2004; Machlus et al., 2009). Thus, we also demonstrate similar observations in whole blood initiate with APTT reagent. These results, along with our observation of significant variations in thrombin generation parameters between apparently healthy individuals, suggest that hypercoagulability due to pathologically altered levels of coagulation factors may be discernable using this chip.

Short-term (three day) storage of chips containing predeposited reagents at 4 °C was also possible without significant change in thrombin generation parameters. Investigation of more lengthy storage times and different storage conditions are also of interest, and will be the subject of future studies. The ability to prepare assay chips off-site and store them for later use would facilitate high-throughput, bedside testing by non-laboratory-trained personnel.

Finally, as a proof of concept for demonstrating the utility of a multichannel chip for performing TGAs, we showed the ability to differentiate between FVIII- and FIX-deficient plasma samples. This remains to be tested using whole blood from individuals with haemophilia. Such a test may be useful as a low-cost approach to screening for or diagnosing haemophilia in developing countries or areas without access to healthcare facilities for persons with haemophilia.

In this study, chips were optimized for initiation of thrombin generation through the contact pathway of coagulation using predeposited APTT reagent. Although we have found that relipidated TF can mediate thrombin generation in other iterations of the chip

(unpublished data), it is unclear how the presence of TF affects thrombin generation in the current channel design. Thuerlemann and colleagues showed that TF was capable of triggering thrombin generation in whole blood after being dried onto the surface of an amperometric test strip (Thuerlemann et al., 2009). Other clotting activators, including snake venom proteases and activated clotting factors, as well as inhibitors, such as APC, also remain to be tested, but their use in TGAs may be useful. Indeed, initiating thrombin generation with FIXa permits sensitive detection of plasma FVIII levels (Ninivaggi et al., 2011), while differentiation between the prothrombin G20210A mutation, factor V Leiden and protein S deficiency is made possible by comparing TGA parameters in the presence and absence of APC (Hezard et al., 2006).

Haemostasis and thrombosis are variably regulated across different vascular beds, in response to different injuries, and across different pathophysiological states (Ishii et al., 1986; Eilertsen and Osterud, 2004; Kretz et al., 2010; Vaezzadeh et al., 2014; Aird, 2015). Thus, the individual constituents of the coagulation cascade differentially influence thrombin formation according to these and other factors. Initiation of thrombin generation under a single set of experimental conditions does not account for this complexity and heterogeneity. Novel methods are needed to facilitate more comprehensive assessment of coagulation. Here, we 1) report a novel chip-based device that facilitates simultaneous evaluation of multiple TGAs that can be initiated under varying conditions, and 2) as a proof of concept, demonstrate its use by differentiating between FVIII- and FIX-deficient plasma. This provides a new tool that may allow for novel approaches and applications for monitoring thrombin generation in the future.

Chapter 6: Discussion

6.1. Part 1: General discussion

Investigation of pathophysiological mechanisms in thrombosis and haemostasis rely on the availability of established models and methods. The works described in this thesis introduce new tools designed to explore concepts in this field. Chapter 3 characterizes a novel murine arterial bleeding model. We showed that compared with the coagulation cascade, the effects of platelets in haemostasis are differentially pronounced depending on the vascular bed, and that mouse models incorporating arterial bleeding may be more sensitive to platelet defects than those that exclusively evaluate venous bleeding. Chapter 4 introduces a MP capture assay that uses physiological ligands to isolate a distinct MP population from citrated plasma. We showed that procoagulant, P-selectin binding MPs – and also total MP TF activity – were not predictive of recurrence in individuals with unprovoked VTE. Finally, Chapter 5 describes a novel multichannel chip for performing capillary blood TGAs under multiple initiating conditions at the point of care. We characterized the chip and demonstrated as a proof of concept that a multichannel chip-based assay can differentiate between FVIII- and FIX-deficiency.

6.2. Part 2: New insights in haemostasis and thrombosis

6.2.1. Platelet aggregation and thrombin formation differ in the artery and vein

Platelet thrombi are differentially formed and regulated under venous and arterial shear. In flow experiments containing immobilized, lipidated TF and collagen, platelets adhered evenly over the thrombogenic surface, but ultimately concentrated at the trailing

edge of the clot (Colace et al., 2012). Under arterial shear forces, platelets are forced downstream, towards the leading edge of the clot. Likewise, platelet thrombi are observed downstream of the fibrin clot in an arteriole thrombosis model (Falati et al., 2002). GPIb and vWF are important for platelet adhesion at high shear. In a saphenous vein bleeding model, images showed that GPIb-deficient mice failed to form thrombi (or platelet plugs) at the injury site, but instead adhered next to it, demonstrating the importance of this receptor in localizing the platelet plug even under low shear.

In Chapter 3, we showed that high doses of $\alpha_{IIb}\beta_3$ inhibitor prolonged saphenous vein bleeding after transection. Likewise, platelet accumulation in the injured saphenous vein was extremely low in both GPIb-deficient and platelet talin knockout mice, and bleeding was significantly prolonged after a single injury (Getz et al., 2015). Further, β3-integrin knockout mice also had severe bleeding after laceration of the saphenous vein (Broze, Jr. et al., 2001). Thus, platelet plug formation is vital for venous haemostasis even after mild injury. However, compared with arterial haemostasis, we showed that only limited $\alpha_{IIb}\beta_3$-mediated aggregation is needed in the vein; even when a more severe injury is induced. This is supported by findings that FIX-deficient mice and mice expressing low levels of human tissue factor (TF^{low}), which had reduced, but not abolished platelet accumulation, had normal venous bleeding times after a single laser injury, and only moderately prolonged bleeding after repeated injuries (Getz et al., 2015). In addition, platelets may be needed for fibrin formation at high, but not low shear, as they can protect against the dilutional effects of arterial flow (Weiss et al., 1986; Fogelson and Neeves, 2015).

Thrombin promotes haemostasis by forming fibrin, as well as through the activation of platelets and endothelial cells. Fibrin, in turn, promotes haemostasis by forming a barrier between blood and the injured vessel wall and by stabilizing the platelet plug, while platelets and endothelial cells can provide a catalytic surface for the production of additional thrombin. Thus, thrombin inhibition can promote bleeding in different ways. However, the relative contribution of these mechanisms may not necessarily be the same within arteries and veins.

Fibrin was required for stabilizing adherent platelet aggregates under arterial shear rates in a pressure-release system, whereas under venous shear rates, platelet aggregates grew larger in the absence of fibrin (Colace et al., 2012). In contrast, immobilized TF greatly promoted platelet accumulation in the presence of both arterial and venous shear, although, this effect was also more pronounced at arterial shear rates. These results suggest that under venous shear, thrombin-mediated platelet activation may contribute more than fibrin to the formation of a stable platelet plug, while under arterial shear, both platelet activation and reinforcement by fibrin play major roles.

These results also show that there is greater demand for thrombin generation in the high-shear environment of the artery during haemostasis. This was demonstrated by the different bleeding times after similar transection injuries made to the saphenous artery and vein in heparin-treated mice (Vaezzadeh et al., 2014) (Chapter 3). However, a slight extension of the vein injury caused heparin to have a similar dose response in both vessel types, which was not observed when mice were treated with $\alpha_{IIb}\beta_3$ inhibitor. Thus, while there is a greater demand for both thrombin and $\alpha_{IIb}\beta_3$ function within the high-pressure

arterial system, there is less of a discrepancy in the need for thrombin activity. A partial explanation for this disparity may be that fibrin and platelet plug formation are mutually dependent at high shear rates, while at low shear, fibrin formation is largely independent of platelets, whereas platelet plug formation depends on thrombin amplification (Chapter 3).

6.2.2. MPs and thrombin generation are potential global coagulation markers of VTE

MPs are attractive candidates as biological markers of thrombotic disease because their levels in circulation may be diagnostic or prognostic of certain thrombotic pathologies (Rautou and Mackman, 2013). TF-expressing MPs for example, are predictive of VTE in pancreatic and potentially other forms of cancer (Khorana et al., 2008; Tesselaar et al., 2009; van Doormaal et al., 2012; Zwicker et al., 2013). A model of MP-mediated thrombus formation postulates that MPs dock onto P-selectin-expressing platelets or endothelial cells via P-selectin ligands, such as PSGL-1 (Falati et al., 2003; Thomas et al., 2009; Owens and Mackman, 2011). In Chapter 4, we developed a MP capture assay that allowed us to assess this model, clinically. Our study is the first to evaluate these MPs in patients, and demonstrate that P-selectin-binding, procoagulant MPs are not associated with future recurrent events in patients with prior unprovoked VTE. We also found that MP TF activity was not predictive of recurrent VTE in these patients. Taken together with previous findings (Ay et al., 2009; Zwicker et al., 2009; Thaler et al., 2014), there is no evidence that MP TF activity is associated with unprovoked VTE either prior to or after recurrent events, or during the acute phase of a first unprovoked thrombotic event.

Several studies have found that thrombin generation parameters are predictive of VTE recurrence (Hron et al., 2006; Eichinger et al., 2008; Tripodi et al., 2008; Besser et al., 2008; Eichinger et al., 2010), while others found that increased thrombin generation only predicted risk of first thrombotic events, but not recurrence (van Hylckama et al., 2007; van Hylckama et al., 2015). Sample collection and follow-up times varied within and between studies, which may explain some of the disagreement between results. Additionally, in most studies, a minimum of 7 pM TF was used to initiate thrombin generation, which has been shown to have reduced sensitivity to contact pathway-mediated amplification of thrombin generation (van Veen et al., 2008a). Interestingly, studies in which 1, 5, or 7.16 pM TF was used to initiate thrombin generation found a relationship between TGA parameters and risk of recurrent VTE (Tripodi et al., 2008; Besser et al., 2008; Eichinger et al., 2010), whereas two studies in which ≥ 15 pM TF was used only reported association between thrombin generation and first, but not recurrent thromboembolic events (van, V et al., 2007; van, V et al., 2015b) (Table 6-1). Although, one study found an association between recurrence and thrombin generation initiated with 71.6 pM TF. Nonetheless, this hints at the possibility that the pathophysiological mechanisms of thrombin generation may differ during first and recurrent thrombotic events. Specifically, initial VTE could be driven by factors that are that are discernable through extrinsic pathway activation, such as prothrombin or AT levels, while recurrent VTE may involve procoagulant alterations in the contact pathway.

In Chapter 5, we have developed a multichannel chip that may facilitate simultaneous assessment of intrinsic and extrinsic regulation of thrombin generation. We

Table 6-1. Predictive value of TGAs in first or recurrent VTE.

Atherosclerosis Risk in Communities (ARIC), Cardiovascular Health Study (CHS), Vienna Cancer and Thrombosis Study (CATS), Leiden Thrombophilia Study (LETS).

	Predictive of first VTE	Predictive of recurrent VTE	[TF] (pM)	Study population
(Lutsey et al., 2009)	✓		71.6	ARIC, CHS
(Ay et al., 2011)	✓		71.6	CATS
(van Hylckama et al., 2007)	✓	x	17.9	Cancer ecluded, VT history excluded
(van Hylckama et al., 2015)	✓	x	15	LETS, healthy controls
(Hron et al., 2006)		✓	71.6	unprovoked
(Tripodi, et al., 2008)		✓	1	unprovoked
(Besser et al., 2008)		✓	5	spontaneous
(Eichinger et al., 2008)		✓	7.16	unprovoked
(Eichinger et al., 2010)		✓	?	spontaneous

demonstrated that contact pathway-initiated TGA, which is sensitive to increased prothrombin levels can be performed on premade chips at the point of care. This may facilitate the use of TGAs in clinical research by allowing it to be incorporated in the clinical environment as a point of care test. Qualitative tests such as haemophilia diagnosis could be used to initially validate this idea.

6.2.3. General findings in haemostasis research

Our studies in Chapter 3 demonstrate that haemostasis is differentially regulated in arterial and venous beds, and highlights the fact that a complete explanatory model of haemostasis must incorporate this notion. In addition to this, other studies have raised several important questions regarding the generally accepted paradigm of the cell-based model of haemostasis (Neyman et al., 2008; Ivanciu et al., 2014; Getz et al., 2015).

The cell-based model proposes that the activated platelet membrane is the main site of thrombin amplification and propagation during *in vivo* coagulation (Hoffman and Monroe, 2001). According to this model, low levels of FIXa and thrombin generated on perivascular, TF-bearing cells activate clotting factors that are bound to high-affinity binding sites on platelets. As a result, prothrombinase, and consequently thrombin, are principally formed on the platelet surface during thrombin amplification and propagation. The model is based on observations that activated platelets support coagulation *ex vivo* (Hoffman et al., 1995; Baglia and Walsh, 1998; Oliver et al., 1999), and that platelets possess putative receptors for a number of clotting factors including FV (Nesheim et al., 1993), FVIII (Gilbert et al., 2015), FIX (Ahmad et al., 1989), FX (Scandura et al., 1996) and FXI (Ho et al., 2000), which makes them well-suited to support thrombin formation

in vivo. The cell-based model is widely regarded as a useful explanation of physiological haemostasis, and has been used to explain the bleeding diathesis in haemophilia patients, who develop unstable haemostatic clots despite normal extrinsic pathway function. However, aspects of the model have been challenged by a number of recent findings.

First, fibrin localizes to sites that contain TF, rather than within the platelet plug. In flow experiments, fibrin accumulated on immobilized lipidated TF and collagen-rich surfaces, while platelet aggregates developed over top of the fibrin clot (Colace et al., 2012). Moreover, many regions contained fibrin without subsequent platelet deposition. *In vivo* arteriole laser injury models show that fibrin is deposited on endothelial cells, independent of platelet adhesion, or is mostly located at the interface of the platelet plug and vessel wall where it localizes with TF (Falati et al., 2002; Ivanciu et al., 2014). In a saphenous vein laser ablation bleeding model, fibrin appears to form a halo around the platelet plug (Getz et al., 2015), suggesting that fibrin is functioning to stabilize the platelet plug by anchoring it to the periphery of the injury site, or forming a tight seal at the platelet-vessel wall interface, rather than sealing off the entire injury site. The difference between fibrin and platelet localization in the saphenous vein and cremaster arteriole can be attributed to the different shear rates, which have been shown to influence platelet and fibrin deposition (Colace et al., 2012), as well as to the injury type.

Second, thrombin generation can occur on other activated cells besides platelets and is rarely observed within the platelet plug. In an arteriole laser injury model, fluorescently-labeled, injected FVa and FXa were found on the endothelial surface at sites that are both upstream and downstream of the platelet plug, suggesting that

prothrombinase can functions at these sites (Ivanciu et al., 2014). In addition, endothelial cells can support fibrin formation in the absence of platelets (Atkinson et al., 2010). Further, RBCs enhance thrombin formation when added to PRP (Peyrou et al., 1999; Horne et al., 2006; Whelihan et al., 2012). Although only a small fraction of RBCs express PS, they outnumber platelets approximately 10:1, making them a sizeable contributor of PS membrane in blood; particularly within the extravascular space. In addition, our lab has observed that the fluorescent thrombin substrate, T13, is cleaved at the platelet-vessel wall interface in a laser injury model (unpublished data), suggesting that only cells at this site contribute to thrombin formation.

Third, thrombin amplification may precede platelet activation and aggregation in some models. Platelet activation in the arteriole laser injury model relies on thrombin, rather than collagen (Dubois et al., 2007). Haemophilia mice fail to generate a platelet thrombus in this model, suggesting that platelet activation and aggregation are dependent on thrombin amplification through intrinsic tenase (Neyman et al., 2008). The infusion of human FVIII largely corrects fibrin deposition, but not platelet accumulation, which implies that a higher concentration of thrombin is required to generate a platelet thrombus than to make a fibrin clot.

The involvement of thrombin amplification in forming a platelet plug during haemostasis is suggested by the observation that FIX-deficient mice have a smaller platelet plug after hemorrhagic injury than TF^{low} mice, which have normal amplification pathways, but compromised initiation (Getz et al., 2015). Interestingly, FIX-deficient mice also showed 2-3-fold greater fibrin deposition compared to wild type mice after

repeated injuries, while TF^{low} mice had significantly less fibrin. Although this is not inconsistent with the notion that compromised clot structure impairs haemostasis in haemophilia, an alternative explanation is that FIX-mediated thrombin amplification is not necessarily required for fibrin clot formation, but important in forming a stable platelet plug, which is needed for haemostasis in this model. Additionally, the extrinsic pathway seems to provide sufficient levels of thrombin for fibrin formation in this bleeding model, although the quality of clot structure is uncertain and is possibly a contributing factor to bleeding in haemophilia mice following clot disruption. Likewise, fibrin formation occurs maximally during the initial phases of thrombin generation *in vitro* (Brummel et al., 2002).

In the saphenous laser injury bleeding model, platelet talin conditional knockout and IL4R/GPIb transgenic mice had reduced platelet accumulation compared to wild type mice but had substantially more fibrin formation (Getz et al., 2015), further demonstrating an inverse relationship between platelet accumulation and fibrin formation in mice with a normal extrinsic pathway. Most of these mice bled until the predefined endpoint of 900 s, whereas wild type mice achieved haemostasis after approximately 10 s. This is consistent with our findings in which high-dose $\alpha_{IIb}\beta_3$ inhibitor caused maximal bleeding after puncturing the vein (Vaezzadeh et al., 2014) (Chapter 3). Thus, impaired platelet function is strongly correlated with bleeding, and inversely related to fibrin accumulation in the vein.

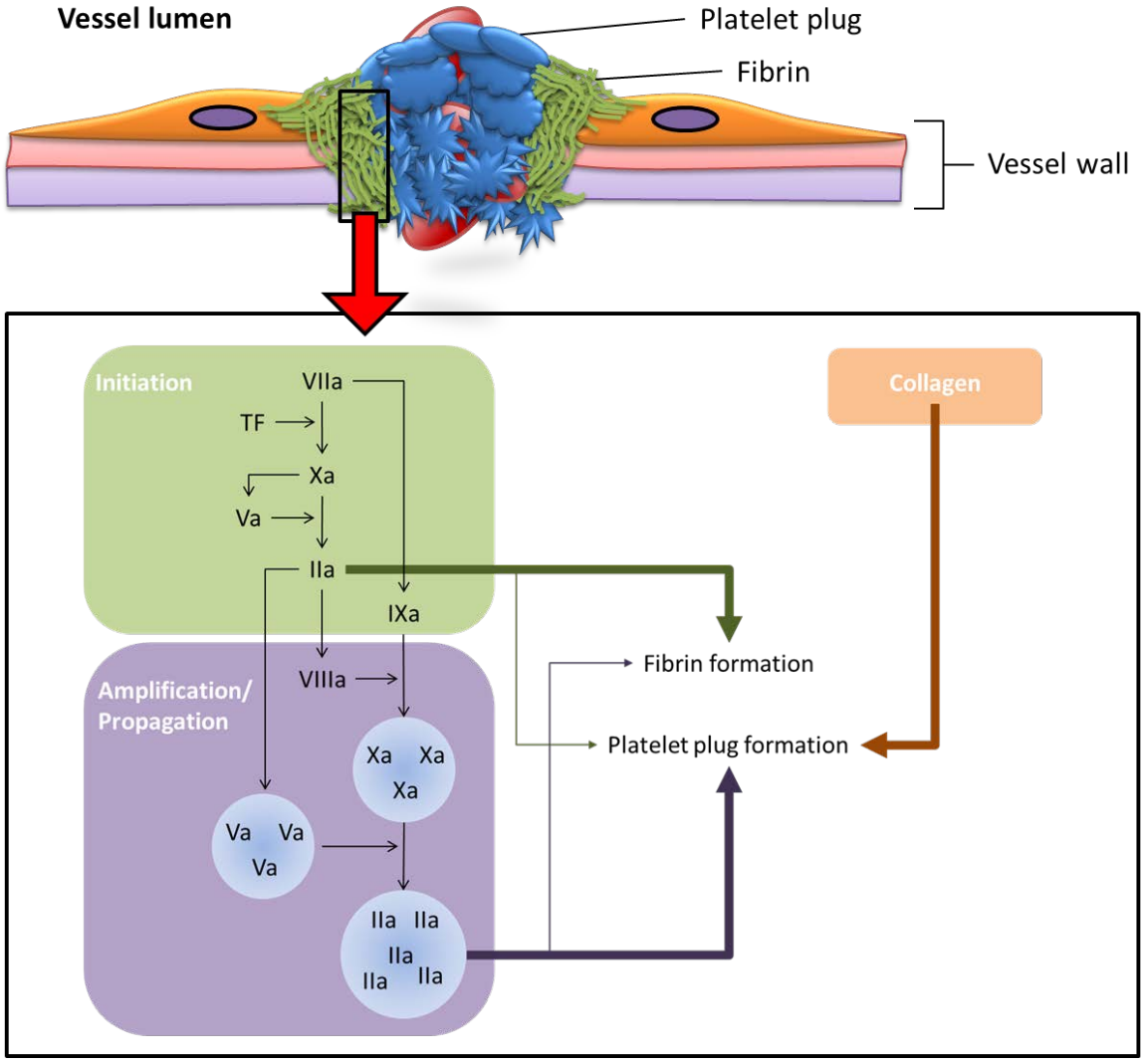
These results provide evidence to postulate a model of haemostasis in which the principal role of thrombin amplification is to mediate the formation of a haemostatic

platelet plug, which is stabilized by a layer of fibrin at the platelet-vessel wall interface (Figure 6.1). An alternative view is that these thrombin-activated platelets in turn alter the structure of the fibrin clot, thus making it better capable of achieving haemostasis. This could be accomplished through promoting thrombin formation as suggested by the original cell-based model, or through a different mechanism, such as protecting against the diluting effects of blood flow (Stalker et al., 2014; Fogelson and Neeves, 2015).

The revised model, in which thrombin amplification is required for platelet plug formation, is consistent with findings showing that FVa corrects platelet thrombus formation in haemophilia mice in an arteriole thrombosis model (Ivanciu et al., 2014). This may be explained by an early burst of thrombin generation caused by the presence of FVa. Since FXa is formed before thrombin-mediated activation of FV, it is reasonable to assume that FVa is rate-limiting during the initiation stage of thrombin generation and that its presence could result in thrombin levels required to induce platelet thrombus formation without FIX. Moreover, haemophilia C is generally associated with a mild bleeding phenotype, unlike severe haemophilia A and B, which cause severe bleeding. This may be explained by observations that FXI enhanced thrombin generation in a FVIIa-initiated system, while the absence of FXI did not affect platelet activation (Oliver et al., 1999). This suggests a potential role for platelets in maintaining haemostasis in haemophilia C, but an impeded role in haemophilia A or B, which show partial inhibition of platelet accumulation (Neyman et al., 2008; Ivanciu et al., 2014). Thrombin-mediated platelet activation in haemostasis can also potentially explain the role of rFVIIa as a haemostatic agent in haemophilia. rFVIIa is thought to function by initiating

Figure 6.1. A revised model of haemostasis.

Evidence from recent studies suggest that thrombin and fibrin are formed at the margins of the platelet plug, where cells at the platelet-vessel wall interface, including endothelial cells (and/or extravascular cells) serve as the primary site of thrombin amplification and propagation. The resulting thrombin burst leads to maximal platelet response, which promotes growth of the platelet plug.


Vessel lumen
Platelet plug
Fibrin
Vessel wall
Initiation
VIIa
TF
Xa
Va
IIa
IXa
VIIIa
Amplification/
Propagation
Xa Xa
Xa
Va Va
Va
IIa IIa
IIa
IIa IIa
Collagen
Fibrin formation
Platelet plug formation

thrombin formation on the platelet surface (Augustsson and Persson, 2014). This could also lead to enhanced platelet activation. This may also explain the ability of rFVIIa to promote haemostasis in some patients with platelet defects who have normal coagulation (Franchini, 2009).

6.2.4. General findings in thrombosis research

Virchow's triad explains venous thrombosis as a failure of homeostatic regulation of the haemostatic system caused by one or more prothrombotic triggers; venous stasis, vessel injury or hypercoagulability (Bagot and Arya, 2008). This highlights the heterogeneity of prothrombotic triggers and mechanisms in VTE. In Chapter 3, we showed that haemostasis can be differentially influenced by thrombin and platelet inhibitors according to vascular conditions (Vaezzadeh et al., 2014). The influence of physiologic milieu is also evident when comparing the efficacy of different treatments in arterial versus venous thrombosis (Franchini and Mannucci, 2008; Castellucci et al., 2013). Further, dabigatran showed improved efficacy compared to warfarin in patients with non-valvular atrial fibrillation (Connolly et al., 2009), but was associated with higher rates of thromboembolic events and bleeding in patients with mechanical heart valves (Eikelboom et al., 2013). Applying this concept to dysregulation of haemostasis in the venous system in light of Virchow's triad would imply that VTE may be variably influenced by different treatments according to the provoking conditions.

Different animal models of thrombosis induce clot formation using various triggers, and this heterogeneity may be reflective of how thrombotic coagulopathies present in patients. Various mechanisms exist depending on how and where thrombosis is

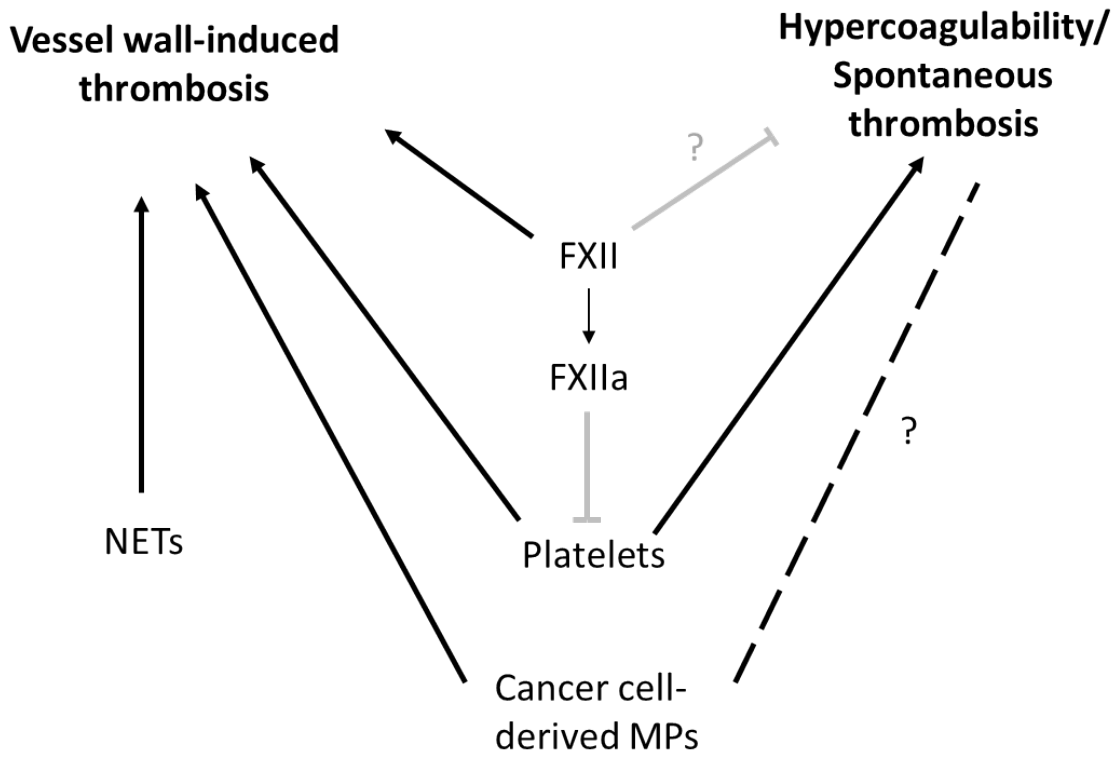
induced. Neutrophils provoke thrombosis in an IVC stenosis model (von Bruhl et al., 2012), but are not influential factors in a spontaneous thrombosis model, in which AT and protein C are systemically inhibited (Heestermans et al., 2016). In addition, FXII promotes thrombosis in models that provoke clot formation by manipulating the vessel wall (ferric chloride, mechanical injury, IVC stenosis, Rose Bengal) (Renne et al., 2005; Cheng et al., 2010; von Bruhl et al., 2012), but has a protective effect in a spontaneous thrombosis model in hypercoagulable mice (Heestermans et al., 2016). FXIIa has been implicated in the progression of immunothrombosis (von Bruhl et al., 2012), but can also inhibit thrombin-mediated platelet activation (Bradford et al., 2000), providing potential mechanisms for its dual effects in these different models (Figure 6.2). Thus, targeting FXII may be useful in mitigating thrombosis under certain conditions, but may potentiate it under others.

6.2.5. Future directions in haemostasis and thrombosis research

The studies described above highlight the need for a revised view of haemostasis and raise several important questions concerning thrombosis and haemostasis. The methods described in this thesis can be used to potentially address several of these questions: Does thrombin initiation or amplification affect platelets or fibrin clots differently in arteries compared to veins (Chapter 3)? If the activated endothelial cell membrane is an important mediator of thrombin generation, are elevated levels of endothelial MPs indicative of a thrombotic state (Chapter 4)? Do the intrinsic and extrinsic pathways cause thrombosis or protect against bleeding through varying effects on platelets and fibrin, and can these be differentiated using a modified TGA (Chapter 5)?

Figure 6.2. The influence of various factors in thrombosis models.

FXII, platelets, cancer cell-derived MPs, and NETs have all been implicated as thrombotic mediators using models that induce thrombosis through vessel wall manipulation (i.e. $FeCl_3$, mechanical injury, IVC stenosis, Rose Bengal). Platelets also promoted spontaneous thrombosis using a model in which AT and protein C were knocked down with small interfering RNA. In contrast, neutrophils did not affect thrombosis in this model, and FXII demonstrated a protective effect. The influence of MPs in this model has not been evaluated. The mechanism through which FXII reduces thrombosis in AT- and protein C-knockdown mice is not known and could not be attributed to altered fibrin structure/fibrinolysis (Heestermans et al., 2016). An inhibitory effect of FXIIa on platelets (Bradford et al., 2000) could be a potential explanation.


Vessel wall-induced thrombosis
Hypercoagulability/ Spontaneous thrombosis
?
FXII
FXIIa
?
NETs
Platelets
Cancer cell-derived MPs

In addition, elucidation of the mechanisms of thrombosis will be important in understanding which effectors can be targeted by potential new antithrombotics, and under what types of prothrombotic conditions. Subsequently, improved classification of these prothrombotic conditions using global coagulation markers, such as MPs (Chapter 4) and thrombin generation assays, initiated using TF as well as other triggers (Chapter 5), could be important in guiding treatment strategies in the future. The following sections of the discussion expand on these and other key issues, and highlight how our newly developed methods can be used to address hypotheses invoked by our own findings and the recent findings of other investigators.

6.3. Part 3. A murine arterial bleeding model, selectin-dependent MP capture assay, and whole blood TGA on a chip are new tools for investigating thrombosis and haemostasis

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6.3.1. A murine arterial bleeding model enables comparison of haemostasis in different vascular beds

The mechanisms through which platelets and thrombin regulate haemostasis in veins or arteries are not clear. Evaluation of venous versus arterial bleeding using haemophilia and TF^{low} mice would help clarify the influence of thrombin initiation and amplification pathways on platelet plug formation. Adapting the model to evaluate bleeding by laser-induced injury and intravital microscopy as previously described (Getz et al., 2015) could help delineate between the effects on platelets and fibrin formation. In addition, reciprocal bone marrow transplant studies between wild type and TMEM16F-

deficient mice, which have impaired PS externalization (Yang et al., 2012), would help determine the roles of haematopoietic and endothelial cell membranes in haemostasis in these different vascular beds.

$\alpha_{IIb}\beta_3$ affects several aspects of platelet function, but its main role is in aggregation. Platelets possess additional mechanisms to facilitate aggregation. The effects of inhibiting other mediators of platelet adhesion and aggregation would provide additional insights into how platelets variably regulate haemostasis in different vascular beds. Further, the importance of different platelet signaling pathways should be examined in venous and arterial haemostasis.

Thrombin promotes haemostasis by forming a dense fibrin mesh and by activating platelets. The relative contribution of clot structure and thrombin-mediated platelet activation in inducing haemostasis in veins or arteries is uncertain. To address this, future studies could investigate the differential effects of FXIII, clot lysis and fibrinogen on arterial and venous bleeding. Evaluating the effects of murine PAR3 and PAR4 on bleeding in the different vascular beds could also help to address this question.

Elucidating the molecular mechanisms that regulate venous and arterial bleeding could have important implications on the development of drugs that can prevent thrombosis without causing life-threatening bleeding. The dose response of UFH, which promotes AT-mediated inhibition of serine proteases was similar in our models of arterial and venous bleeding (Chapter 3). Within the developing clot, Gla domain-expressing coagulation factors, such as FXa have affinity towards activated cell membranes, and may therefore be more protected from the dilutional effects of flow than soluble enzymes.

Thus, it would be interesting to compare the effects of varying doses of direct thrombin and FXa inhibitors on bleeding in arteries and veins. Also, the effects of haemostatic agents on mitigating bleeding caused by platelet and coagulation impairments could be modeled in both the artery and vein. This could have clinical relevance, since the haemostatic agent, rFVIIa is used to treat patients with both platelet and coagulation defects, and these patients exhibit different bleeding diatheses (George et al., 1990; Bolton-Maggs and Pasi, 2003; Mulder and Llinas, 2004).

Finally, conduit arteries contain a thick layer of vascular smooth muscle cells in the vessel wall, which regulates constriction and dilation of the artery. The femoral artery is a superficial artery and can therefore be clearly visualized and potentially video recorded under a surgical microscope. In this way, vessel diameter during the course of bleeding can potentially be monitored. It may therefore be possible to evaluate the effects of vasospasms on haemostasis and therefore better evaluate the differential influence of receptors common to haematopoietic cells and vascular smooth muscle cells on haemostasis.

The effects of vascular tone on haemostasis in the artery and vein could be evaluated using mice deficient in various NO synthases. In addition, $P2Y_{12}$ receptors are richly expressed on vascular smooth muscle cells and can regulate vasoconstriction (Wihlborg et al., 2004). One study compared the effects of different $P2Y_{12}$ inhibitors on ADP-induced vasoconstriction. The authors showed that ticagrelor, but not clopidogrel or prasugrel inhibited ADP-induced constriction in rat VSMCs (Grzesk et al., 2012). The

effects of pharmacologic $P2Y_{12}$ inhibitors on vessel constriction during haemostasis in a murine bleeding model have not been evaluated.

6.3.2. A selectin-dependent MP capture assay is a new tool for investigating the association between circulating MPs and thrombosis

As reviewed in Chapter 1, conventional flow cytometric measurement of MPs through detection of fluorescent labeling or multi-labeling is fraught with limitations. Functional assays enable amplification of procoagulant signals such as PS expression and TF through prothrombinase and FXa generation assays, respectively. Improved methods to immobilize such MPs that bear particular surface markers of interest may provide a sensitive method to quantify different circulating MP populations.

The use of immobilized P-selectin allows physiological ligand binding under shear, ensuring that captured MPs express functional P-selectin ligands. In addition, efficiency of capture depends on the rate of interactions between MPs and immobilized ligands, which can be enhanced through agitation. Tethering between P-selectin and PSGL-1 demonstrates a biphasic interaction that is governed by shear forces. Threshold levels of shear mediate the formation of catch bonds between the two molecules, which reaches maximal efficiency at an optimal shear (McEver and Zhu, 2010). Thus, orbital shaking within a 96-well plate may improve selectin-mediated MP capture through dual mechanisms; by increasing interactions between MPs and ligands, and by increasing binding efficiency (Gomes et al., 2014). Indeed, in Chapter 4, we found that procoagulant activity of MPs captured in P-selectin-coated wells increased in response to elevated orbital shaking, whereas that of non-specifically-bound MPs in IgG-coated wells did not.

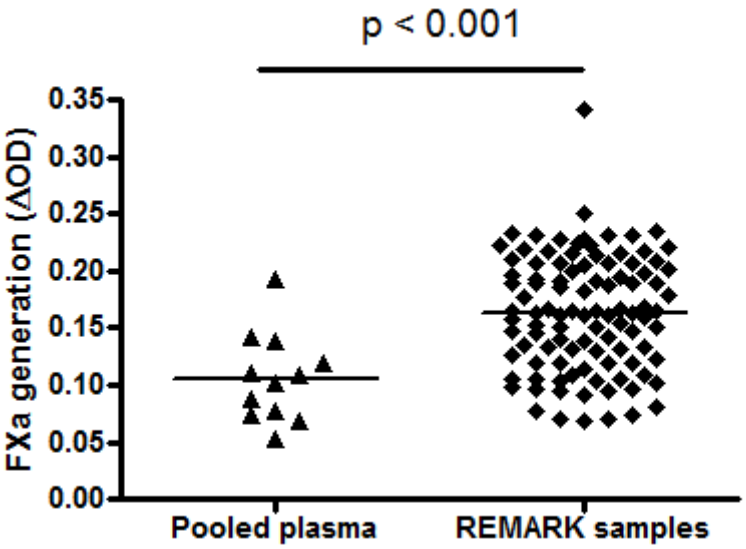
Thus, the studies performed in Chapter 4 provide a MP capture assay that is optimized for sensitivity to selectin-mediated interactions.

A pancreatic cancer cell line that coexpresses TF and PSGL-1 has been used to demonstrate P-selectin/PSGL-1-dependent mechanisms of thrombosis in animal models (Thomas et al., 2009). In addition, soluble P-selectin was found to be a risk factor in cancer-associated thrombosis (Ay et al., 2008). Soluble P-selectin was also shown to cause shedding of procoagulant MPs, potentially through a mechanism dependent on binding to PSGL-1 (Hrachovinova et al., 2003). Thus, evaluation of P-selectin-binding as well as P-selectin-expressing MPs may be of interest in cancer patients. Also, evaluation of MPs in cancer-associated VTE studies, such as AVERT, a study in which cancer patients are treated with apixaban to prevent VTE, or REMARK (McShane et al., 2005), a study in which patients with cancer-associated VTE are followed and tested for potential biomarkers of VTE recurrence, could be performed. We found higher levels of non-specifically-captured procoagulant particles in patients enrolled in REMARK compared with pooled plasma samples from healthy individuals (Figure 6.3). However, since these were non-specifically captured, it is not clear whether they represent MPs or other procoagulant components. Additional studies are also needed to compare these samples with plasma from individual healthy controls following an identical plasma isolation protocol.

As suggested by recent findings, endothelial cells play important roles in haemostasis and thrombosis because they are important sites of prothrombinase activity and fibrin formation (Ivanciu et al., 2014). However, the endothelium does not easily

Figure 6.3. MP levels in REMARK plasma samples.

One hundred plasma samples obtained at different times from 32 patients enrolled as part of REMARK were incubated in human IgG-coated wells, which were subsequently washed and evaluated for capture of procoagulant particles. The mean of the REMARK samples was significantly higher than that of pooled plasma samples from healthy donors.


p < 0.001
FXa generation (ΔOD)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Pooled plasma
REMARK samples

lend itself to direct evaluation. Endothelial MPs, which may be released upon cell activation, may thus be useful surrogates of endothelial cell activity or dysfunction. The association between endothelial cell MPs and VTE or other thrombotic states is controversial, as a number of studies have identified increased endothelial MPs in patients compared to healthy controls, while others have failed to find a difference (Dignat-George et al., 2004; Chirinos et al., 2005; Bidot et al., 2005; Flores-Nascimento et al., 2009; Bal et al., 2010; Campello et al., 2011; Ye et al., 2012). Most of these studies have relied on conventional flow cytometry to address this issue. Since endothelial MPs express P- and E-selectin, the selectin-based capture assay may be useful in addressing this issue. Ligands, such as ESL-1 or PSGL-1 may be immobilized onto surfaces, while Fab fragments can be used to block interactions with other counterreceptors.

6.3.3. A point of care, whole blood TGA on a chip could facilitate research and clinical assessment of thrombosis and haemostasis

TGAs have potential utility as screening tests for thrombophilia and haemophilia (Chapter 5), but can have more meaningful impacts on clinical decision-making if they can be shown to reliably predict patient outcomes and help define treatment strategies. Indeed, TGAs predict bleeding in haemophilia patients, and can estimate replacement factor levels in blood (Dargaud et al., 2005; Pike et al., 2015). In addition, thrombin generation parameters were associated with increased risk of bleeding in patients with low FIX, who had normal INRs while receiving warfarin (Dargaud et al., 2013). As described previously, several studies have found thrombin generation parameters to be

predictive of either initial or recurrent VTE (Tans et al., 2003; van Hylckama et al., 2007; Eichinger et al., 2008; Tripodi et al., 2008; Lutsey et al., 2009; Ay et al., 2011).

Lack of standardized methods and reagents however, likely contributes to a high degree of interlaboratory variability (van Veen et al., 2008b; Dargaud et al., 2010). In addition to different commercial assays, as well as variable TF concentrations and sources, phospholipid concentrations can significantly affect assay results, as elevated levels of inactive clotting factors may saturate binding sites on lipid vesicles and inhibit thrombin generation (Machlus et al., 2009). Despite attempts to standardize low TF-initiated TGAs, thrombin generation parameters vary widely within the normal population, and a range of values for normal thrombin generation has not been defined (Brummel-Ziedins et al., 2005a; Danforth et al., 2012). Cut-offs for defining elevated TGA parameters vary greatly between studies (Hron et al., 2006; Tripodi et al., 2008; van, V et al., 2015a). Finally, TGAs are cumbersome and not conducive to point of care testing, which reduces some of their potential clinical value. Thus, a number of unresolved issues still limit the clinical utility of TGAs.

In Chapter 5, we demonstrated a proof of concept for a low-cost chip-based device that initiates thrombin generation after the introduction of non-anticoagulated capillary blood, which may help to address several of these issues. The ability to perform TGAs in this way expands the potential clinical application of the test to include bedside testing that can be performed by non-laboratory-trained personnel. This can facilitate collection of data from a large number of donors within a short period of time, which can help establish values for normal and elevated thrombin generation. Indeed, the results suggest

that discrimination between healthy individuals and hypercoagulable blood is possible with the use of the chip.

Whole blood testing also provides a more physiologic milieu, in which the influence of blood cells and platelets are also evaluated. These have been shown to variably influence thrombin generation in different individuals (Allen et al., 2004), and thus, may be important discriminating factors with potential clinical relevance. The use of whole blood also precludes the need to add an external source of phospholipid vesicles, thereby limiting inter-laboratory variation and potential experimental artifacts.

We found that while intra- and inter-individual coefficients of variation were sufficient for discriminating between healthy individuals, assays occasionally showed large variations between channels, suggesting that further optimization and characterization are required. Flow-mediated effects are a likely source of variation. Using fluorescence microscopy to visualize channels, correlations between convective mass transfer of the substrate and the appearance of cleaved product at the site of signal detection can provide insights into the events occurring on the chip. This may enable identification of methods for normalizing results or reveal the need for alternative strategies of reagent deposition. Embedding reagents in a dissolvable film, or immobilizing activators to the surface of channels could also be investigated. An alternative strategy is to exclude results in which there is too much variability between duplicate channels. Finally, ongoing testing of different materials and channel geometries should be continued.

Ultimately, chips should be validated using whole blood from specific patient cohorts, including haemophilia patients. In Chapter 4, we used plasma samples from patients enrolled in the DODS trial to determine whether procoagulant MPs predicted recurrent VTE. TGAs have been found to predict recurrence of VTE in patients with unprovoked events. Plasma samples and buffy coats from DODS can be evaluated with the chip as a preliminary investigation into whether it could be useful as a prognostic tool in unprovoked VTE. Thrombin generation has also been shown to predict VTE in cancer patients (Ay et al., 2011). Thus, evaluating the chip using samples from trials such as REMARK (McShane et al., 2005) could also be helpful. Subsequently, the chip can be incorporated into point of care clinical trials, in which capillary blood testing is embedded into routine clinical situations for later evaluation, enabling bulk data collection.

While diagnosis and management of haemophilia A and B in developed countries enables improved quality-of-life and normalized lifespan for those with these bleeding disorders, the vast majority of persons with haemophilia in the world remain untreated or undiagnosed (Bolton-Maggs, 2006). The World Federation of Haemophilia lists as part of its "Cornerstone Initiative" program, a mandate to "develop or improve diagnosis capacity" in countries with minimal levels of care. A lab on a chip device to identify FVIII and FIX deficiencies using finger prick whole blood thrombin generation may be a low-cost approach to diagnose individuals with haemophilia A or B. Thus, testing thrombin generation in persons with haemophilia using the chip would further validate the test for haemophilia screening or diagnostics, which could support an existing clinical need. Finally, in regions where factor dose-management, rather than diagnosis is an issue,

a point of care method of monitoring circulating factor levels would be useful. To this end, we have observed that plasma levels of rFVIII may correlate with chip-based thrombin generation (Figure 6.4).

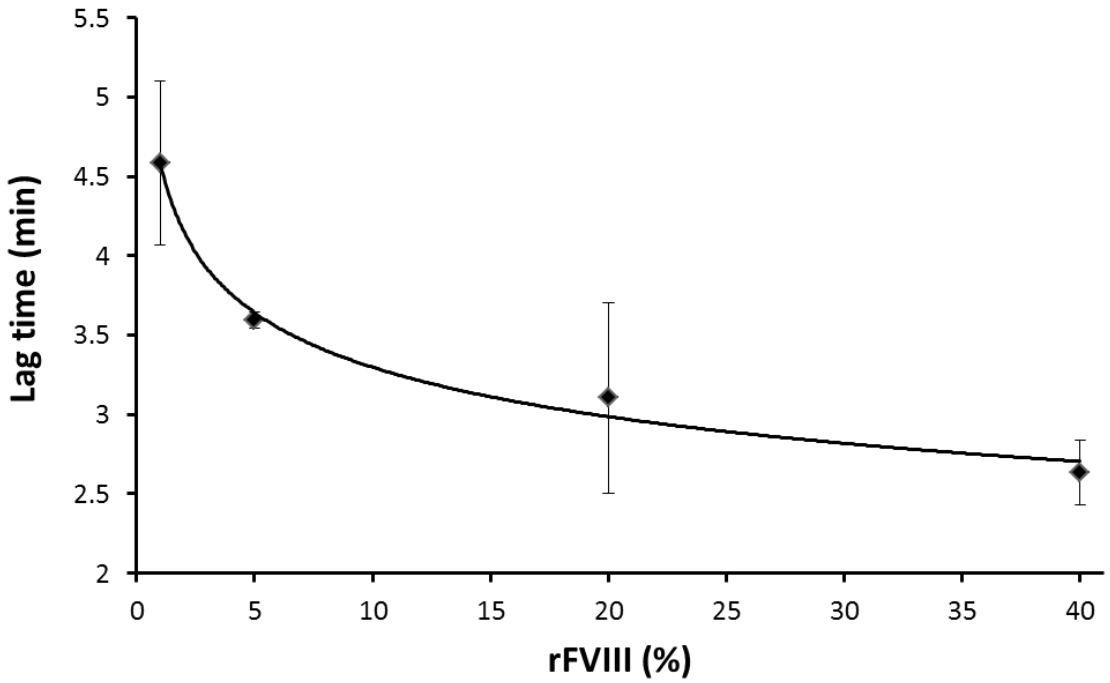
6.3.4. A TGA on a chip allows a more comprehensive approach to evaluating the coagulation cascade in whole blood

TGAs triggered by high concentrations of TF measure the effects of the extrinsic pathway, but are not influenced by variations in contact pathway proteins. The effects of FVIII and FIX levels on thrombin generation are most discernible at TF concentrations up to 5 pM (van Veen et al., 2008a), while even lower (< 1pM) concentrations are required to discriminate between varying FXI levels (Pike et al., 2015). However, the use of low TF concentrations is associated with greater variation in thrombin generation parameters. In addition, the signal strength obtained from the use of low TF may not be sufficient to generate a thrombin generation curve. The absence of standardized TF concentrations is thus a major source of inter-laboratory variation.

The use of contact activators as an alternative trigger of thrombin generation can sensitively measure the effects of contact pathway proteins with good signal strength, but does not permit measurement of the extrinsic pathway. In Chapter 5, we showed that differentiation between FVIII- and FIX-deficient plasma is possible through systematic evaluation of thrombin generation under different experimental conditions in the presence of APTT reagent. Further, the effects of the protein C pathway can only be identified by comparing thrombin generation curves obtained in the presence and absence of

Figure 6.4. Correlating plasma FVIII levels with chip-based TGA.

Citrated FVIII-deficient plasma was reconstituted with varying concentrations of rFVIII, recalcified, and evaluated using a chip-based TGA. Thrombin generation was initiated using APTT reagent, which was predeposited inside of the channels.


Lag time (min)
5.5
5
4.5
4
3.5
3
2.5
2
0
5
10
15
20
25
30
35
40
rFVIII (%)

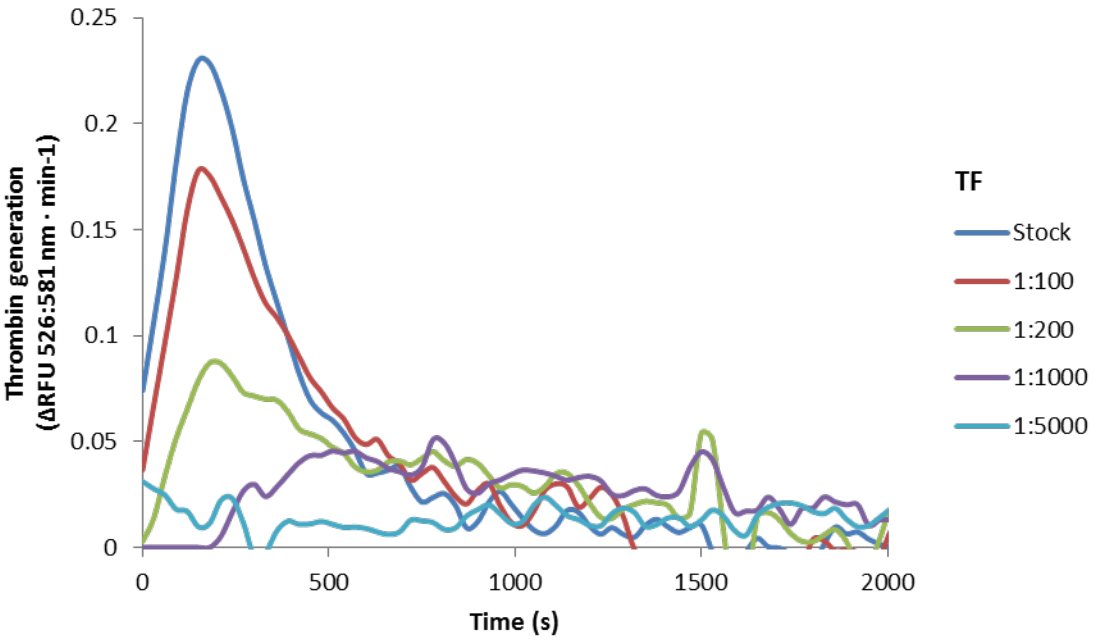
exogenous, soluble TM or APC (Hezard et al., 2006). The differential influence of platelets on thrombin generation may be aided by the use of platelet activators or inhibitors in whole blood or PRP (Allen et al., 2004; Duckers et al., 2010). Lastly, prothrombin levels are linearly correlated with ETP and peak thrombin (Chapter 5) (Butenas et al., 1999; Allen et al., 2004), which suggests that TGAs performed in the presence of high concentrations of prothrombin may be a potential strategy to amplify signal strength in individuals with low thrombin generation.

Although there are advantages to performing TGAs under multiple experimental conditions, data regarding the influence of variable experimental conditions on thrombin generation in different patient cohorts and healthy donors is scarce. A point of care TGA performed on a chip offers a novel approach to rapidly accumulate patient data, which will facilitate investigation into the utility of performing different types of TGAs. Other methods of whole blood thrombin generation have been described (Tappenden et al., 2007; Thuerlemann et al., 2009; Ninivaggi et al., 2012), but none have been tailored and optimized for obtaining multiple curves. In a 384-well plate format, our lab has used 12 µl blood samples to generate single thrombin generation curves, whereas 10 µl or less can be used to obtain four thrombin generation curves on a chip.

In Chapter 5, deposited reagents included APTT reagent, rFVIII and rFIX. Tests have also been performed in the presence and absence of EDTA (not shown). In a single-channel design containing glass surfaces, we demonstrated that lipidated TF can also be used to perform TGAs (Figure 6.5). However, this and other initiators and inhibitors will

Figure 6.5. Whole blood thrombin generation initiated with predeposited TF.

Varying concentrations of relipidated TF were predeposited in single channels along with the thrombin substrate, ‘T13.’ Channels were made using glass slides to form the floor and ceiling, and double-sided tape was used for the side walls.


0.25
0.2
0.15
0.1
0.05
0
Thrombin generation
(ΔRFU 526:581 nm · min-1)
0
500
1000
1500
2000
Time (s)
TF
Stock
1:100
1:200
1:1000
1:5000

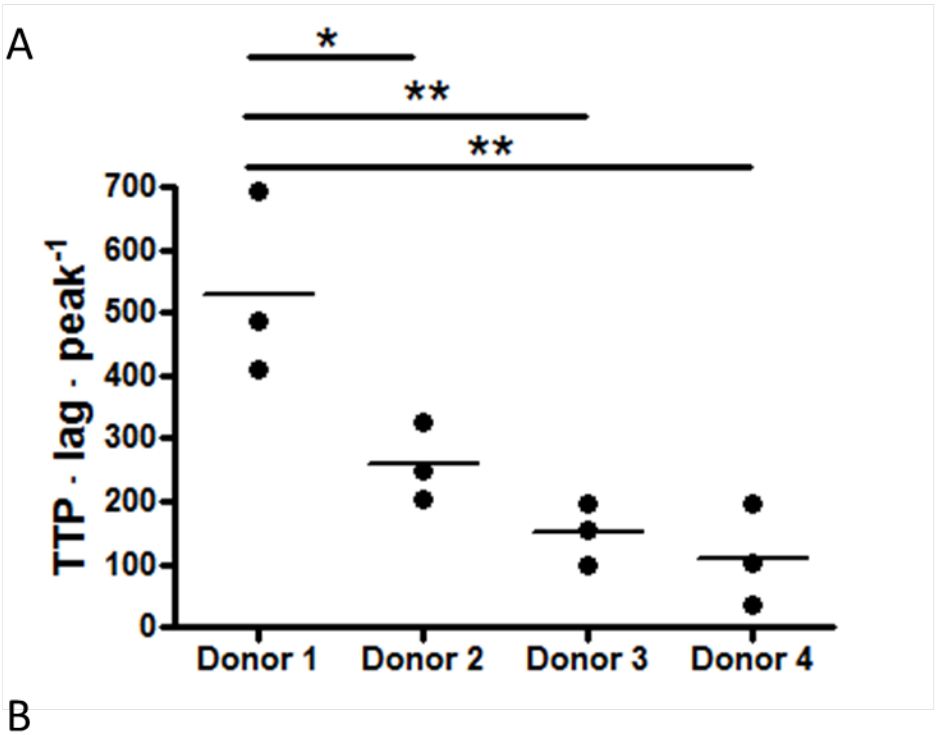
need to be tested and optimized in the current iteration of the chip in order to fully exploit the advantages of a multichannel chip design.

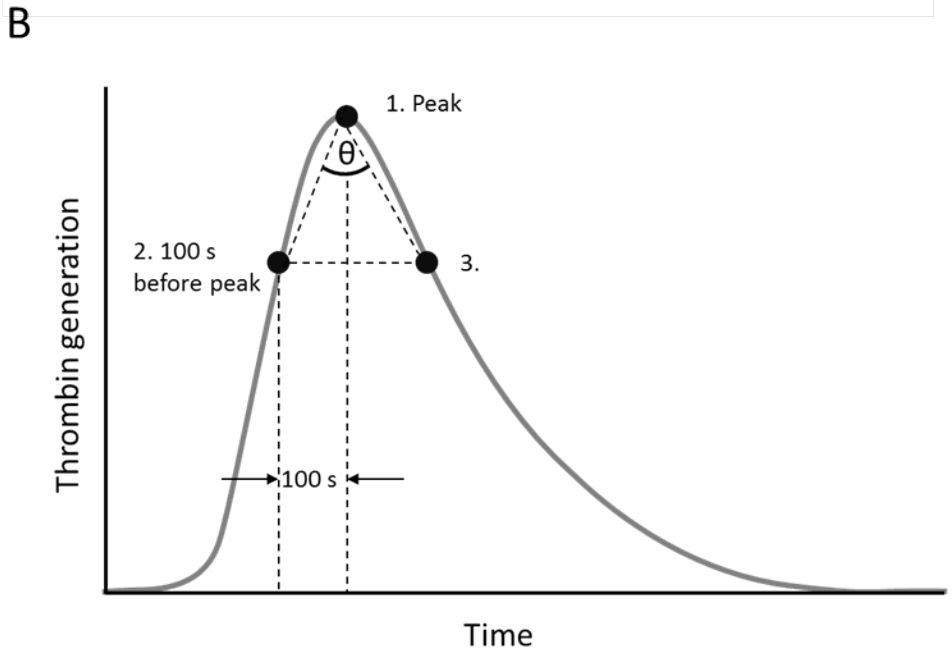
Comparing relative change in TGA parameters before and after addition of APC enabled differentiation between factor V Leiden, protein S deficiency, and the prothrombin mutation G20210A (Hezard et al., 2006). Similar ratioing may be useful for other conditions and may allow some form of internal standardization to limit the effects of external TF. Additionally, thrombin generation parameters may be combined into a single score or value obtained through an algorithm (Tripodi et al., 2009). For instance, the product of the lag time and time to peak can be divided by peak thrombin in order to potentially further discriminate patients with different thrombin-generating capacities (Figure 6.6A). A novel parameter in which the time to maximum rate was multiplied by an angle derived from the peak of the thrombin generation curve (Figure 6.6B) was able to linearly correlate thrombin generation with plasma rFVIII at certain concentrations.

All standard TGA parameters except for ETP only consider events until the time at which thrombin generation peaks, but ignore the termination phase. Identification of new, useful parameters that provide added levels of discrimination, on the other hand, is limited by the rate at which they are discovered. A novel approach could be to incorporate machine learning algorithms into evaluation of thrombin generation curves, thereby considering important aspects of the thrombin generation curve that may otherwise go unnoticed. This could be most useful for analyzing complex profiles consisting of multiple thrombin generation curves obtained through various initiating conditions. As a proof of concept for this, we prepared 50 sets of paired curves that

Figure 6.6. Possible new parameters for evaluating thrombin generation curves.

(A) Combining thrombin generation parameters such as the TTP, lag time and peak thrombin into a single measure of thrombin generation may improve discrimination between thrombin generation profiles. (B) Identification of the angle θ (using *x* and *y* units that are on the same order of magnitude) offers information about the transition between the propagation and termination phases of thrombin generation.


A
*
**
**
700
600
500
400
300
200
100
0
TTP - lag - peak-1
Donor 1
Donor 2
Donor 3
Donor 4


B
1. Peak
θ
2. 100 s
before peak
3.
100 s
Thrombin generation
Time

resembled the thrombin generation profiles of individuals with APC resistance, and 50 pairs of curves that resembled the profiles of normal controls (Figure 6.7). Using these as samples, IBM visual recognition software (https://visual-recognition-demo.mybluemix.net/) was able to correctly classify new sample curves as 'APC resistant-like' or 'normal.'

6.3.5. Combining these methods can help lead to a better understanding of haemostasis and thrombosis

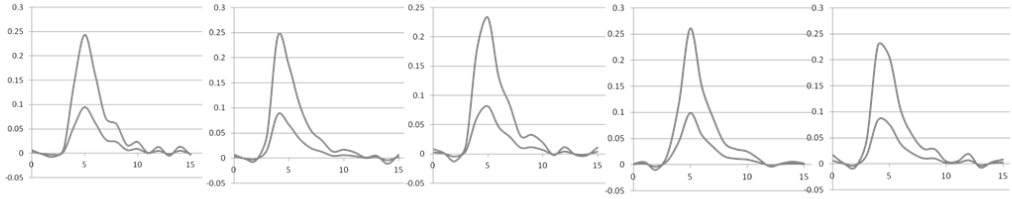
Correlating whole blood thrombin generation with bleeding phenotypes in transgenic and drug-treated mice can help determine how a TGA on a chip can be used to predict bleeding. Blood obtained from a mouse during bleeding model experiments can be evaluated using a TGA to determine how thrombin levels released from the site of injury change over time. Although some mice may have limited bleeding, the use of a chip can allow several analyses to be performed. In addition, using the MP capture assay, different populations of MPs may be evaluated in bleeding and thrombosis models.

It has been proposed that some populations of MPs may be used for therapeutic purposes in haemophilia (Gross and Vaezzadeh, 2010). MPs quantification using a solid-phase capture assay could be used for dosing in bleeding models. Finally, combining the concepts of a hydrophilic channel for performing TGAs with a solid-phase MP capture assay may enable the generation of a simpler method of MP isolation and quantification. Microfluidic channels enable maximized surface-to-volume ratios, potentially allowing many analytes to contact the channel walls within a short period of time. Immobilizing receptors or antibodies onto the surface of a long channel might allow for the capture,

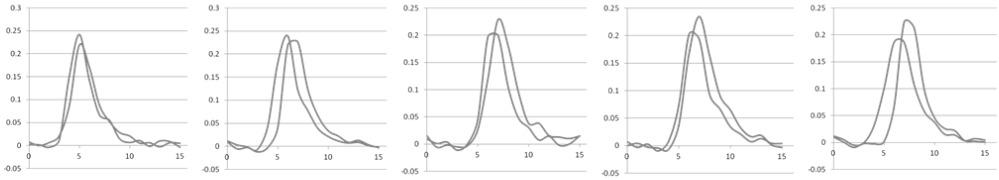
Figure 6.7. Proof of concept for classifying thrombin generation profiles using visual recognition software.

Previously-obtained thrombin generation curves were modified to create fifty pairs of curves that resemble thrombin generation profiles in the presence and absence of APC. An additional set of 50 pairs were generated to reflect APC resistance. Examples are shown in (A) and (B). These samples were used to characterize the two groups using IBM visual recognition software. New sample curves representing (C) normal plasma and (D) APC resistance were generated to test the ability of visual recognition software to correctly classify thrombin generation profiles.

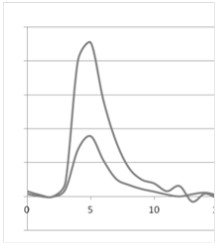
A. **"Normal" samples**



B. **"APC-resistance" samples**



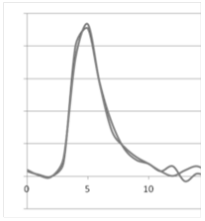
C.



Classification:
"Normal"

Confidence score:
65%

D.



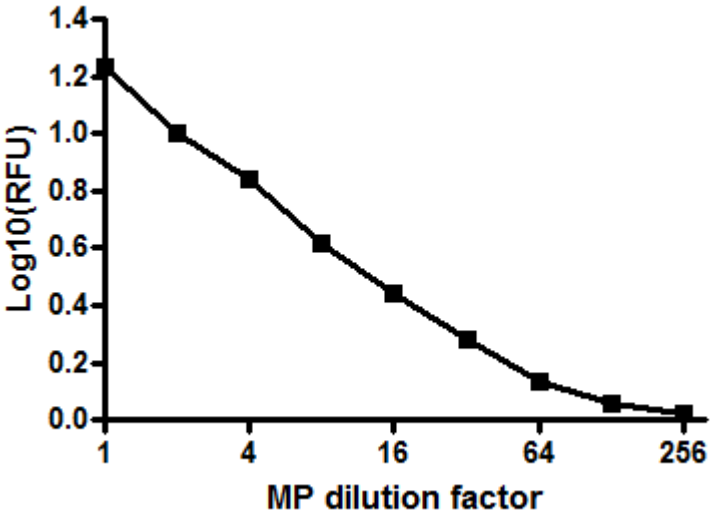
Classification:
"APC resistance"

Confidence score:
75%

concentration, and washing of MPs from plasma samples without the need for centrifugation. This may be enhanced by the capture of MPs onto the surface of beads that remained fixed inside the channel. Indeed, literature data (Falati et al., 2003), and preliminary findings from our lab show that capture of MPs onto antibody- or receptor-coated beads enables detection of low concentrations of MPs (Figure 6.8). Thrombin generation can be performed to measure MPs inside the channel.

Figure 6.8. Capture and detection of MPs using receptor-coated beads.

Calcium ionophore-generated WEHI274.1 MPs labeled with a fluorescent dye were captured in solution onto P-selectin-coated polystyrene beads and measured by flow cytometry. According to preliminary data, this method allowed for concentration-dependent detection of MPs diluted up to 256 times from the original stock concentration. Axes are plotted on a logarithmic scale.


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Log10(RFU)
1
4
16
64
256
MP dilution factor

Chapter 7: Concluding Remarks

Thrombosis and haemostasis are directed by complex mechanisms – the elucidations of which have important implications in the treatment of thrombotic and hemorrhagic disease. The body of work presented in this thesis adds novel findings to the literature, including insights about 1) the relative contributions of platelets and coagulation in venous and arterial haemostasis, 2) the involvement of procoagulant MPs in recurrence of unprovoked VTE, and 3) contact pathway-activated thrombin generation in whole blood. Beyond this, each study also offers a new clinical or investigative tool – namely, a murine arterial bleeding model, a P-selectin-dependent functional MP capture assay, and a point of care TGA on a chip – with which future studies can evaluate thrombosis and haemostasis.

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