Impact of Thrombomodulin and Thrombin-Activatable Fibrinolysis Inhibitor on the Anticoagulant and Profibrinolytic Effects of Rivaroxaban

Justin John William Garabon

University of Windsor

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Impact of Thrombomodulin and Thrombin-Activatable Fibrinolysis Inhibitor on the Anticoagulant and Profibrinolytic Effects of Rivaroxaban

By

JUSTIN J.W. GARABON

A Thesis
Submitted to the Faculty of Graduate Studies through the Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada

2016

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Impact of thrombomodulin and thrombin-activatable fibrinolysis inhibitor on the anticoagulant and profibrinolytic effects of rivaroxaban

by

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June 17, 2016
DECLARATION OF ORIGINALITY

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication.

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I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.
Rivaroxaban is a direct factor Xa inhibitor, recently implemented as a favorable alternative to warfarin in anticoagulation therapy. Rivaroxaban effectively reduces thrombin generation, which plays a major role in the activation of thrombin activatable fibrinolysis inhibitor (TAFI). Activated TAFI (TAFIa) has an antifibrinolytic role and therefore we hypothesized that rivaroxaban would induce more rapid clot lysis. *In vitro* clot lysis assays were used to explore this hypothesis. Additionally, these assays were used to determine if the profibrinolytic effects of rivaroxaban could be modulated by TAFI levels or a stabilizing T325I TAFI polymorphism. Rivaroxaban was shown to decrease thrombin generation, resulting in less TAFI activation, thus enhancing lysis. These effects were also shown to be less substantial in the presence of greater TAFI levels or the more stable I325 protein. These findings suggest a role for TAFI levels and the T325I polymorphism in the pharmacodynamics and pharmacogenomics of rivaroxaban.
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TABLE OF CONTENTS

DECLARATION OF ORIGINALITY ........................................................................iii

ABSTRACT........................................................................................................iv

ACKNOWLEDGEMENTS.....................................................................................v

LIST OF FIGURES ..............................................................................................ix

LIST OF APPENDICES........................................................................................x

LIST OF ABBREVIATIONS/SYMBOLS.................................................................xi

CHAPTER 1: INTRODUCTION..............................................................................1

1.1 Hemostasis ..................................................................................................1

1.2 Coagulation ..................................................................................................3

1.3 Regulating coagulation ..............................................................................9

1.4 Fibrinolysis ..................................................................................................11

1.5 Regulating fibrinolysis ..............................................................................14

1.6 Thrombin-activatable fibrinolysis inhibitor ..............................................16

1.6.1 General properties of TAFI .................................................................17

1.6.2 The genetic and non-genetic regulation of TAFI ..................................18

1.6.3 Quantifying TAFI ..................................................................................20

1.6.4 TAFI activation and thrombomodulin ...............................................22

1.6.5 TAFIa inhibition and inactivation .......................................................25

1.6.6 TAFIa function ......................................................................................28

1.7 Virchow’s triad and thrombosis .................................................................29

1.7.1 Venous Thrombosis .............................................................................34

1.7.2 Arterial Thrombosis .............................................................................39
1.8 TAFI as a risk factor for thrombosis .......................................................... 44
1.9 Anticoagulant Therapy ................................................................................. 46
1.9.1 Rivaroxaban: Structure and Function ....................................................... 48
1.9.2 Clinical pharmacology of rivaroxaban ..................................................... 51
1.10 Rationale, Hypothesis and Research Objectives ......................................... 58

CHAPTER 2: MATERIALS AND METHODS .......................................................... 59
2.1 Cell Culture .................................................................................................... 59
2.2 Recombinant TAFI Expression Vector Construction ..................................... 59
2.3 Recombinant TAFI Expression and Purification ............................................ 60
2.4 Hipp-Arg Activity Assay ................................................................................ 61
2.5 Rivaroxaban .................................................................................................. 62
2.6 PCPS Vesicles ................................................................................................ 63
2.7 TAFI-deficient Plasma (TDP) ....................................................................... 63
2.8 Clot Lysis Assay ............................................................................................. 64
2.9 Plasmin Generation Assay .............................................................................. 65
2.10 Thrombin Generation Assay ......................................................................... 65
2.11 Statistical Analysis ........................................................................................ 66

CHAPTER 3: RESULTS ......................................................................................... 67
3.1 Rivaroxaban and TM increase clot formation latency .................................... 67
3.2 Rivaroxaban and TM decrease clot formation rate ........................................ 70
3.3 The TAFI-dependent prolongation in clot lysis time is modified by TM and rivaroxaban ................................................................................................................... 73
3.4 Rivaroxaban and TM both contribute to thrombin generation impairment ................................................................. 77
3.5 TAFI levels and the T325I variant are capable of modulating the profibrinolytic effects of rivaroxaban .......................................................... 80
3.6 Rivaroxaban reduces the TAFI-attributed reduction in plasmin generation .............................................................. 83
3.7 Rivaroxaban does not directly inhibit TAFIa activity ........... 86

CHAPTER 4: DISCUSSION ................................................................. 87
4.1 Future directions ................................................................. 96
4.2 Conclusions ..................................................................... 99

REFERENCES/BIBLIOGRAPHY ..................................................... 100

APPENDIX ................................................................................ 131

CHAPTER 5: OPTIMIZATION OF THE CHANDLER LOOP ASSAY: A WHOLE BLOOD MODEL OF FIBRINOLYSIS .................................................. 131
5.1 The Chandler loop assay ..................................................... 131
5.2 Materials and methods ....................................................... 133
5.2.1 Rivaroxaban .................................................................. 133
5.2.2 Alexa-488 Labelled Fibrinogen ....................................... 134
5.2.3 Chandler Loop Assay ..................................................... 134
5.3 Optimization of Conditions ............................................... 135

VITA AUCTORIS ...................................................................... 149
# LIST OF FIGURES

**Figure 1.1** The pathways of coagulation.................................................4

**Figure 1.2** TAFI and the mechanisms of fibrinolysis..............................16

**Figure 1.3** Structural requirements of thrombomodulin.............................24

**Figure 1.4** Key components of TAFI structure........................................28

**Figure 1.5** Virchow’s triad......................................................................31

**Figure 1.6** Venous vs. arterial thrombosis..............................................35

**Figure 1.7** Atherogenesis......................................................................41

**Figure 1.8** Three dimensional binding of rivaroxaban to Factor Xa..........48

**Figure 2.1** Purification of recombinant TAFI...........................................59

**Figure 3.1** The effect of rivaroxaban and TM on clot formation latency........67

**Figure 3.2** The effect of rivaroxaban, TM and TAFI on clot formation rate...70

**Figure 3.3** The effect of rivaroxaban, TM and TAFI on clot lysis.................74

**Figure 3.4** The effects of rivaroxaban and TM on thrombin generation......77

**Figure 3.5** The role of rivaroxaban, TAFI levels and the T325I variant on clot characteristics..........................................................80

**Figure 3.6** The role of rivaroxaban, TAFI levels and the T325I variant on plasmin generation.................................................................83

**Figure 3.7** The effects of rivaroxaban on Hipp-Arg hydrolysis by TAFIa.......84

**Figure 4.1** The relationship between rivaroxaban and fibrinolysis.............89

**Figure 5.1** CPI inhibits TAFIa, resulting in greater lysis of a whole blood clot..128

**Figure 5.2** The initial replication of Mutch *et al.* showed erratic results......132

**Figure 5.3** Chandler loop experiments showed an increase in lysis when TAFIa was inhibited by PTCI..............................................................133

**Figure 5.4** Rivaroxaban increases clot lysis..............................................134

**Figure 5.5** Confirmation of the proper fluorescence readout......................136
Figure 5.6 Inter- and intra-individual variability…………………………………140

Figure 5.7 Direct comparison of rivaroxaban and PTCI effects showed inconclusive results…………………………………………………………141

LIST OF APPENDICES

Chapter 5: Optimization of the Chandler loop assay: A whole blood model of fibrinolysis
# LIST OF ABBREVIATIONS/SYMBOLS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAFR</td>
<td>anisylazoformyl-arginine</td>
</tr>
<tr>
<td>ACS</td>
<td>acute coronary syndrome</td>
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<tr>
<td>ADP</td>
<td>adenosine disphosphate</td>
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<td>activated protein C</td>
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<td>AUC</td>
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<td>C/EBP</td>
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<td>platelet glycoprotein Ibα</td>
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<td>HBS</td>
<td>HEPES-buffered saline</td>
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<td>HBST</td>
<td>HEPES-buffered saline/0.01% (v/v) Tween 80</td>
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<tr>
<td>HK</td>
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<td>TBS</td>
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<td>TM</td>
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<td>HEPES</td>
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<td>$K_{IV}$</td>
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<td>$K_{on}$</td>
<td>kinetic association rate constant</td>
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<tr>
<td>$K_{off}$</td>
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<td>KLF</td>
<td>Kruppel-like transcription factor</td>
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<td>leech carboxypeptidase inhibitor</td>
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<td>low-molecular weight heparin</td>
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<td>matrix metalloproteinase 9</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>NOAC</td>
<td>non-vitamin K antagonist oral anticoagulant</td>
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<td>non-ST-segment elevation myocardial infarction</td>
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<tr>
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<td>prekallekrein</td>
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<tr>
<td>PTCI</td>
<td>potato tuber carboxypeptidase inhibitor</td>
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<tr>
<td>RBC</td>
<td>red blood cell</td>
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embolism

Serpins  
serine protease inhibitors
sCD40L  
soluble CD4 ligand
sc-tPA  
single-chain tissue plasminogen activator
sc-uPA  
single-chain urokinase-type plasminogen activator
SNP  
single nucleotide polymorphism
STEMI  
ST-segment elevation myocardial infarction
TAFI  
thrombin-activatable fibrinolysis inhibitor
TAFIa  
activated thrombin-activatable fibrinolysis inhibitor
TAFIai  
inactivated thrombin-activatable fibrinolysis inhibitor
TdP  
TAFI-deficient plasma
TF  
tissue factor
TFPI  
tissue factor pathway inhibitor
TCI  
tick carboxyypeptidase inhibitor
TM  
thrombomodulin
TNF-α  
tumor necrosis factor-alpha
TXA2  
thromboxane
tPA  
tissue-type plasminogen activator
tc-tPA  
two-chain tissue plasminogen activator
tc-uPA  
two-chain urokinase-type plasminogen activator
uPA  
urokinase-type plasminogen activator
uPAR  
urokinase-type plasminogen activator receptor
VCAM-1  
vascular cell adhesion molecule-1
VKA  
vitamin K antagonist
VT  
venous thrombosis
VTE  
venous thromboembolism
vWF  
von-Willebrand factor
CHAPTER 1: INTRODUCTION

1.1 Hemostasis

The process of hemostasis is an efficient protection mechanism that is utilized during times of vascular injury (1). When a blood vessel is damaged, the hemostatic response inhibits blood flow at the site of injury, thus averting excessive blood loss, while maintaining blood flow adjacent to the site of damage. The cessation of blood flow is followed by the gradual healing of the damaged tissue. This entire process is intricately regulated and involves a delicate balance between factors that contribute to coagulation (clot formation), fibrinolysis (clot dissolution), and the healing process (1, 2).

The endothelium of a blood vessel plays a critical role in promoting blood fluidity and discouraging sporadic hemostasis within a healthy vessel by producing various molecules that are vasoprotective or thromboresistant (3). In order to inhibit platelet activation at the interface between the platelet and vessel wall, ecto-adenosine disphosphatase (ADPase) is expressed on the endothelial cell surface, while prostacyclin (PGI$_2$) and nitric oxide (NO) are secreted from the cells in a paracrine manner (3). The endothelium protects against coagulation of blood proteins by expressing cell surface proteins such as thrombomodulin (TM), protein S, heparin-like molecules and tissue factor pathway inhibitor (TFPI) (3). The endothelium can also promote fibrinolysis by producing tissue-plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) and secreting them into the blood (3). Although these thromboresistant mechanisms conspire to prevent clotting under typical conditions, blood homeostasis is altered when vascular damage occurs.
When the endothelium is compromised, hemostasis progresses through various mechanisms. First, injury of the blood vessel initiates vasoconstriction, which functions to limit the loss of blood by reducing the flow rate and the volume of blood entering that particular area (4). Vasoconstriction is initiated in most vessels by a sympathetic reflex to the pain stimulus, which causes the contraction of the smooth muscle layer within the vessel walls (5). Humoral factors such as histamine, kinins, serotonin and thromboxanes can also contribute to vasoconstriction, and these are commonly released from activated platelets (6).

As a consequence of vascular injury, the subendothelium and its components are exposed to the blood, creating a surface that allows for the development of a platelet plug. Platelets are small, anuclear cell fragments derived from megakaryocytes and are an important aspect of hemostasis (7). The platelet plug is the first hemostatic structure, created to form a physical barrier to protect against blood loss from the damaged endothelium, and evolving through three general stages: initiation, extension, stabilization by the fibrin network (8). The initiation of platelet plug formation occurs when platelets adhere to exposed subendothelial components such as collagen (9), or von Willebrand factor (vWF), which acts as a structural bridge between collagen and the platelet under conditions of higher blood flow (10). The process of platelet adhesion results in a monolayer of activated platelets covering the exposed subendothelium (8, 11). Once this occurs, the extension phase can ensue, functioning to increase the size of the platelet plug. Locally generated thrombin contributes to this process by cleaving protease activated receptors (PAR) 1 and PAR 4 on the platelet surface, resulting in platelet activation (12, 13). This thrombin is important to the platelets that are near the TF-
bearing cells, as the combination of contact- and thrombin-mediated platelet activation creates a dense core of platelets with heightened activation, held together via $\alpha_{\text{IIb}}\beta_3$ integrins (11). The activated platelets within the core also begin releasing adenosine diphosphate (ADP) and thromboxane $A_2$ (TXA$_2$), which activate new platelets through binding to platelet surface receptors, namely the P2Y and TP receptors, respectively (14, 15). These newly activated platelets form a loosely adherent shell of less activated platelets around the previously formed platelet core (11). A platelet plug becomes stabilized as a consequence of the procoagulant tendencies of the activated platelets, including factor Va (FVa) production (13, 16) and enhanced binding to coagulation zymogens, proteases and cofactors, promoted by surface receptor expression and exposure of negatively charged membrane phospholipids (3, 17). These characteristics are vital to the role of platelets within the coagulation process.

1.2 Coagulation

The process of coagulation generally functions in parallel to the spatiotemporal regulation of platelet activation and function, ultimately creating a stable hemostatic plug supported by a crosslinked fibrin network. The process of coagulation has classically been viewed as a successive series or “cascade” of proteolytic, zymogen-to-enzyme conversions ultimately resulting in the production of the serine protease, thrombin and subsequently, the fibrin network (Fig. 1.1) (18, 19). Although this cascade had been historically understood to proceed through two distinct lineages, the “intrinsic” and “extrinsic” pathways, more recent evidence suggests that these two processes are closely related, non-redundant and are significantly influenced by cellular contributions (20-22).
Figure 1.1 The pathways of coagulation. The coagulation process involves intricate communication between an extrinsic, tissue factor (TF) pathway and an intrinsic, contact-mediated pathway, ultimately resulting in the formation of a fibrin clot. Tissue damage commonly initiates the “initiation phase of coagulation via the extrinsic pathway. This is accomplished through TF exposure and subsequent Factor X (FX) activation by the TF/VIIa complex. FXa, along with its cofactor FVa, can then convert small amounts of prothrombin to thrombin. This thrombin generation initiates the “amplification” of coagulation through positive feedback mechanisms that incorporate the classically defined intrinsic pathway of coagulation. This feedback allows for a massive burst in thrombin concentration, allowing thrombin to effectively cleave fibrinogen, ultimately resulting in the formation of a fibrin clot network. Coagulation factors are indicated in green and regulatory proteins of the coagulation process are indicated in red. Figure adapted from (23).
In general, the process of coagulation contributes to hemostasis through three non-exclusive phases: initiation, amplification and propagation (22). The initiation of coagulation commonly relies on the exposure of tissue factor (TF) bearing cells to the circulation following endothelial damage (24). In this “extrinsic” lineage of coagulation, TF on the subendothelial cell surface is a cofactor that complexes with activated factor VII (FVIIa), forming a “tenase” complex that performs optimally in the presence of negatively charged phospholipids and calcium (16, 25, 26). This complex is able to activate minute amounts of circulating factor X (FX) to FXa (25). As mentioned previously, when platelets bind to collagen in the subendothelial matrix, they become activated. A consequence of this activation is the secretion of partially activated factor V (FVa) from the platelet α granules (27). In addition to this FVa, thrombin (28) and FXa (29) activate FV in the plasma. FVa is a cofactor that binds the TF-bearing cell or platelet surface, which accelerates the binding of FXa to FVa, resulting in the “prothrombinase” complex (16, 30). This complex requires the presence of negatively charged membrane phospholipids and calcium (31). At this point, the prothrombinase complex can activate small amounts of prothrombin (FII) to thrombin (FIIa) (16).

This limited thrombin generation is sufficient to initiate fibrin formation (vide infra); moreover, it is critical to the “amplification” phase of the coagulation response as it functions in multiple ways to prepare for the large-scale thrombin generation during the propagation phase. First, this thrombin enhances the extent of activation in the platelets bound to the damaged endothelium, consequently advancing their procoagulant abilities (32). This minute thrombin generation also begins to promote the activation of other
coagulation proteins known as being a part of the “intrinsic” coagulation pathway, namely factor XI (FXI), and factor VIII (FVIII) (33, 34). These factors, along with FV, are activated on the platelet surface during this phase (33).

As a result of these actions, the system is prepared for mass thrombin generation and multiple actions contribute to this. During the initiation phase, a small amount of FIXa is activated by the TF/FVIIa complex (35). This FIXa, supplemented by additional FIXa created by platelet-bound FXIa (36), can then bind to its cofactor, FVIIIa, which requires the negatively charged phospholipids on the platelet surface and calcium, resulting in the formation of another “tenase” complex (16). This activates more FX, allowing ample FXa to rapidly form prothrombinase complexes on the platelet surface, providing the machinery required to generate substantial amounts of thrombin (16). Thrombin can generate further positive feedback by activating platelets (37), FV (38), FVIII (39) and FIX (40). As a consequence of these processes, the massive burst in thrombin generation becomes sufficient to effectively cleave fibrinogen, thus creating the structurally supportive fibrin network that sustains the hemostatic plug (16).

In addition to the coagulation factors mentioned here, the classical model of the coagulation “cascade” includes the “contact pathway”, which incorporates high-molecular weight kininogen (HK), prekallekrein (PK) and factor XII (FXII) (41, 42). Previous studies have shown that FXII deficient mice showed no bleeding tendencies, but had a reduced occurrence of thrombotic events (43). It was then concluded that FXII contributed to pathological thrombosis, but was not necessarily required for the process of hemostasis (43). FXII can be activated through various methods under conditions favoring thrombosis. For example, bacterial proteoglycans and endotoxin released during
sepsis-induced disseminated intravascular coagulation (DIC) can activate FXII (44, 45). Moreover, collagen, misfolded protein aggregates, negatively charged surfaces of activated blood cells and polyphosphates released during active platelet degranulation can also initiate FXII autoactivation (45, 46). Moreover, glass surfaces have traditionally been used to initiate this pathway and although not found within the body, indwelling glass catheters have been known to be associated with pathological thrombosis (47).

From here, activated FXII (FXIIa) can activate inactive prekallikrein to kallikrein, which functions by activating more FXII along with cleaving high molecular weight kininogen into bradykinin, which is an effective, hypotensive molecule (41). FXIIa then continues the previously mentioned coagulation process by activating FXI (48). FXIIa is also known to activate small amounts of FVII (49), thus providing a contribution to extrinsic coagulation mechanisms, as well.

The intrinsic and extrinsic pathways of coagulation are both influential in generating thrombin. The extent of this process and the rate at which it occurs will significantly affect fibrin polymerization and thus, the integrity of the fibrin meshwork (50). Fibrinogen is a large protein (340 kDa) that is found within the plasma at concentrations of 6-12 µM. Fibrinogen is structurally comprised of two sets of three distinct polypeptide chains; Aα, Bβ, and γ (51). The amino-termini of these six chains converge at a central “E” nodule within fibrinogen (52). The “D” nodule is comprised of the carboxyl-terminal ends of the Bβ and γ chains (52). The carboxyl-termini of the Aα chains interact with the E nodule (53). Cleavage of fibrinogen begins when thrombin binds to the E nodule (54, 55) and cleaves off the amino-termini of the Aα chains, releasing a peptide segment called fibrinopeptide A (FpA) (56). This permits
spontaneous, non-covalent interactions between E and D domains (E:D association), resulting in the formation of double-stranded protofibrils during a “lag” phase (50, 57, 58). These protofibrils are then able to aggregate laterally with one another, which provides the characteristic increase in absorbance of clotting plasma (59). Although not essential for lateral aggregation, the amino-terminus of the Bβ chain can subsequently be cleaved by thrombin, resulting in the release of fibrinopeptide B (FpB) and the promotion of further lateral aggregation (60). The physical properties of the resulting fibrin network created during this process depend on immediate conditions such as pH, ionic strength, calcium levels, polyphosphate, fibrin and fibrinogen binding proteins, and most importantly, thrombin and fibrinogen levels (61-67). For example, thick unbranched networks of fibrin are created when low amounts of thrombin are generated in the presence of constant fibrinogen levels. Alternatively, thin fibrin fibers organized in dense and highly branched networks are formed when high amounts of thrombin are generated at constant fibrinogen levels (63, 64). The latter fibrin network structure is also produced when thrombin levels are normal, but fibrinogen levels are elevated (64-66). These variables controlling the process of fibrin network formation ultimately affect clot integrity and resilience to fibrinolytic degradation. Networks consisting of thick, unbranched fibers are typically unable able to handle greater amounts of stress and strain and are more susceptible to clot lysis (66, 68, 69). The dense, highly branched networks, however, are more durable and robust when subjected to fibrinolysis (66, 68, 69). The last significant contribution made to clot structure is performed by activated factor XIII (FXIIIa). After the fibrin network has formed, FXIII can bind to the fibrin, then become activated to FXIIIa by thrombin (70). FXIIIa is a transglutaminase that facilitates
crosslinking reactions between α chains, γ chains and α-γ combinations in order to fortify the clot structure (71, 72). Lastly, FXIIIa provides clot stability by incorporating fibronectin into the fibrin meshwork (73). This fibronectin increases clot fiber thickness and promotes platelet adhesion and aggregation (74). This process is crucial to ensuring that the fibrin clot is not prematurely degraded via fibrinolysis.

1.3 Regulating coagulation

The process of coagulation requires extensive regulation in order to function properly. If coagulation is impaired, inappropriate bleeding may result, as seen in hemophilia (2, 75). Alternatively, an overzealous coagulation process can produce prothrombotic conditions (2, 75). There are various molecules and pathways that contribute towards the negative regulation of coagulation.

The protein C pathway significantly contributes to anticoagulation under certain circumstances (76). Protein C is a single chain protein present as an inactive zymogen in circulation (77). The conversion of protein C to activated protein C (APC) is catalyzed upon the endothelial cell membrane by thrombin in complex with TM, a transmembrane cofactor that increases the ability of thrombin to activate protein C by more than 1000-fold (78, 79). The endothelial protein C receptor (EPCR) is also capable of amplifying this activation of protein C by approximately 20-fold (80). The anticoagulant function of APC is mediated by its ability to cleave and inactivate coagulation proteins FV/FVa and FVIIIa on the cell surface (78, 79). Certain aspects of these processes require the cofactor, protein S (81-83). This cofactor has a strong affinity for phospholipid
membranes that are negatively charged, allowing it to draw APC closer to the membrane and optimally position its active site for FV/FVa and FVIIIa inactivation (84, 85).

The function of APC also has a certain level of regulation within the circulation. There are several inhibitory agents that are able to react slowly with APC, giving APC a half-life of 20-25 minutes in plasma (86, 87). These include protein C inhibitor (PCI), plasminogen activator inhibitor 1 (PAI-1), α₁-proteinase inhibitor (α₁-P1), and α₂-macroglobulin (86-89). APC can also be removed from the bloodstream through endocytosis of the APC/EPCR complex (90, 91).

Tissue factor pathway inhibitor (TFPI) is a proteinase inhibitor that is capable of regulating the process of coagulation, more specifically the extrinsic pathway, as the name implies. It can inhibit various coagulation factors via a structural domain (Kunitz domain) that mimics the substrates of these factors. TFPI functions by inhibiting FXa, most efficiently when it is found within the prothrombinase complex (92-94). Additionally, TFPI can inhibit the TF/FVIIa complex (93, 95). Inhibition of this complex by TFPI requires a quaternary complex between TFPI, TF/FVIIa and FXa (93, 95). The requirement of Xa within this complex makes TFPI a negative inhibitor of the coagulation process as some Xa must be generated before it can perform its function (92).

A specific serine protease inhibitor (serpin) that is very pertinent to the process of endogenous anticoagulation is antithrombin III (AT III) (96). AT III is capable of strongly inhibiting thrombin and FXa (97). Additionally, FIXa, FXIa and XIIa are also less frequent targets of AT III (98, 99). The ability of AT III to inhibit thrombin and FXa is weak when it is alone (100, 101). When heparin or other heparin-like
glycosaminoglycans are provided by the endothelial cell membrane, they act as cofactors that enhance the inhibitory effects of AT III by over 1000-fold (100, 101). Due to the potent effect of heparin on AT III activity, heparin has been employed as an anticoagulant therapeutic (102). The importance of AT III is outlined by the fact that an AT III deficiency results in the highest risk for venous thromboembolism (VTE) out of all known inherited thrombophilias (103).

1.4 Fibrinolysis

Once the coagulation process has generated a fibrin network deposit and has effectively inhibited excessive blood loss from a point of injury, the process of fibrin degradation (fibrinolysis) must be activated in order to facilitate the healing process (104). Additionally, basal fibrinolytic activity functions to ensure the removal of any spontaneously formed fibrin deposits (105). The most important functional component of fibrinolysis is plasmin, the protease that cleaves the fibrin network, but many other regulatory components contribute to this process as a whole (106).

Plasmin is the activated form of the zymogen plasminogen and the process of plasminogen activation can be mediated by two glycoproteins. The major intravascular activator of plasminogen is tissue plasminogen activator (tPA) (107). Endothelial cells are responsible for the synthesis and secretion of tPA into circulation (108), where it has a very short half-life of approximately 5-10 minutes (109). Although tPA is produced in the single chain form (sc-tPA), plasmin is capable of cleaving tPA at the Arg275-Ile276 peptide bond, creating a double chain form (tc-tPA) (110). This functions as a positive feedback mechanism as the two-chained tPA has greater activity and affinity towards
plasminogen (111). The plasminogen activating ability of either form of tPA is fairly weak when in solution, but when tPA is bound to fibrin, it can activate plasminogen over 400-fold more efficiently (111, 112).

The other major endogenous activator of plasminogen is urokinase plasminogen activator (uPA). This glycoprotein is also produced in the single-chain form (sc-uPA) by endothelial cells, among others such as macrophages and some tumor cells (113, 114). The activity of this plasminogen activator does not improve in the presence of fibrin, as it does for tPA (115). However, similar to tPA, sc-uPA can be cleaved at the Lys158-Ile159 peptide bond (116) in order to yield a two-chained form (tc-uPA) with a plasminogen activating ability of about 100-fold greater than that of the single-chain form (117, 118). Additionally, the membrane-bound specific uPA receptor (uPAR) promotes plasminogen activation by localizing uPA to the membrane (119). Although tPA and uPA are the two major activators of plasminogen, up to 15% of plasminogen activation can be attributed to other activators such as kallikrein, FXIa, and FXIIa (120-123).

Plasminogen is the proenzyme form of plasmin and it is produced in the liver and is generated at a plasma concentration of approximately 1.5 µM (124). The structure of plasminogen includes five homologous “kringle” domains. These triple-loop structures are functionally crucial as the first (K1) and fourth (K4) kringle domains are each capable of binding lysine with a high and low affinity, respectively (125, 126). The function of plasminogen can be influenced by fibrin, inhibitors or cell surface receptors, and each of these interact with the lysine binding domains found on the kringle structures (125). Plasminogen becomes activated to plasmin when it is cleaved at the Arg560-Val561 bond (127). In circulation, plasminogen has a glutamic acid (Glu) residue on its amino-
terminus (Glu-plasminogen) (128, 129). This form of plasminogen can be proteolytically converted by plasmin to a version that contains a lysine (Lys) residue on its amino terminus (Lys-plasminogen) (128, 129). The Lys-plasminogen form has a greater affinity for fibrin and cellular receptors, and can be activated more effectively than Glu-plasminogen. Lys-plasminogen can be activated 11- and 9-fold more effectively than Glu-plasminogen by tPA and uPA, respectively (119, 130, 131).

As plasmin is formed, it can begin to lyse the fibrin network, effectively solubilizing the clot. First, plasmin begins by partially degrading the carboxyl-terminal area of α and β chains in the D domain of crosslinked fibrin (132, 133). Following this, plasmin continues to cleave connections between D and E domains of the fibrin crosslinks, eventually releasing the recognizable “D-dimer” fibrin degradation product (132, 133). The initial, partial degradation of crosslinked fibrin is critical to the acceleration of this entire process. When this occurs, carboxyl-terminal lysine residues become exposed. These residues are responsible for docking plasminogen and tPA to the clot, as well as facilitating the Glu-Lys plasminogen conversion (130). They also provide sites for binding of plasmin to fibrin, which results in the protection of plasmin from its main inhibitor α2-antiplasmin (vide infra) (134). Therefore, exposure of these residues creates extensive positive feedback to plasmin activation. In addition to the aforementioned mechanisms of plasminogen activation, various cell surface plasminogen receptors can also contribute to this process. For example, the annexin 2, α-enolase, glycoprotein IIb/IIIa and integrin αMβ2 receptors are capable of potentiating plasminogen activation on the surface of endothelial cells, monocytes, platelets, and leukocytes, respectively (135).
1.5 Regulating fibrinolysis

Just as it is important to regulate the extent to which coagulation occurs, the fibrinolytic pathways must also be carefully regulated. Hyperfibrinolysis could result in premature fibrin clot lysis or inadequate clot durability, which could result in hemophilia-like bleeding tendencies after trauma (136). A potent method of fibrinolytic regulation occurs via the inhibition of plasmin, which is commonly accomplished by certain circulating serpins (99). A member of this group that is responsible for significant amounts of plasmin inhibition is α2-antiplasmin. With a half-life of approximately 2.6 days and a plasma concentration of approximately 2.5 g/L, α2-antiplasmin is readily available to complex with plasmin (137). This inactive complex is subsequently cleared from circulation within the liver. Plasmin can also be inhibited by α2-Macroglobulin, which is a non-serpin found in platelet α granules and is produced by endothelial cells and macrophages (106). Moreover, this protein only exhibits 10% of the inhibitory activity that α2-antiplasmin is capable of, thus its role in plasmin inactivation is less important (138). Another serpin with a minor role in plasmin inhibition is proteinase nexin (139).

An additional method of fibrinolytic regulation is through the impediment of plasminogen activation. Plasminogen activator inhibitor (PAI) proteins are members of the serpin family that inhibit the functions of both tPA and uPA. Of the two major PAIs, PAI-1 is the most important and efficient inhibitor of plasminogen activation and is produced by endothelial cells, platelets, monocytes, macrophages and adipocytes (140). A complete deficiency of this critical glycoprotein results in a moderately severe bleeding
disorder (141). PAI-2, which only has a significant presence in plasma during pregnancy, is also capable of tPA and uPA inhibition, but to different degrees than PAI-1 (142). PAI-2 can inhibit the two-chain forms of tPA and uPA, but is a much less effective sc-tPA inhibitor, and is not capable of inhibiting sc-uPA at all (143). In addition to the PAIs, a minor contributor to the downregulation of plasminogen activation is c1-esterase inhibitor, which is capable of direct tPA inhibition (144).

A reduction in fibrinolytic activity can result from the function of FXIIIa. After thrombin activates FXIII, the structural reinforcement that FXIIIa provides to the fibrin architecture provides a form of protection from the digestive enzyme, plasmin (145). Moreover, as FXIIIa carries out crosslinking reactions, it can also reduce the extent of plasminogen activation (146). As previously noted, plasminogen and tPA contain lysine binding domains that facilitate their interactions with fibrin. The crosslinking reaction performed by FXIIIa reduces the exposed lysine residues available for plasminogen and tPA, thus affecting the positive feedback mechanisms of plasminogen activation (147). In addition to fibrin, α2-antiplasmin is also a substrate for FXIIIa activity and FXIIIa crosslinks α2-antiplasmin to the fibrin structure, holding within the clot, effectively increasing its ability to inhibit plasmin activity (148).

Lastly, thrombin-activatable fibrinolysis inhibitor (TAFI) also contributes to the downregulation of fibrinolysis (Fig. 1.2). TAFI exerts its function in a manner analogous to that of FXIIIa. The exposed lysine residues on the fibrin network are efficiently removed by the activated form of TAFI (TAFIa), thus abolishing the aforementioned positive feedback mechanisms of plasminogen activation (149).
Figure 1.2 TAFI and the mechanisms of fibrinolysis. When the coagulation process generates sufficient amounts of thrombin necessary to initiate fibrinogen cleavage and fibrin aggregation, the process of fibrinolysis begins. Fibrin acts as a cofactor for the process of plasminogen activation. As plasmin begins to degrade fibrin, C-terminal lysine residues become exposed on the surface (Fibrin’). These residues facilitate a strong positive feedback response for plasminogen activation. When TAFI is activated, it removes these exposed lysine residues, abolishing this strong positive feedback, resulting in the attenuation of fibrinolysis. Eventually, fibrin becomes degraded into fragments known as fibrin degradation products (FDPs).

1.6 Thrombin-activatable fibrinolysis inhibitor

The discovery of TAFI was made by four different research groups. In 1989, Hendriks et al. discovered unstable basic carboxypeptidase activity in serum that could not be attributed to carboxypeptidase N (CPN), another basic carboxypeptidase found in
plasma (150). This activity disappeared after two hours at 37°C, thus attracting the name “unstable carboxypeptidase” or CPU (150). Around the same time, a carboxypeptidase was discovered by Campbell and Okada that displayed a functional preference for substrates that contained arginine, as opposed to lysine (151). Consequently, they named the enzyme arginine carboxypeptidase (CPR) (151). In 1991, Eaton et al. discovered a protein which they named plasma procarboxypeptidase B (152). They found that this protein bound to plasminogen in plasma and after analyzing the cDNA sequence from the gene encoding this protein, found that it was homologous to the pancreatic procarboxypeptidase B (152). Two years later, Bajzar and Nesheim discovered that the profibrinolytic potential of protein C could be attributed to the reduction in the thrombin-mediated activation of a specific enzyme that functioned against fibrinolysis (153). This created the name thrombin-activatable fibrinolysis inhibitor (TAFI) (153). Although they reported that this enzyme was in fact identical to plasma procarboxypeptidase B found by Eaton et al. (153), amino acid sequence analysis ultimately determined that CPU, CPR, plasma procarboxypeptidase B and TAFI were all the same protein (154, 155).

1.6.1 General properties of TAFI

TAFI is primarily synthesized in the liver (152) and by megakaryocytes (156, 157) as a prepropeptide consisting of 423 amino acids and can potentially be N-glycosylated at four sites in the activation peptide (Asn22, Asn51, Asn63, Asn86) (152). Cleavage of the 22-amino acid signal sequence from “proTAFI” would theoretically result in a 401 amino acid protein with a molecular weight of 45,999 Da, but the extent of glycosylation causes TAFI to appear as a $M_r \approx 60,000$ band during sodium dodecyl
sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (158). Cleavage of the signal sequence results in mature TAFI being secreted from the cell, creating two pools of TAFI. The greatest proportion of TAFI can be found in circulation, where plasma levels of TAFI range from 73-275 nM or 4-15 µg/mL (159, 160). Alternatively, less than 0.1% of total TAFI is stored as an alternatively glycosylated population that is stored within the α-granules of platelets (approximately 50 ng per 10 platelets) and can be released upon platelet activation (156, 157). Although this is a small amount, the local concentrations of this population of TAFI can be compounded by higher orders of platelet recruitment, thus implying the functional relevance of platelet-derived TAFI.

1.6.2 The regulation of TAFI production

*CPB2* is the gene that encodes the TAFI protein. This gene has a chromosomal location of 13q14.11 and is transcribed mainly in the liver (154). The gene itself is comprised of approximately 48 kilobase pairs that include 11 exons (161). Transcription of this gene from approximately 11 different initiation sites results in 5’-untranslated regions (UTR) ranging from 9-46 base pairs; the promoter lacks a consensus TATA sequence (161). These transcriptional properties are characteristic of several other liver-derived coagulation factors, including FVII, FIX, FX, FXII and protein C (161). Potential regulation of *CPB2* transcription can also occur via a CCAAT/enhancer binding protein (C/EBP) binding site and a glucocorticoid response element within the promoter region (162). As a result of three different polyadenylation sites, *CPB2* transcripts can have a 390, 423 or 549 nucleotide 3’-UTR, consequently providing the transcript with less stability as the size of the 3’-UTR increases (163). There has been a total of nineteen
SNPs detected in the \textit{CPB2} gene, with ten in the 5’ flanking region, three in the 3’ flanking region and six in the coding region (164). Most of the SNPs in the coding region correspond to silent mutations, but two (+505G/A and +1040C/T) correspond to Thr147Ala and Thr325Ile substitutions, respectively (165, 166). The T147A polymorphism does not affect any of the functional characteristics of TAFI/TAFIa (167). Contrary to this, an Ile substitution at residue 325 in the TAFI protein accounts for a two-fold increase in the stability of the active TAFIa species, translating to a higher antifibrinolytic activity (1.6-fold) (167).

Ultimately, the 3’ flanking SNPs of \textit{CPB2} have the potential to regulate transcript stability, the 5’ flanking SNPs can alter transcript regulation and the SNPs within the coding region may affect protein production (168). The potential effect of the 3’-flanking SNPs on the production of TAFI has been proven as there have been strong associations made between these SNPs (but not the 5-flanking SNPs) and plasma TAFI levels (169-171). However, only 25% of the variation of TAFI levels within plasma can be attributed to the known polymorphisms (168).

The remaining 75% of TAFI regulation can be attributed to numerous non-heritable contributions. First, the production of TAFI has been shown to be under hormonal influence. Glucocorticoids lead to increased expression of \textit{CPB2} mRNA (172), and although the exact effect remains to be elucidated, sex hormones have been shown to affect TAFI levels as well (168, 173-176). However, more conclusive studies have shown that in women, plasma TAFI becomes elevated with age (177) and throughout the duration of pregnancy (178-180). Likely due to its contribution to inflammatory regulation, TAFI levels are also affected by situations involving inflammation and
disease. On a cellular level, Interleukins (IL-1β and IL-6 promote larger, less stable $CPB2$ transcripts, thus reducing mRNA abundance (163, 172). Inflammatory disorders such as celiac disease, Behcet’s disease, inflammatory bowel disease and Reiter’s syndrome have been associated with increased levels of plasma TAFI, but the exact mechanisms that drive these associations are undetermined (181, 182). To further complicate this relationship, other studies have shown contradictory results to this, reporting decreased TAFI levels during endotoxemia (183) and a short-term TAFI decrease, followed by a later significant increase was found in mice models (184). Increased TAFI levels have also been seen in other diseases such as diabetes (185, 186), nephrotic syndrome (187), lung cancer (188) and for certain events, including kidney transplantation (189), peritoneal dialysis (190), and under elevated levels of low density lipoprotein (LDL)-cholesterol (191). Alternatively, as a consequence of affecting the source of TAFI expression, liver disease is associated with reduced plasma TAFI concentrations (192).

### 1.6.3 Quantifying TAFI

Throughout the years of TAFI research, the quantification of this protein has been challenged by several issues. These difficulties may contribute to the large range of TAFI concentrations detected in plasma to date. Although numerous strategies exist to measure TAFI levels, each with their own advantages and disadvantages, they each fall within three main categories: immunological, enzymological or functional. The standard immunological-based method for TAFI measurement is an enzyme-linked immunosorbent assay (ELISA) (193, 194). Although these methods have the potential to
be automated, there are various problems associated with TAFI detection. Both commercial and “in house” antibodies have been developed, but for some, the relative extent of activity towards TAFI, TAFIa and the inactivated form of TAFI (TAFIai, vide infra) differ between one another (195). Moreover, some antibodies are unable to distinguish between TAFIa and TAFIai, thereby allowing for TAFI activation to be assessed, but not necessarily levels of functionally active TAFIa (196). The T325I polymorphism also introduces variability into these assays as antibodies can react with each isoform differently, often binding to the I-325 variant to a lesser extent (197, 198).

TAFI can also be measured by taking advantage of the enzymatic activity of TAFIa basic carboxypeptidase activity. Two substrates that are commonly used for this purpose are anisoylazoformylarginine (AAFR) and hippuryl-arginine (Hipp-Arg) (168, 199). Each of these colorimetric substrates are useful in purified systems, but are not optimal for use in blood or plasma samples as they contain carboxypeptidase N, which is also capable of hydrolyzing these substrates (200). Recently, a sensitive TAFI specific substrate, Bz-o-cyano-Phe-Arg was privately developed, but it is used in conjunction with high performance liquid chromatography, making it a very labor intensive alternative (201).

Lastly, TAFI levels can be assessed using functional methods that quantify the physiological function of TAFI. This is most commonly done by monitoring clot formation and lysis turbidimetrically in plasma and assessing the ability of TAFIa to prolong the time required for clot lysis (202). By using this method, greater effects are able to be detected when the more stable Ile325 variant is present. Recently, thromboelastography has been used for the same purpose (203). Although less sensitive
than plasma clot lysis assays, this method uses more physiologically relevant whole blood clots.

1.6.4 TAFI activation and thrombomodulin

The structure of the TAFI protein contains a segment (Phe1-Arg92) called the activation peptide. Cleavage at Arg92 removes the activation peptide, which allows substrates to access the underlying active site. Although the reaction proceeds slowly, thrombin is capable of cleaving the Arg92 residue of TAFI, creating TAFIa (152, 204). Plasmin is also capable of activating TAFI, at an efficiency that is 8-fold greater than thrombin alone (205). Moreover, when a glycosaminoglycan (GAG) is present, plasmin-mediated TAFI activation is enhanced 16-fold (205). The most potent activator of TAFI is the complex of thrombin and thrombomodulin. This complex mediates TAFI activation with an efficiency that is 10-fold greater than plasmin/GAG, which is about 1250-fold greater than thrombin alone (204). Strong evidence suggests that thrombomodulin, through binding to both thrombin and TAFI, is able to present the Arg92 residue of TAFI in a more efficient orientation for cleavage by thrombin (206). Although the magnitude of TAFI activation is significantly different between thrombin, plasmin and the thrombin/thrombomodulin complex, various experiments show that they all play a role in physiological TAFI activation, including the use of monoclonal antibodies to block Arg92 cleavage by each respective activator (202, 207, 208). Moreover, it has been shown that hindering the intrinsic pathway of coagulation via inhibition of FVIIIa, FIXa, FXa or FXIa results in less TAFI activation and a reduction in the prolongation of clot lysis time, without contributions from the thrombin/TM complex (209, 210).
Thrombomodulin is a transmembrane glycoprotein that can be found on the surface of blood vessel endothelial cells. Soluble forms of this protein can also be generated by enzymatically cleaving the protein off the membrane surface (211). The amino-terminus of thrombomodulin contains a lectin-like domain. The rest of the structure consists of six endothelial growth factor (EGF)-like repeats (EGF1-6), a region rich in serine and threonine residues, and a transmembrane domain that separates these structures from a small cytoplasmic tail (212). EGF 5-6 is the region responsible for binding thrombin, specifically via the anion-binding exosite I of the thrombin protein (Fig. 1.3). Moreover, EGF 4-6 is structurally required for TM to facilitate protein C activation (213). In order for TM to facilitate the activation of TAFI, EGF domains 3-6 are required (214).
Figure 1.3 Structural requirements of thrombomodulin. The structural characteristics of thrombomodulin are outlined here, with emphasis on the EGF domains. The domains required for thrombin binding, protein C activation and TAFI activation are labelled accordingly. Figure modified from (215).
TM levels can vary greatly, depending on specific conditions at any given time. Normally, the amount of soluble TM that can be found within the plasma ranges from approximately 0.04 to 0.7 nM (216, 217). Under pathologic conditions, such as during vascular damage, infections, sepsis and/or inflammation, soluble levels can reach about 4.4 nM (218-222). When including the contribution of membrane-bound TM from the endothelium, effective TM concentrations can range from approximately 1-100 nM in vasculature. Throughout circulation, the high end of this range is found in the capillary bed or microvasculature where surface to volume ratio is at a maximum (217, 223-225). The lower end of this spectrum can be found in large vessels (226).

The levels of TM within circulation greatly contribute to the function of TAFI, in part because thrombin/TM is the most potent TAFI activator. Additionally, a biphasic pattern exists with regard to TM concentration and TAFI activation (227). At lower concentrations of TM, TAFI activation is optimal and the resulting effect is antifibrinolytic. However, as TM levels rise, the thrombin/TM complex begins to activate greater amounts of protein C. As a result, APC diminishes the coagulation response, providing less available thrombin for coagulation and TAFI activation, ultimately evoking an anticoagulant and profibrinolytic effect (227).

1.6.5 TAFIa inhibition and inactivation

To date, an endogenous TAFIa inhibitor has yet to be discovered in humans, however, several exogenous and synthetic inhibitors have been characterized. The lysine analog 2-guanidinoethylmercaptopussuccinate (GEMSA) and zinc-chelating agents such as o-phenanthroline and ethylenediaminetetraacetic acid (EDTA) may be used to inhibit
TAFIa function (202, 228). Additionally, carboxypeptidase inhibitors from potato tuber (PTCI), leeches (LCI) and ticks (TCI) are used to inhibit TAFIa (229-231). Certain reversible inhibitors of TAFIa are capable of exerting a biphasic effect on the function of this enzyme. At certain low concentrations, these inhibitors will actually stabilize TAFIa, leading to a paradoxical antifibrinolytic effect. For example, a 1 mM concentration of GEMSA or 100 nM concentration of PTCI causes TAFIa to prolong clot lysis time from 100 minutes to 350 minutes and 240 minutes, respectively (232).

The aforementioned TAFIa inhibitors are not present under physiological conditions and there are no known endogenous inhibitors of TAFIa. Consequently, the regulation of TAFIa enzymatic activity relies exclusively on its intrinsic instability (150). When in its zymogen form, the activation peptide of TAFI plays a role in maintaining stability of the protein. The original hypothesis was that the Val35/Leu39 residues of the activation peptide interacted with Tyr341 to stabilize the zymogen (Fig. 1.4). However, a recent paper by Zhou et al. created a TAFI variant with residues 1-73 deleted that was just as stable as the wild-type protein, indicating that the Ala74-Arg92 region is important for this stability (158, 233, 234). Without the activation peptide, the intrinsic instability of TAFIa results in a half-life of approximately 10 minutes at 37°C and approximately 2 hours at room temperature. Alternatively, at 0°C, TAFIa is stable indefinitely (158). The short half-life of TAFIa within circulation is not due to proteolytic cleavage of the enzyme, but rather to a conformational change (235). The region of TAFIa within residues 296-350 (234) plays an important part in the stability of the enzyme and is called the “dynamic flap”. The active site of TAFIa becomes disrupted when this region undergoes irreversible changes as a result of random movement. The indefinite stability
of TAFIa at 0°C supports this notion, as this movement would be minimized at such a low temperature. Additionally, the stabilizing effects of the reversible inhibitors previously mentioned can be attributed to their ability to reduce the flexibility of the dynamic flap (234, 236-238). Several unnatural mutations within this area have been found to result in a stabilizing effect, thereby increasing the overall stability of TAFIa. Ceresa et al. (239) combined each of these known mutations and constructed TAFI Ser305Cys/Thr325Ile/Thr329Ile/His333Tyr/His335Gln (TAFI-CIIYQ). This combination of substitutions results in a 180-fold increase in stability, which is the greatest observed for any construct to date (239). After TAFIa has been thermally inactivated to TAFIai, proteolytic cleavage by thrombin can occur at Arg302 and cleavage by plasmin can occur at Arg302, Lys327 or Arg330 (240, 241). Moreover, at high concentrations of plasmin, TAFIa may be cleaved by plasmin at Arg302, Lys327 or Arg330 prior to any conformational changes occurring, resulting in a population of fragmented, inactive TAFIa species (241).
Figure 1.4 Key components of TAFI structure. Several important structural characteristics of TAFI are outlined in the context of the TAFI crystal structure. The activation domain is coloured blue and the instability region is coloured orange. The Zn$^{2+}$ ion found within the active site of TAFI is indicated. The inset highlights the key interactions between residues in the activation domain (Val35 and Leu39) and in the instability region (Tyr341) that contribute to the stability of the TAFI zymogen. Adapted from (234).

1.6.6 TAFIa function

As a basic carboxypeptidase, TAFIa functions by cleaving lysine and arginine residues from the carboxyl-termini of peptide substrates via a Zn$^{2+}$-ion-catalyzed hydrolysis reaction (164). In this reaction mechanism, the Zn$^{2+}$ ion is coordinated by His159, Glu162, and His288, while residues Arg235, Tyr341, and Asp348 contribute to substrate binding. Arg235 and Tyr341 interacts with the carboxylate group of the arginine or lysine substrate, while the basic side chain of the substrate forms a salt bridge
with Asp348. The Zn$^{2+}$ and the Glu363 residue are then able to activate a water molecule, which is required to hydrolyze the peptide bond, via a nucleophilic attack on the carbonyl carbon of this bond (164).

The physiological function of TAFIa is exerted by hydrolyzing the exposed carboxyl-terminal lysine residues from fibrin that has been partially degraded by plasmin (149). As previously stated, these lysine residues are critical to the positive feedback mechanisms of the fibrinolytic process. Plasminogen and tPA bind to the fibrin surface through interactions with these lysine residues, which allows tPA to facilitate plasminogen activation more efficiently. Additionally, these lysine residues stimulate the conversion of Glu-plasminogen to Lys-plasminogen, a form of plasminogen that is activated by tPA more efficiently (149). Lastly, plasmin can bind to the fibrin surface via exposed lysine residues, which protects this enzyme from inactivation by α₂-antiplasmin (134). Thus, by removing an integral part of these feedback mechanisms, TAFIa effectively attenuates fibrinolysis, consequently stabilizing the fibrin network in a clot.

1.7 Virchow’s triad and thrombosis

In the 1700’s, Rudolph Virchow developed the concept that the pathophysiological mechanisms of thrombosis were driven by a “triad” of abnormalities (Fig 1.5). These three components consisted of flaws in vascular endothelium function, blood constituents, and blood flow or shear (242, 243). Thrombotic risk is consequently dictated by combinations of these factors. Two main processes contribute to the first member of this triad, vessel wall function. Integrity of the vascular endothelium is critical because platelets and leukocytes are recruited to the site of vascular injury via exposed
adhesion molecules. Here, these cells accumulate, display TF and form a functional procoagulant surface (24). For example, the greater the presence of TF in an atherosclerotic plaque, the more thrombogenic a plaque will be (244). Additionally, venous thromboembolism (VTE) can be associated with an increase in TF production or a decrease in TM production, caused by inappropriate expression of proinflammatory mediators, including cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin 1β, as well as bacterial lipopolysaccharide (66, 245-247). Various cells, such as platelets, erythrocytes, endothelial cells, leukocytes, megakaryocytes and even tumor cells, are able to release microparticles into the circulation, which are not confined to the vessel walls (248). Although the exact function of microparticles in thrombosis and hemostasis is not completely understood, several pathogenically relevant associations have been revealed over recent years. For example, microparticles derived from leukocytes are rarely found in healthy individuals. However, individuals suffering from diseases such as diabetes (249), sickle cell disease (250) and cancer (251, 252) produce elevated levels of this derivation of microparticle. Furthermore, associations have been made in cancer patients between venous thromboembolism and tumor-derived microparticles (253, 254). Microparticles have also been shown to increase their expression of procoagulant factors such as TF or phosphatidylserine in situations involving factors such as inflammation or stasis (255-258). This activity was shown to spike before an event of venous thrombosis in some patients (252).
Figure 1.5 Virchow’s Triad. A schematic representation of the three components of Virchow’s triad. Virchow stated that hypercoagulability of the blood, endothelial damage, and blood stasis are critical factors in the development of thrombosis.

The second factor of Virchow’s triad is procoagulant activity within blood plasma. This activity is mainly due to the elevation of coagulation factor levels (hypercoagulabilities). For example, a mutation (G20210A) in prothrombin is associated with elevated prothrombin levels (hyperprothrombinemia) and consequently, a risk for venous thrombosis (259). Under these circumstances, when coagulation is initiated, the rate and peak of thrombin generation are heightened (260, 261). This effect on thrombin generation leads to an increased risk for thrombosis because it results in a complex network of fine fibrin fibers (262) that is further protected by an elevated level of activated TAFI (263). Another genetic abnormality known as the FV Leiden mutation results in the production of a FV variant that is resistant to cleavage by APC, effectively
diminishing that particular anticoagulation mechanism (264). Another blood characteristic contributing to coagulopathy is faulty synthesis, formation or function of fibrinogen or fibrin. An overproduction of fibrinogen (hyperfibrinogenemia) is a risk factor for venous and arterial thrombosis (265-267) as during coagulation, the formation of elevated amounts of fibrin is accelerated, providing a dense fibrin network. The result is a robust, stable fibrin network more resistant to lysis (66). Moreover, there are numerous genetic mutations associated with the expression of fibrinogen variants that function improperly (dysfibrinogenemia), resulting in densely packed fibrin networks resistant to fibrinolysis (268). Various prothrombotic disorders such as acute coronary syndrome, hyperhomocysteinemia or diabetes have also been associated with a process called “acquired dysfibrinogenemia”. Here, fibrinogen is targeted by unfavorable posttranslational modifications such as oxidation, nitration, glycation or homocysteinylation, which results in a modification of the fibrin network and an increase in network stability (268).

The last component of Virchow’s triad is the role of blood flow, which is mainly defined by shear stress and rate. In terms of blood flow through the vasculature, shear stress is defined as the tangential force exerted by the blood on the surrounding endothelium (269). Shear stress is calculated as the product of shear rate and blood viscosity, meaning that shear stress is proportional to the rate of blood flow through a vessel. The diameter of the endothelium is a determining factor of the velocity of blood flow through a vessel. Blood vessels are capable of regulating their respective diameter, thus providing a method of shear stress control. Under normal conditions, the majority of arterial vessels maintain a shear stress of 5-10 dynes/cm² (270, 271), which is said to
counteract coagulation, thrombosis, inflammation and hypertrophy, while promoting fibrinolysis (271, 272). During extended periods of immobility or after trauma, venous blood flow can become significantly reduced or even static, which diminishes the shear stress experienced by the vessel wall. This stress reduction contributes to the association of stasis with a risk of venous thrombosis. For example, the endothelium contains transcription factors, such as Kruppel-like transcription factor (KLF), which are expressed in response to shear stress. With increasing shear stress, KLF expression increases, which functions to induce the expression of antithrombotic and anti-inflammatory proteins such as thrombomodulin (273-275). Alternatively, when stasis reduces shear stress, KLF expression is downregulated, which allows for an increase in expression of procoagulant factors such as TF and vascular cell adhesion molecule-1 (VCAM-1) (276, 277). The significant increase in blood pressure seen in individuals with hypertension can also be prothrombotic, as this pressure can stimulate endothelial cells to increase TF activity (278) and the mechanical damage associated with this pressure can also elevate levels of microparticles derived from both platelets and the endothelial surface (279).

The rate of flow or shear rate for the blood flowing through a vessel also has important implications in thrombotic processes, as this dictates the rate at which contributing agents, such as platelets or coagulation factors, are brought towards and away from a site of interest, amongst other things (280). For example, the rate at which fibrinogen is brought to a point of initiated coagulation dictates the extent of fibrin production and polymerization of that site. Increasing shear rate at these locations reduces the amount of fibrin present and causes a reduction in protofibril extension and
lateral aggregation (281). The opposite can be seen under conditions of low shear rate (281). Moreover, low flowing conditions during the formation of the fibrin network provides a condition where fibrin aggregates are not mechanically disrupted, but are still able to create well branched linkages of thick fibers, thus creating a stable network more resistant to lysis (282-284).

Shear rate can be significantly influenced by a stenotic region within a blood vessel. Although under normal conditions shear rates within veins are approximately 10-100 s\(^{-1}\) and 500-1500 s\(^{-1}\) within the arteries. Stenosis can spike shear rates towards 70,000-250,000 s\(^{-1}\) in extreme cases (285, 286). Furthermore, a stenosis can disturb the flow of blood, which may result in a region of oscillatory blood flow and low shear stress immediately downstream from the blockage. Consequently these locations, called “recirculation zones”, retain blood materials for longer periods of time (287). This flow pattern also has important implications on platelet function. As platelets and leukocytes pass through the high-shear stenotic region they become activated, thus promoting their aggregation; this can have negative consequences, especially if they become subsequently stalled in a recirculation zone (287-289).

1.7.1 Venous Thrombosis

Although introduced centuries ago, Virchow’s triad remains a very relevant concept. An important part of this work was the premise that each aspect of the triad contributes to the development of various thrombotic disorders, and that the risk for such disorders becomes compounded by the influence of multiple triad facets. There are many pathological conditions associated with venous/arterial thrombosis and each can be
related in some way to the factors described by Virchow.

Venous thrombosis is the formation of an occlusive clot at a location within the venous system. The progression of and risk factors that contribute to VT are different from that of arterial thrombosis, mainly exemplified by the fact that venous thrombi are formed under much lower shear stress and are fibrin rich. The most common form of VT is deep vein thrombosis (DVT), which commonly occurs in the legs. There are many possible negative consequences of VT such as pulmonary or systemic embolisms, which will be discussed further at the end of this section.

One mode of VT formation is through the classic injury-induced coagulation process commonly associated with surgery. Aside from this, it is rare to see any endothelial injury associated with the onset of VT (290). As such, there likely are alternative mechanisms contributing to VT development (Fig. 1.6). Evidence shows that the valves found within venous circulation are an important contributor to VT, as they are frequently sites of thrombus formation (290-292) and elevated valve numbers within the body are associated with increase VT development (293). The most important implication of these valves is that stasis is a common characteristic of valve sinuses (294). Without the proper replenishment of flowing blood, these sinuses can develop hypoxic conditions and can be associated with an increase in hematocrit (295). This hypoxic state can contribute to thrombosis by activating the endothelial cells in the proximity, causing a downregulation in endothelial surface anticoagulant proteins (226, 296, 297) or by upregulating endothelial procoagulant proteins such as tissue factor (297-299). Moreover, hypoxia is also capable of inducing the expression of vWF, E-selectin and P-selectin on the endothelium (300-302). P-selectin and E-selectin are adhesion molecules that bind to
their respective ligands in order to mediate cell-cell interactions (303). This activity could potentially facilitate the recruitment of platelets, leukocytes or microparticles derived from leukocytes, which have been shown to play a role in thrombus formation (304, 305). Thrombus formation has also been shown to depend on P-selectin and TF function, which are characteristic of monocyte-derived microparticles (255, 305-308). To recapitulate, venous thrombosis most commonly develops in the following general sequence: blood flow stasis creates a hypoxic environment that causes endothelial activation and modulates endothelial expression to favor coagulation. This activated endothelium is then capable of recruiting microparticles as a source of TF, resulting in the initiation of coagulation. Fibrin formation is followed by the recruitment of platelets, which expand the thrombus size. This sequence is supported by venous thrombus structure which consists of a red, RBC and fibrin rich segment that resides adjacent to the endothelium, which engulfs white, platelet rich portions (290, 291). This is also explains why antiplatelet medications are less effective towards VT than those that target coagulation (309).
Venous versus arterial thrombosis. Thrombi formed in the veins (A) and arteries (B) differ in terms of their initiation, progression and architecture. The valves found within veins greatly contribute to the formation of thrombi. Normal, laminar blood flow is disrupted by valves, creating areas of recirculating blood and a potentially hypoxic environment. Hypoxia can, in turn, activate the epithelium, creating a range of procoagulant effects, including tissue factor (TF) expression. TF can then begin the extrinsic pathway of coagulation, causing the development of a fibrin network that traps many red blood cells, hence the formation of a red clot. Conversely, in arterial thrombosis, an atherosclerotic plaque plays an important role. This plaque narrows the vessel, causing rapid flow adjacent to the stenosis, followed by an area of disrupted flow and increased stasis due to recirculating blood. The abundant inflammatory mediators in combination with the high shear stress over the stenosis contribute to platelet activation.
Any lesions or erosions of the plaque’s fibrous cap expose the inner thrombogenic core. These conditions create a favorable environment for platelet adhesion and aggregation, thus creating a platelet-rich, white clot. Figure adapted from (243).

The known risk factors for VT represent all three components of Virchow’s triad. As previously mentioned, increased levels of coagulation factors such as FVIII, vWF, FVII, prothrombin or the antifibrinolytic protein, TAFI. The incidence of mutations such as factor V Leiden or prothrombin 20210A can also be associated with elevated VT risk (310-312). Furthermore, a lack of or deficiency in various anticoagulant proteins will also contribute to VT risk (312-316). There is a strong correlation between VT risk and older age. This may be caused by a range of factors including an age-dependent increase in procoagulant proteins and an increase in body mass, which is important as obese individuals have elevated levels of FVIII and IX (317, 318). Moreover, older individuals are more prone to infection and have reduced physical activity, especially when associated with illness, which creates a state of stasis (317). Cancer is also associated with a six to ten-fold increase in VT risk, which may be attributed, in part, to the fact that TF-bearing microparticles are released from tumor cells (319, 320). Cancer related surgeries, as well as hip and knee replacement surgeries pose a heightened risk for VT, due to the incidence of venous damage and procedure/immobility-based stasis (321, 322). Consequently, about 80% of individuals who undergo hip or knee replacement surgery and do not receive anticoagulant prophylaxis will experience DVT (323). One of the greatest risk factors for VT is the previous occurrence of a thrombotic event as about 25% of these events occur in individuals who have already suffered one (324). Consistent with Virchow’s triad and the synergistic nature of multiple contributions to thrombosis, it
is well known that the aforementioned factors dramatically increase risk when present simultaneously. For example, the FV Lieden mutation results in a seven-fold increase in VT risk and the use of an oral contraceptive is associated with a four-fold increase. Consequently, with both of these characteristics present, an individual will have a 35-fold increase in VT risk (312).

In the event that an individual establishes conditions sufficient to initiate VT, several consequences may arise. A common subsequent event to DVT is a pulmonary embolism (PE). Many times, these events are classified collectively as venous thromboembolism (VTE). This condition occurs when a developing venous thrombus, or pieces of this thrombus, detach from its site of origin and travel throughout the blood stream, at which point the thrombus is called an embolus. This embolus is then capable of travelling towards the heart and lungs and can potentially create a blockage in the arteries within the lungs, thus establishing a pulmonary embolism (325). Depending on the size and severity of the embolism, the mortality rates of such an event range from approximately 1% to as high as 80% (326).

1.7.2 Arterial Thrombosis

There are many components that play a role in the progression of atherosclerosis from a normal endothelium to an atherothrombotic event (Fig. 1.7). To initiate this process, the normal endothelium becomes activated by risk factors such as the accumulation of lipoproteins in the intima of the blood vessel (327). Once this occurs, the endothelial cells begin to recruit inflammatory leukocytes through the production of chemoattractants and adhesion molecules (328, 329). The accumulation of lipids then
continues and lipoproteins become modified. As monocytes are recruited to the area, they differentiate into macrophages (330, 331). These macrophages are then able to engulf the modified lipoproteins, which causes them to become plaque-forming foam cells (332, 333). During this time, the activated endothelium and leukocytes that are already present in the area secrete more inflammatory cytokines to further amplify leukocyte recruitment and initiate the migration and proliferation of smooth muscle cells (334). As the growth of this atherosclerotic plaque continues, the presence of high levels of inflammatory mediators causes an increase in the production of tissue factor and proteinases that degrade the cellular matrix (335, 336). If these proteinases threaten the structure of the fibrous cap containing the lipid rich core of the plaque, a rupture may occur, resulting in contact between blood and the abundant source of procoagulant factors inside the plaque (Fig. 1.6) (337-339). Although less frequent than rupture, the physical erosion of the superficial plaque layer can also expose the constituents of the inner core of the plaque (340). Intra-plaque components such as collagen are then able to mediate the adhesion to and aggregation of platelets. Subsequent to the recruitment of platelets, the TF from the plaque core initiates coagulation. Due to the order of these events and the high shear nature of the arterial environment, a “white” platelet-rich thrombus is developed (341). This thrombus is associated with a great risk of negative consequences, such as acute coronary syndromes, as it may occlude the vessel or the thrombus may break off (embolize), posing a risk for stroke. Lastly, after the formation of a thrombus, the initiation of the healing response may cause collagen and smooth muscle cells to accumulate upon the plaque, causing a fortified, often calcified, fibrous plaque that creates stenosis (342-344).
Figure 1.7 Atherogenesis. The initiation and progression of atherosclerosis requires several steps that lead to a risk of rupture and thrombus formation. Briefly, as LDL begins to accumulate under the endothelium in the intima of the blood vessel, it becomes modified. This is associated with the activation of the endothelium, causing it to attract inflammatory leukocytes, such as monocytes. Monocytes can cross the endothelium to reach the modified lipoproteins and engulf them, forming foam cells. As foam cells accumulate and inflammation propagates, increased levels of tissue factor (TF) and various proteases will accumulate in the core of the plaque. The proteases threaten the integrity of the plaque, potentially leading to a rupture. Prothrombotic material such as collagen and TF can initiate a thrombus in the exposed blood. Adapted from (257).

There are numerous risk factors associated with the progression and pathogenesis of atherosclerosis, many of which differ from the risk factors for VT. Some of these risk factors are influential in causing this disease, but others may just be associated with its pathology (345). In general, a greater risk is associated with older age, obesity, immobility, or elevated serum cholesterol, LDL-cholesterol and triglycerides, for example (346). More specifically, many studies have shown that increased levels of C-reactive protein is a strong, independent risk factor for adverse cardiovascular events following atherosclerosis (347-350). Some studies have shown the role of this protein in promoting receptor-mediated uptake of low-density lipoprotein (LDL) by macrophages (351) and initiating a proinflammatory response from endothelial cells (352). With
inflammation being such a critical aspect of atherosclerosis, it is logical that risk can be associated with certain inflammatory mediators such as soluble CD40 ligand (sCD40L) (353, 354). This protein, which can be released from platelets, can induce the release of proinflammatory cytokines from vascular cells (355). A greater risk of atherothrombosis is also associated with elevated levels of other inflammatory mediators such as the myeloid-related protein 8/14 complex, which has been shown to induce proinflammatory cytokines and adhesion molecules in endothelial cells (356).

Elevated levels of fibrinogen are one of the risk factors that both VT and arterial thrombosis (AT) share (265), as it contributes to a procoagulant state within circulation. Moreover, increased levels of the antifibrinolytic protein PAI-1 is another AT risk factor that can also contribute to VT risk (357, 358).

The importance of the structure of a blood vessel is also outlined by the fact that stiffness of the aorta is an independent risk factor for unfavorable cardiovascular events, as it facilitates hypertension and consequent shear stress (359). Increased levels of matrix metalloproteinase 9 (MMP-9) are associated with greater aortic stiffness and consequently, are also associated with coronary artery disease and other unfavorable cardiovascular events (353, 359, 360). Lastly, any event that results in endothelial dysfunction and/or disturbed shear stress contributes to plaque instability, thus increasing its risk for rupture (361). This is a brief list of specific risk factors associated with atherosclerosis and AT, all of which are somehow relevant to Virchow’s triad. These factors facilitate the progression of atherosclerosis, but the greatest danger of atherosclerosis is associated with plaque rupture and subsequent thrombus formation.
A great amount of morbidity and mortality occurs when atherosclerosis develops within the coronary arteries (coronary heart disease (CHD)), as there is a great risk that it will lead to an occlusion within these vessels, subsequently resulting in reduced blood flow to the heart (myocardial ischemia). These events can be collectively classified as acute coronary syndromes (ACS) and include both unstable angina and myocardial infarction. Unstable angina is the least severe event and it occurs when the coronary artery is partially or intermittently occluded by the thrombus. Non-ST-segment elevation myocardial infarction (NSTEMI) occurs when the partial or intermittent occlusion occurs for an extended period of time, causing some heart tissue damage. The most severe event, ST-segment elevation myocardial infarction (STEMI) occurs when the thrombus completely occludes the coronary artery, resulting in a great amount of tissue damage (362, 363).

Another leading cause of mortality is ischemic stroke, which occurs when an occlusive thrombi inhibits blood flow to portions of the brain. The extent of damage caused in these situations depends on the severity of the occlusion and the duration of persistence. Ischemic stroke can be classified into two categories, thrombotic and embolic. In a thrombotic stroke, an artery within the brain develops atherosclerosis and a thrombus forms from the plaque, immediately restricting blood flow in that location. Alternatively, an embolic stroke occurs when an atherosclerotic plaque ruptures somewhere else in the body, but the resultant thrombus breaks free, travels to the brain and occludes a smaller vessel. Additionally, another type of embolic stroke is associated with a condition called atrial fibrillation (AF) (364-367). AF is known as the most common cardiac arrhythmia, supported by a 1% prevalence. Ultimately, the irregular
heartbeat causes disruptions in blood flow within the heart. With contributions of other components of Virchow’s triad, the formation of a thrombus in the left atrium is a common result of this condition, at which point the thrombus can be pumped to the cerebral circulation to initiate a stroke (368-371).

Atherosclerosis is also able to create conditions where the flow of blood is restricted in locations outside of the coronary and intracranial arteries. These situations comprise peripheral artery disease (PAD) and include events in a range of arteries in the upper extremities, mesenteric circulation, renal circulation, and most commonly, the lower extremities (372). In the most severe of these cases, the tissues will become ischemic, resulting in ulceration and gangrene, sometimes requiring amputation (372).

1.8 TAFI as a risk factor for thrombosis

The potent antifibrinolytic effects of TAFIa make it a logical culprit for thrombotic risk. It could even be hypothesized that the more stable TAFI variant (Ile325) would further contribute to this risk. Numerous studies to date have explored the association of both TAFI levels and genotype with various thrombotic diseases. Although greater TAFI levels are generally found to be a risk factor for certain disorders, the genotypic contributions remain controversial. Elevated TAFI levels have been potentially implicated in arterial thrombosis, including premature peripheral artery disease (373) and coronary artery disease (374). Additionally, greater TAFI level have also been associated with increased risk of ischemic stroke (375, 376). Associations between venous thrombosis and plasma TAFI concentrations have also been reported. At elevated
concentrations, plasma TAFI is associated with deep vein thrombosis (377), recurrent venous thromboembolism (378) and even cerebral venous thrombosis (379).

Evidence pertaining to the contributions of TAFI genotype has been much more variable and in some cases contradictory. The most notable example of this is the Ala147Thr polymorphism in the TAFI protein. The Thr147 allele has been found to be associated with protection against myocardial infarction in one study (380), while being associated with an increase in angina risk in another (381). A third study showed no relationship between genotype and coronary heart disease (382). The role of the Thr325Ile polymorphism in thrombotic disease has also been controversial, possibly due to the fact that the more stable Ile325 variant is commonly associated with lower plasma levels (383, 384). One study provided evidence that the Ile325 variant was associated with a decrease in myocardial infarction risk, without a correlation with TAFI levels (383). Conversely, another study showed that TAFI levels are associated with cardiovascular disease and that the Ile325 variant provides a risk for cardiovascular disease in young patients, especially coronary heart disease (384). In support of the latter study, Ile/Ile325 homozygosity has been found to be associated with a 25% increase in risk for coronary heart disease (385). Furthermore, the Ile325-encoding allele has also been shown to be associated with poor outcomes in stroke therapy, as it creates conditions more resistant to fibrinolytic therapy (386).

Clearly, the research within this area must continue in order to make firm conclusions regarding the influence of TAFI on thrombotic disease. In general, there seems to be a pathogenic role for TAFI, but more studies are needed to verify this and to explore all potential confounding variables that could contribute to the discrepancies seen.
In the literature thus far.

1.9 Anticoagulant Therapy

In the past, the management of venous and arterial thrombosis has been a difficult process, due to the nature of the available anticoagulant therapeutics. For many years, heparin was used for short-term anticoagulant applications. As previously mentioned, heparin is capable of binding antithrombin III, facilitating a strong inhibition of thrombin and FXa (100). Unfortunately, this treatment must be administered parenterally, making it unsuitable for long-term treatment. Additionally, it has unpredictable effects and may pose a risk for heparin induced thrombocytopenia. Due to the fact that heparin is prepared from animal tissue, it can induce a significant immune response, causing a great reduction in platelet count (thrombocytopenia). The platelets remaining after this event are commonly prone to aggregation, thus thrombosis is a common result (387). To improve on these pitfalls, low-molecular-weight heparin (LMWH) and fondaparinux were developed, which reduced the risk of heparin-induced thrombocytopenia, relieved the need for monitoring and allowed for subcutaneous injections (388). Heparin preparations are fractionated to collect LMWH, which is more stable and displays activity selective to the inhibition of FXa. Fondaparinux is a synthetic pentasaccharide developed based on the structure of LMWH, which eliminates the risk for heparin-induced thrombocytopenia (389). Although these improvements were made, these agents still had a tendency to accumulate within patients with renal impairment, lacked antidotes and required daily injections, which is not feasible for a long term treatment regime (388). Other derivatives of these compounds, such as bivalirudin, had more beneficial
characteristics, such as a shorter half-life and direct thrombin inhibition but were still associated with many of the same drawbacks, including parenteral administration (390).

Vitamin K antagonists present as an orally administered alternative to the anticoagulant treatments discussed above. Vitamin K is an important cofactor that is required for glutamic acid carboxylation, which is a critical process required by various coagulation factors to become biologically active (391). Unfortunately, these agents have a very slow onset, requiring the need to initiate antithrombotic treatment with LMWH or fondaparinux, followed by a transition to VKAs by very carefully adjusting the dosage of these simultaneous treatments (392). Furthermore, VKAs require a constant dosage monitoring to maintain efficacy and safety as their effects can be modulated by many factors such as genetic polymorphisms affecting VKA metabolism, vitamin K intake from the diet, and numerous drug-drug interactions (393). Newer developments have introduced drugs such as AVE5026, which is an ultra-low molecular weight heparin that is specific for the inhibition of FXa (394). Although this treatment displays fast onset and utilizes a fixed dosage, it is still a subcutaneously administered drug. FXa and thrombin are both very suitable targets, as thrombin inhibition reduces fibrin formation and platelet activation and FXa inhibition greatly inhibits thrombin generation. However, a major weakness of indirect FXa inhibitors such as AVE5026 and fondaparinux and indirect thrombin inhibitors such as heparin and LMWH, is that the complexed and fibrin bound factors cannot be inhibited effectively (395, 396). Fibrin bound thrombin and FXa, as well as FXa within the prothrombinase complex are all crucial to thrombin generation, thus presenting a fault in the efficacy of these indirect inhibitors.

Regardless of the progression of the aforementioned antithrombotic treatments,
there was a great need for fast-acting, predictable, direct-target inhibitors with little food and drug interactions. This need was met with the development of various inhibitors classified as new oral anticoagulants, now known as non-vitamin K antagonist oral anticoagulants (NOACs). Several NOACs were developed in recent years, such as the direct thrombin inhibitor, dabigatran, and the direct FXa inhibitors rivaroxaban and apixaban. These drugs are the most advanced NOACs and are all fairly comparable in terms of efficacy and safety (see (397-399)).

1.9.1 Rivaroxaban: Structure and Function

In 1998, Bayer launched a Factor Xa program with the goal of developing a new orally administered direct FXa inhibitor that would advance the resources available for anticoagulation (400). In 1999, after screening over 200,000 compounds and completing two medicinal chemistry programs to optimize both potency and bioavailability of the drug, BAY 59-7939 was discovered (400). This 435.88 g/mol compound, now known as rivaroxaban (5-chloro-N-([(5S)-2-oxo-3-[4-(3-oxomorpholin-4-yl)phenyl]-1, 3-oxazolidin-5-yl]methyl]thiophene-2-carboxamide) achieves these optimal functions through a mechanism of action facilitated by its unique structure (Fig. 1.8) (401). Two critical hydrogen bonds are formed between rivaroxaban and Gly219 residue of FXa. A weak hydrogen bond forms between this residue and the chlorothiophene-carboxamide NH group and a strong hydrogen bond connects to the carbonyl oxygen from the (S)-oxazolidinone core. These bonds effectively support the oxazolidinone core, which allows the L-shape of this core to direct the chlorothiophene and morpholinone moieties into the S1 and S4 pockets, respectively. The chlorothiophene moiety is the most critical
for factor Xa inhibition, as it contains a chlorine that interacts with the Tyr228 residue at the bottom of the S1 pocket via its aromatic ring (401). In other FXa inhibitors, this interaction within the S1 pocket was accomplished through a bond between Asp189 and an arginine mimic of the compound, such as an amidine. The unique Cl-Tyr228 bond mediated by rivaroxaban allows it to potently inhibit FXa while avoiding the impaired bioavailability associated with more basic compounds (402). This is due to the fact that more basic compounds are less lipophilic, thus impairing their ability to permeate the gastrointestinal tract epithelia (402). Moreover, the high potency of rivaroxaban is attributed to the fact that upon entering the binding site, it displaces a water molecule, creating an entropically and enthalpically favorable reaction (401). Binding of rivaroxaban to the active site of FXa occurs very rapidly and is reversible, defined by a kinetic association rate constant ($k_{on}$) of $1.7 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ and a kinetic dissociation constant ($k_{off}$) of $5 \times 10^{-3} \text{ s}^{-1}$ (403). The inhibition of FXa is dependent on the concentration of rivaroxaban and has an inhibitory constant ($k_i$) of 0.4 nM (404). In addition to the inhibition of free FXa, rivaroxaban is capable of inhibiting prothrombinase-bound and clot-bound FXa with half-maximal inhibitory concentrations (IC$_{50}$) of 2.1 nM (404) and 75 nM respectively (405). The inhibitory function of rivaroxaban is specific to FXa, as it was shown to be over 10,000-fold more selective for FXa than other relevant serine proteases, which was characterized with an IC$_{50}$ of over 20 µM (404). When FXa is inhibited, thrombin generation is ultimately hindered. Rivaroxaban has been shown to delay the initiation of thrombin generation and diminish the burst of thrombin produced during the propagation phase (406, 407). When applied to in vivo animal models, rivaroxaban was capable of reducing thrombus formation in venous thrombosis models.
for rabbits and rats (404, 408). Rivaroxaban was also able to effectively reduce thrombus formation in arterial thrombosis models within rats, rabbits and mice (404, 409). These results allowed for rivaroxaban research to continue into the clinical setting.

Figure 1.8 Three dimensional binding of rivaroxaban to Factor Xa. The structure of rivaroxaban is critical to its unique Factor Xa (FXa) inhibition mode. The two
dimensional structure of rivaroxaban is indicated in white and the important moieties are noted in orange. The three dimensional structure of rivaroxaban is shown in green, in the context of binding FXa. When binding FXa, hydrogen bonds support the oxazolidinone core, allowing the L-shape of the drug to direct the chlorothiophene moiety into the bottom of the S1 pocket, where it interacts with Tyr228 of FXa. This is the most important interaction for FXa inhibition and because this group is not a basic arginine mimic with a hindered ability to cross the gastrointestinal epithelium, the chlorothiophene also provides rivaroxaban with a superior bioavailability. Figure adapted from (401) and (410).

1.9.2 Clinical pharmacology of rivaroxaban

Rivaroxaban is administered orally and achieves a high bioavailability of 80-100% for a 10 mg dose (411). A beneficial characteristic of rivaroxaban in comparison to preceding oral anticoagulants is a rapid onset of action, as a maximum plasma concentration (C_{max}) is reached between two and four hours, correlating with a maximal FXa inhibition after three hours post-administration (412, 413). The C_{max} is typically around 600 nM and concentrations remain above 200 nM for approximately 12 hours post-treatment. Furthermore, as patients become older and/or have decreased renal output, the C_{max} value can increase to about 1000 nM (414). The rapid action of rivaroxaban removes the need or the parenteral bridging required for previous slow-acting anticoagulants (415). The half-life of this drug in young, healthy individuals is five to nine hours and increases to 11-13 hours in elderly subjects (411). Furthermore, multiple doses in healthy adults failed to result in any significant accumulation and rivaroxaban clearance was not significantly affected by age, sex, body weight, diet (416, 417), or ethnicity (411, 418, 419), greatly reducing the need for routine monitoring of coagulation and dose adjustment. However, under fasting conditions, higher dosages were met with non-linear dose proportional increases in C_{max} and the area under the
concentration-time curve (AUC) (420), which could be returned to a linear relationship when doses were taken with food (411). Once rivaroxaban is administered, approximately one third of the dose remains as unchanged drug and undergoes active renal secretion to be eliminated in the urine (411, 421). The remaining two thirds is metabolically degraded via cytochrome P450, cytochrome P3A4, cytochrome P2J2 and can be transported extracellularly by P-glycoprotein (P-gp) (411, 422). Subsequently, half of these pharmacologically inactive metabolites is excreted via the hepatobiliary route and the other half is secreted via the kidneys (411, 421). Consequently, both inhibitors and promoters of these metabolic enzymes should not be co-administered with rivaroxaban, as they can cause clinically relevant increases and decreases in rivaroxaban AUC, respectively (411, 422). Furthermore, plasma concentrations of rivaroxaban may also become elevated in subjects with moderate renal impairment (423), leading to greater FXa inhibition and bleeding risk (411).

In phase I clinical studies, the effects of rivaroxaban on thrombin generation were still maintained after 24 hours, thus supporting a once-daily administration of the drug (412, 424). The dosage amounts of rivaroxaban were initially determined with four phase II clinical trials based on the prevention of VTE in patients undergoing total hip or knee replacement surgery, called the Oral Direct Factor Xa (ODIXa) program. These studies determined that a daily 10 mg dose of rivaroxaban would provide a good benefit-risk balance (425-428) but twice daily 10 and 20 mg doses also provided an increased efficacy, with a slight increase in major bleeding.

Subsequent to the phase II dosage finding studies, numerous clinical trials have been implemented to determine the efficacy and safety of rivaroxaban throughout a range
of applications. This section will briefly discuss the most important completed clinical trials supporting the currently approved indications for rivaroxaban, as well as clinical trials in progress, aimed at expanding these approved applications. Beginning in 2006, four studies were conducted to assess the “regulation of coagulation in orthopaedic surgery to prevent deep vein thrombosis and pulmonary embolism (RECORD)”. The first study, RECORD1 (NCT00329628) determined that a once-daily 10 mg oral dose of rivaroxaban was significantly more effective than a once-daily 40 mg subcutaneous dose of enoxaparin (LMWH) for extended thromboprophylaxis in patients undergoing elective total hip arthroplasty. The safety profiles of these drugs were also found to be similar to one another (429). The same results were shown in RECORD2 (NCT00332020) when the dosage of rivaroxaban was provided long-term and the enoxaparin was provided at 40 mg once daily for only 10-14 days (430). The RECORD3 (NCT00361894) trial assessed short-term once-daily dosages of 40 mg enoxaparin and 10 mg rivaroxaban for thromboprophylaxis following total knee arthroplasty, which continued to show the superiority of rivaroxaban in efficacy, while displaying similar bleeding rates between the two drugs (431). Lastly, RECORD4 (NCT00362232) also explored the same endpoints, except a twice-daily dosage of 30 mg enoxaparin was used. The superiority of rivaroxaban was also supported by this study (432). These studies were influential in the approval of rivaroxaban for prophylaxis of deep vein thrombosis, which may lead to pulmonary embolism (VTE), in patients undergoing both hip and knee arthroplasty.

In 2007, a new group of studies was initiated, concerned with evaluating the treatment of VTE for both acute phase and extended time periods (EINSTEIN). In both EINSTEIN DVT (NCT00440193) and PE (NCT00439777) the treatment strategies were
the same. Patients received a 15 mg dose of rivaroxaban twice daily for 3 weeks, followed by 20 mg once daily, or they received the traditional 1 mg/kg enoxaparin once daily, overlapping with a vitamin-K antagonist such as warfarin or acenocoumarol. Here, in terms of efficacy for both indications, rivaroxaban proved to be a non-inferior single-drug alternative to traditional treatments, while displaying a similar bleeding incidence (433, 434). The extended use of rivaroxaban was then tested in EINSTEIN EXT (NCT00439725), where 20 mg doses were continued for patients who had already received 6-12 months of anticoagulation therapy, showing that this treatment option was superior to placebo in the prevention of recurrent VTE, without a significant increase in major bleeding events (433, 434). These studies were important for the approval of rivaroxaban for the treatment of DVT and PE, as well as for reducing the risk of these events following an initial 6 months of anticoagulation therapy for VTE. An ongoing trial called EINSTEIN CHOICE (NCT02064439) has expanded on this study. Indefinite anticoagulation with vitamin K antagonists such as warfarin is difficult to maintain, due to the difficulties associated with monitoring and managing dosage. In these instances, taking aspirin, an antiplatelet agent, has been shown to be an easy alternative that has benefits over no anticoagulation at all. EINSTEIN CHOICE is currently investigating if a once daily dose of rivaroxaban is superior to aspirin in these cases by monitoring recurrent VTE events in patients who have previously completed 6-12 months of anticoagulation therapy (435). Another ongoing EINSTEIN study has targeted a different population of patients by comparing the efficacy and safety of rivaroxaban in children 6-17 years old with acute VTE (NCT02234843).
As previously mentioned, hip or knee arthroplasty is not the only significant risk factor for VTE. Since cancer elevates the risk for VTE events, a trial (NCT02583191) was initiated in 2016 to explore the efficacy and safety of rivaroxaban in cancer patients for the treatment of acute VTE, in comparison to the current standard, LMWH. Medically ill patients who experience frequent hospitalization are also at risk for VTE, mainly due to immobility. In 2007, the MAGELLAN (NCT00571649) study was launched to compare rivaroxaban to enoxaparin in these patients. Rivaroxaban was found to be non-inferior to enoxaparin under the standard-duration treatment and was also shown to decrease VTE risk compared to placebo under extended duration treatment, with an increase in bleeding risk (436). An ongoing study, called MARINER (NCT0211564), was launched in 2014 and is currently exploring the prophylactic efficacy and safety of rivaroxaban in patients similar to those in the MAGELLAN study, but only including those that have a high VTE risk and continue treatment 45 days after they have been discharged from the hospital (437).

Shortly after the RECORD studies began in 2006, clinical trials exploring the use of rivaroxaban for applications outside of VTE began. The ROCKET AF (NCT00403767) trial studied the prevention of stroke or systemic embolism in patients with non-valvular AF and found that rivaroxaban was non-inferior to warfarin. Rivaroxaban treatment was not associated with any increases in major bleeding and was associated with less frequent intracranial and fatal bleeding (438). This study provided the evidence required for the approval of rivaroxaban for reducing the risk of stroke and systemic embolism in patients with non-valvular atrial fibrillation. Due to the fact that several NOAC options are available for these applications, a 2016 study compared their
efficacies to one another. It was shown that direct FXa inhibitors, rivaroxaban and apixaban, and direct thrombin inhibitor, dabigatran, all showed similar effectiveness in stroke prevention in patients with AF, with apixaban having a slightly lower gastrointestinal bleeding risk (439).

Around the same time as the ROCKET AF trial initiation, the implications of rivaroxaban for patients with recent ACS were explored. To begin, the ATLAS ACS TIMI 46 (NCT00402597) trial studied the use of rivaroxaban in patients with recent ACS to reduce the occurrence of death, MI, repeated MI, stroke and ischemia. The ultimate goal of this study was to determine the optimal dose and dose regimen for this application in terms of safety (440). The dosages selected here were applied to a continuation study in 2008 called ATLAS ACS-TIMI 51 (NCT00809965). In this study, low doses (2.5 mg or 5 mg) of rivaroxaban (or placebo) were given in combination with aspirin or in combination with aspirin plus an additional antiplatelet agent (clopidogrel). Overall, treatment involving rivaroxaban resulted in a reduction in the risk for death due to cardiovascular causes such as MI or stroke. Although rivaroxaban was not associated with a greater risk for fatal bleeding, it increased the risk for major bleeding and intracranial hemorrhage. However a more favorable benefit-risk ratio was determined for the lower 2.5 mg dose of rivaroxaban (441). An ongoing study called GEMINI-ACS-1 (NCT02293395) is studying a variation of the treatment options explored in the ATLAS studies. Specifically, rivaroxaban in combination with the antiplatelet drug clopidogrel will be compared to the standard dual antiplatelet therapy provided to patients after ACS (aspirin + clopidogrel) (442). Another ongoing study, initiated in 2013, is exploring a more specific study population (PIONEER AF-PCI) (NCT01830543). Patients with
advanced atherosclerosis commonly undergo surgeries to artificially widen blood vessels with plaque buildup using small tubing (percutaneous coronary intervention [PCI]).

PIONEER AF-PCI explores combinations of rivaroxaban with single or dual antiplatelet therapy in comparison to warfarin plus dual antiplatelet therapy in patients that undergo PCI and have AF (443).

As apparent in some of the results developed from these clinical trials, the use of rivaroxaban and other NOACs allow for a similar or heightened efficacy in comparison to traditional anticoagulant therapy regimens, however, sometimes an increase in bleeding risk was seen as well. Thus, the introduction of NOACs has prompted the development of various acute treatments to use in the case of a bleeding event. One important clinical trial (NCT00168077) initiated in 2005 showed that a concentrate of prothrombin was able to control rapid hemorrhaging in an emergency anticoagulant reversal setting (444). A study performed in The Netherlands (NTR2272) also showed a prothrombin concentrate to perform equally as well in similar situations involving the reversal of rivaroxaban-mediated anticoagulation (445). Moreover, in 2015, a clinical trial (NCT02220725) was initiated to determine the efficacy of Andexanet alfa in reversing the anticoagulant activity of direct FXa inhibitors, such as rivaroxaban. This drug is a FXa molecule with a modified catalytic site that abolishes its procoagulant activity, causing it to sequester rivaroxaban and its anticoagulant effects. It was shown that Andexanet could reverse rivaroxaban- or apixaban-dependent anticoagulant effects within minutes of administration and was not associated with any toxic effects (446).
1.10 Rationale, Hypothesis and Research Objectives

Rivaroxaban has many antithrombotic indications, generally including the prophylaxis and treatment of VTE, as well as the prevention of stroke and recurrent ACS (411). Rivaroxaban exerts an anticoagulant function through the direct inhibition of FXa, which results in a subsequent reduction in thrombin generation (407). In addition to participating in the activation and positive feedback of the coagulation cascade, thrombin is also an important activator of TAFI (152). The primary function of TAFI is to attenuate clot lysis by impairing positive feedback mechanisms associated with plasmin generation (149). TAFI levels can vary within plasma between approximately 75-275 nM, which for the most part, correlates directly with TAFIa production and thus, antifibrinolytic potential (159, 160). Furthermore, CPB2 has a common polymorphism that codes for a T325I substitution in the TAFI protein. I325 TAFIa is twice as stable, resulting in a greater antifibrinolytic potential (167). Therefore, due to the fact that rivaroxaban decreases thrombin generation, which is important for the activation of TAFI, it is hypothesized that rivaroxaban will diminish TAFI activation, thus providing a profibrinolytic aspect to the antithrombotic function of rivaroxaban, and that this function can be modulated by greater TAFI levels and the I325 variant.

The specific objectives designed to explore these hypotheses are:

1. To determine the effect that rivaroxaban has on TAFI activation and fibrinolysis, through the use of an in vitro clot lysis assay.

2. To evaluate the contributions of TAFI levels and the T325I polymorphism to the profibrinolytic effects of rivaroxaban in an in vitro clot lysis assay.
CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Culture

The HEK293 cell line was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were grown in Modified Eagle’s Medium (MEM) (Gibco, Mississauga, ON, Canada) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% antibiotic/antimycotic (Gibco). These cells were maintained at 37°C and 5% CO₂.

2.2 Recombinant TAFI Expression Vector Construction

The cDNA from wild-type TAFI was amplified from a pNUT-based expression plasmid (158) and inserted into the pcDNA4A-his vector using PstI and AgeI restriction sites. This yielded an expression vector encoding a recombinant form of TAFI with a carboxyl-terminal 6X histidine tag. The primers used were as follows: sense (5’- AAA CTG CAG TTG GGA TGA AGC TTT GC -3’) (PstI site underlined); and anti-sense (5’- GGA CCG GTA ACA TTC CTA ATG ACA TGC CAA G -3’) (AgeI site underlined). The AgeI site allowed for an in-frame fusion of the TAFI-encoding open reading frame with the 6X His tag.

Site directed mutagenesis was used to introduce the T325I mutation in the full length TAFI-pcDNA4A-his template (10 ng/µL) using Quikchange II Mutagenesis Kit as per manufacturer’s protocol (Agilent Technologies, Life Technologies, Santa Clara, CA). The oligonucleotide primers consisted of T325I sense (5’-TAC CTG GTA TTT TTA CTA ATT TTC TCA ATA GCA CGA ACT GCT TCA C-3’) and T325I anti-sense (5’-
GTG AAG CAG TTC GTG CTA TTG AGA AAA TTA GTA AAA ATA CCA GGT A-3’ (mutagenic nucleotides underlined). The mutagenesis reaction used the following conditions: 30 seconds at 95°C, 18 cycles of 30 seconds at 95°C, 1 minute at 60°C, and 6 minutes at 68°C. *DpnI* digestions and bacterial transformation were carried out according to the manufacturer’s protocol. The identity of all clones was verified by DNA sequence analysis (Robarts Research Institute DNA Sequencing Facility; London ON).

### 2.3 Recombinant TAFI Expression and Purification

HEK293 cells were transfected with plasmid constructs containing sequences encoding wild-type TAFI and Thr325Ile TAFI using polyethylenimine. Zeocin (Gibco) (3 mg/mL) was used to select for cell lines stably expressing these variants. The secreted recombinant proteins were purified using His-tag purification methods. Briefly, a binding buffer (TRIS pH 7.9, 0.5 M NaCl, 1 mM 2-mercaptoethanol, 5 mM imidazole, 10% glycerol) was passed over a nickel-sepharose column, followed by conditioned medium harvested from the stable cell lines, supplemented with the same components as the binding buffer. A wash buffer consisting of binding buffer with 10 mM imidazole was then passed over the column, then the recombinant protein was eluted with binding buffer containing 400 mM imidazole. The eluted fraction was then extensively dialyzed in a storage buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, pH 7.9). The dialyzed fraction was then concentrated using Amicon Ultra 15 mL centrifugation filters (EMD Millipore, Billerica, MA, USA). Protein purity was assessed via SDS-PAGE followed by silver staining (Fig. 2.1). Protein concentration was quantified via absorbance at 280 nm
(ε = 2.64 mL/mg*cm) using a Spectramax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA, USA). Protein was stored at -70°C until use.

![Image of chromatography purification of recombinant TAFI](image)

**Figure 2.1 Chromatography purification of recombinant TAFI.** Purification was conducted over Ni²⁺-Sepharose columns. Column fractions were subjected to SDS-PAGE followed by silver staining. Molecular weight standards are noted on the left. BF: conditioned medium before it was passed over the column; FT: unbound fraction; W: wash fraction; E: elution fraction.

### 2.4 Hipp-Arg Activity Assay

TAFI was activated to TAFIa by incubation with CaCl₂ (5 mM), thrombin (1 nM) and rabbit-lung thrombomodulin (5 nM) in HBS containing 0.01 % (v/v) Tween-80 (HBST) for 10 minutes at room temperature. The reaction was stopped by inhibiting
thrombin with phenylalanylprolylarginyl chloromethylketone (PPAck; 1 μM final concentration) and placing the reaction on ice. A dilution of the resultant TAFIα solution was made (100 nM, final) and 50 μL was added to a well in a half-volume 96-well microplate. 50 μL of a 2x concentrated mixture containing the TAFIα substrate hippuryl-L-arginine (150 μM, final), PPAck (1 μM, final), and rivaroxaban (800 nM, final) or DMSO (1%, final) was added to each well to complete the 100 μL total reaction volume. Substrate hydrolysis was measured via absorbance at 254 nm using a Spectramax Plus 384 plate reader (Molecular Devices). The slope of initial reaction velocity, as calculated by SoftMax Pro software (Version 5.4, Molecular Devices), was used to measure TAFIα activity. These reactions were completed to verify that rivaroxaban does not directly inhibit TAFIα function.

2.5 Rivaroxaban

Rivaroxaban was a generous gift from Bayer Pharma AG (Wuppertal, Germany). The powder form of purified rivaroxaban was dissolved in 100% dimethyl sulfoxide (DMSO) to a final concentration of 1 mM and stored at -80°C until use. Quality control NMR studies (data not shown) showed the expected drug composition after one year of storage. All experiments containing rivaroxaban were completed using separate stocks further diluted in 100% DMSO in order to obtain a final value of 1% DMSO in all reactions. All experiments containing 0 nM rivaroxaban included DMSO at 1% of the final reaction volume.
2.6 PCPS Vesicles

The procedure for vesicle creation was modified from the “Morrissey Lab Protocol for Preparing Phospholipid Vesicles (SUV) by Sonication (2001)”, provided at avantilipids.com (Avanti Polar Lipids, Alabaster, AL, USA). L-α-Phosphatidylcholine (PC, 2.08 µmol) (Sigma-Aldrich, Oakville, ON, Canada) and L-α-phosphatidylserine (PS, 0.52 µmol) (Avanti Polar Lipids) were mixed together in a 13 x 100 mm glass tube to make a 80:20 molar PC:PS ratio. The solvents were then dried under vacuum for one hour. The tube was then filled with nitrogen gas and HBS buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) was added to the dried lipid layer and incubated at room temperature for one hour. The hydrated phospholipids were then resuspended by vortexing. The resulting solution was subjected to sonication in a waterbath for 20-30 minutes, at which point the mixture turned from milky to only slightly hazy. This was an indication that the small, unilamellar vesicles were formed, then they were stored at 4°C until use, for a maximum of 7 days.

2.7 TAFI-deficient Plasma (TDP)

A 2 mL MA-T4E3-Sepharose column (Laboratory for Pharmaceutical Biology, KU Leuven, Belgium (198) was equilibrated using HBST. Fresh-frozen, citrated, pooled normal human plasma (Innovative Research, Novi, MI, USA) was passed through a 0.2 µm filter (Nalgene, Thermo Scientific, Waltham, USA) and then added to the column to deplete it of TAFI. Once the plasma passed through, the column was washed with HBST, then the column-bound TAFI was eluted using 0.2 M glycine (pH 3). This plasma was passed over the column until it was determined to be deficient of TAFI, as verified by
clot lysis assay. TAFI deficiency was achieved once clot lysis times were equal between reactions in the presence or absence of thrombomodulin. TDP aliquots were then stored at -80°C until use.

2.8 Clot Lysis Assay

TAFI-deficient plasma (50 µL) was supplemented with rivaroxaban (0-900 nM) and rabbit thrombomodulin (Haematologic Technologies, Essex Junction, VT, USA) (0-5 nM, final) and recombinant TAFI (0-100 nM, final) were added in various combinations. Each combination was also supplemented with synthetic PCPS vesicles (10 µM, final) and the mixture was completed to 92 µL with a HEPES buffer (25 mM HEPES, 137 mM NaCl, 3 mM CaCl₂, 3.5 mM KCl, 0.1% BSA, pH 7.4). Clotting was initiated by adding this mixture to a microtitre plate well containing a 2 µL microdrop of t-PA (0.2 ng/mL, final) and a 6 µL microdrop containing CaCl₂ (17 mM, final) and thromboplastin-DS (Pacific Hemostasis, Middletown, VA, USA) (1/10⁴, final dilution), creating a final reaction volume of 100 µL. Turbidity was then measured via absorbance at 405 nm in a Spectramax Plus 384 plate reader (Molecular Devices) at 37°C. Latency of clot formation was designated as the time corresponding to the midpoint between maximum and minimum absorbance during the clot formation period. Clot formation rate was designated as the slope (absorbance/time) of the line of best fit during the clot formation period, as calculated by the Softmax Pro software (Version 5.4, Molecular Devices). Clot lysis time was designated as the time corresponding to the half-maximum point of absorbance during the clot lysis period, minus the clot formation latency time.
2.9 Plasmin Generation Assay

This assay was conducted under the same conditions as the clot lysis assay, with the addition of 0.6 mM H-D-Val-Leu-Lys-4-aminomethylcoumarin (HD-VLK-AMC) (Bachem, Torrance, CA, USA) fluorogenic plasmin substrate. Reactions were completed in Fluorotrac 200, 96-well, black, fluorescence plates (Greiner, Monroe, NC, USA) and read at 390/480 excitation/emission wavelengths using a SpectraMax Plus M5e microplate reader (Molecular Devices). Plasmin generation latency was designated as the point when fluorescence began to increase significantly from baseline levels. Plasmin generation peak was designated as the slope of the line of best fit over the inflection point of the curve. This inflection point indicates the maximum rate of substrate hydrolysis, corresponding to the greatest presence of plasmin.

2.10 Thrombin Generation Assay

This assay was conducted under the same conditions as the clot lysis assay, with the addition of 0.5 mM Z-Gly-Gly-Arg-7-Amino-4-methylcoumarin (Z-GGR-AMC) (Technothrombin TGA Kit, Technoclone, Vienna, Austria), a fluorogenic thrombin substrate. Clotting was initiated with thromboplastin-DS at a final dilution of 1/10^3 (Pacific Hemostasis). Reactions were completed within Fluorotrac 200, 96-well, black, fluorescence plates (Greiner) and read at 360/460 excitation/emission wavelengths using a SpectraMax Plus M5e microplate reader (Molecular Devices). Thrombin generation peak was designated as the maximum slope of the raw fluorescence vs. time curve, indicating the point in time with the greatest amount of thrombin present. Thrombin generation latency was designated as the time, in seconds, taken to reach this point.
Concentrations of thrombin over time were calculated using a thrombin activity calibration curve and Technothrombin TGA evaluation software (Technoclone), as per the manufacturer’s protocol.

2.11 Statistical Analysis

Statistical analyses were performed with SPSS software, version 23.0 (SPSS Inc., Chicago, Illinois). All comparisons were performed by one-way ANOVA using a Tukey post-hoc analysis. Statistical significance was assumed at p < 0.05.
CHAPTER 3: RESULTS

3.1 Rivaroxaban and TM increase clot formation latency

Clot formation time was measured throughout physiologically relevant ranges of both rivaroxaban and thrombomodulin (TM). This was done in the absence (Fig. 3.1 A) or presence of recombinant wild-type (WT) TAFI (Fig. 3.1 B) or T325I TAFI (Fig. 3.1 C). Independent experiments (Fig. 3.1 D) included the absence or presence of WT and T325I TAFI under the same conditions, all completed with the same PCPS preparation. Throughout this analysis, it was shown that neither variant of TAFI had any significant effect on clot formation latency. Conversely, rivaroxaban was able to significantly delay clot formation. For the most part, a significant delay was accomplished at 200 nM rivaroxaban. Additionally, TM also had a significant effect on clot formation latency. The majority of the time, this significant delay was not seen until the highest concentration (5 nM) of TM. It was also apparent that the delay in clot formation caused by TM was much more pronounced when rivaroxaban was present.
Figure 3.1: The effect of rivaroxaban and TM on clot formation latency. Clot lysis assays were performed under titrated concentrations of rivaroxaban and TM. 0-5 nM TM and 0-800 nM rivaroxaban were titrated in the absence of TAFI (A), or the presence of 10 nM wild-type TAFI (B) or T325I TAFI (C). Additional experiments (D) included titrations of 0-5 nM TM and 0-600 nM rivaroxaban with or without the presence of each physiological TAFI variant at 10 nM. These conditions were tested with the same preparation of PCPS in order to compare the effects of each TAFI variant. The amount of time required to reach the point of half-maximal turbidity was designated as the clot formation latency. The data represent means ± standard error of the mean for three independent experiments. Daggers: P<0.05 compared to 0 nM TM of same concentration of rivaroxaban; asterisks: P<0.05 compared to 0 nM rivaroxaban of same concentration of TM.

3.2 Rivaroxaban and TM decrease clot formation rate

The rate of clot formation was also measured throughout the clot lysis assays performed in the presence of physiologically relevant ranges of both rivaroxaban and TM. This was done in the absence (Fig. 3.2 A) or presence of recombinant wild-type (WT) TAFI (Fig. 3.2 B) or T325I TAFI (Fig. 3.2 C). Independent experiments (Fig. 3.2 D) included the absence or presence of WT and T325I TAFI under the same conditions, all completed with the same PCPS preparation. When reactions with and without TAFI are directly compared (Fig. 3.2 D), TAFI seems to have a minor effect on clot formation rate in certain situations involving greater levels of rivaroxaban. Otherwise, the effects of rivaroxaban and TM on clot formation rate are similar to the effects seen on clot formation latency. These results also show that rivaroxaban has a significant effect on clot formation rate, causing significant reductions at the 200 nM level. Moreover, 5 nM TM is capable of decreasing the rate of clot formation in the presence of rivaroxaban, which as seems to become more apparent as rivaroxaban levels increase.
Figure 3.2: The effect of rivaroxaban, TM and TAFI on clot formation rate. Clot lysis assays were performed under titrated concentrations of rivaroxaban and TM. 0-5 nM TM and 0-800 nM rivaroxaban were titrated in the absence of TAFI (A), or the presence of 10
nM wild-type TAFI (B) or T325I TAFI (C). Additional experiments (D) included titrations of 0-5 nM TM and 0-600 nM rivaroxaban with or without the presence of each physiological TAFI variant at 10 nM. These conditions were tested with the same preparation of PCPS in order to properly compare the effects of each TAFI variant. Clot formation rate was designated as the slope (absorbance/time) of the line of best fit during the clot formation period, as calculated by the Softmax Pro software. The data represent means ± standard error of the mean for three independent experiments. Daggers: P<0.05 compared to 0 nM TM of same concentration of rivaroxaban; asterisks: P<0.05 compared to 0 nM rivaroxaban of same concentration of TM; number signs: P<0.05 compared to no TAFI at that particular combination of rivaroxaban and TM concentrations.

3.3 The TAFI-dependent prolongation in clot lysis time is modified by TM and rivaroxaban

Lysis time was measured via clot lysis assay in the presence of rivaroxaban and TM within physiologically relevant ranges. This was done in the absence (Fig. 3.3 A) or presence of recombinant wild-type (WT) TAFI (Fig. 3.3 B) or T325I TAFI (Fig. 3.3 C). Independent experiments (Fig. 3.3 D) included the absence or presence of WT and T325I TAFI under the same conditions, all completed with the same PCPS preparation. When these reactions were completed in the absence of TAFI, rivaroxaban was generally able to significantly reduce the clot lysis time at 400 nM amounts, regardless of the amount of TM present in the system. A similar pattern was seen in the reactions that contained TAFI for the low concentrations of TM that were not capable of inducing sufficient amounts of TAFI activation to create a significant lysis-prolonging-effect. For the most part, when TM levels reached 0.5 nM, the reactions containing any form of TAFI had significantly increased clot lysis times. In the presence of TAFI, without rivaroxaban, as TM levels increased, clot lysis time increased, corresponding to greater amounts of TAFI activation. Notably, there was a relationship between the extent of lysis time prolongation and the
stability of the respective TAFI variants, with the more stable variants having a greater lysis time that was not as sensitive to the presence of increasing concentrations of rivaroxaban. As rivaroxaban was added at greater concentrations, the reactions containing 5 nM TM began to decrease in clot lysis time. Specifically, in Fig. 3.3 D, it can be noted that at 600 nM rivaroxaban, for the WT TAFI variant, there is no significant difference between 0.5 and 5 nM TM, but a difference remains for the T325I variant. For the naturally-occurring TAFI variants, however, the profibrinolytic effect mediated by rivaroxaban is continually apparent at the greater concentrations (0.5 – 5 nM) of TM, although a significant reduction in clot lysis time may not be seen until higher concentrations of rivaroxaban were added. Furthermore, at 5 nM TM and 300 nM rivaroxaban, rivaroxaban significantly reduced clot lysis time in the WT TAFI variant, but not the T325I variant.
Figure 3.3: The effect of rivaroxaban, TM and TAFI on clot lysis. Clot lysis assays were performed under titrated concentrations of rivaroxaban and TM. 0-5 nM TM and 0-800 nM rivaroxaban were titrated in the absence of TAFI (A), or in the presence of 10 nM wild-type TAFI (B) or T325I TAFI (C). Additional experiments (D) included titrations of 0-5 nM TM and 0-600 nM rivaroxaban with or without the presence of each physiological TAFI variant at 10 nM. These conditions were tested with the same preparation of PCPS in order to properly compare the effects of each TAFI variant. Clot lysis time was designated as the time corresponding to the half-maximum point of absorbance during the clot lysis period, minus the clot formation latency time. The data represent means ± standard error of the mean for three independent experiments. Daggers: P<0.05 compared to 0 nM TM of same concentration of rivaroxaban; asterisks: P<0.05 compared to 0 nM rivaroxaban of same concentration of TM; number signs: P<0.05 significant differences between 0.5 nM and 5 nM TM at that particular rivaroxaban concentration.

3.4 Rivaroxaban and TM both contribute to thrombin generation impairment

The fluorogenic thrombin substrate, Z-GGR-AMC, was used to measure thrombin generation in the context of a clot lysis assay. This was done in the absence or presence of both 5 nM TM and 100 nM rivaroxaban to assess their direct effects on the production of thrombin. When monitoring thrombin generation latency (Fig 3.4 A) and peak thrombin levels (Fig 3.4 B), the same patterns can be seen. These experiments show that in the absence of rivaroxaban, TM does not significantly affect thrombin generation. Conversely, rivaroxaban is able to significantly diminish thrombin generation, regardless of TM levels. When rivaroxaban is present, however, TM is then capable of further impairing thrombin generation. Each of these effects can be easily observed from inspection of the thrombogram (Fig. 3.4 C). This representation is also useful in noting that without rivaroxaban, thrombin generation occurs in a rapid burst that is just as quickly inhibited. Moreover, in the presence of rivaroxaban, or both rivaroxaban and TM,
thrombin generation occurs at a much slower rate that is sustained for longer periods of time.
Figure 3.4: The effects of rivaroxaban and TM on thrombin generation. Clot lysis assays were performed in the presence of Z-GGR-AMC, a fluorescent thrombin substrate. Raw fluorescence values were monitored over time in order to assess the generation of thrombin. Reactions were performed in the presence or absence of both 5 nM TM and 100 nM rivaroxaban. Thrombin generation latency (A) was calculated as the time required to reach the maximum slope of the fluorescence vs. time curve. The peak of thrombin generation (B) was designated as the value of the maximum slope of the fluorescence vs. time curve. The data represent means ± standard error of the mean for three independent experiments. Daggers: P<0.05 compared to 0 nM TM of same concentration of rivaroxaban; asterisks: P<0.05 compared to 0 nM rivaroxaban of same concentration of TM. A thrombogram (C) (concentrations of thrombin over time) was constructed using a thrombin activity calibration curve and Technothrombin TGA evaluation software. The data represent a mean of three independent experiments.

3.5 TAFI levels and the T325I variant are capable of modulating the profibrinolytic effects of rivaroxaban

The effects of increasing levels of both TAFI and rivaroxaban were assessed using a clot lysis assay. Both components were included within physiologically relevant ranges of concentrations, and no additional TM was present. Here, it was shown that TAFI, even at higher levels, has no effect on both clot formation latency (Fig. 3.5 A) or clot formation rate (Fig. 3.5 B). The significant effects of rivaroxaban on clot formation latency and clot formation rate were once again observed in these experiments. Rivaroxaban was still able to significantly increase clot formation latency and significantly decrease clot formation rate at a concentration of 200 nM. The most significant interactions between TAFI and rivaroxaban function occurred in the context of clot lysis (Fig. 3.5 C). Without rivaroxaban present, both variants of TAFI were shown to significantly prolong clot lysis to greater extents as their concentration increased. When comparing the two TAFI variants, the antifibrinolytic effects of the I-325 protein
were significantly greater than that of WT TAFI in some cases (0 nM rivaroxaban and 100 nM TAFI; 200 nM rivaroxaban and 75 nM TAFI; 400 nM rivaroxaban and 100 nM TAFI; and 600 nM Rx and 75 nM TAFI) and although not statistically significant, this trend was consistent throughout the remaining TAFI and rivaroxaban concentrations. As rivaroxaban was introduced, significant reductions in clot lysis time occurred. Generally, as rivaroxaban concentrations reached 400 nM, the lower amounts of TAFI (50 and 75 nM) were no longer capable of significantly prolonging clot lysis time, in comparison to no TAFI being present. Furthermore, significant differences in the ability to prolong clot lysis were seen between the WT and T325I TAFI variants. First, at 200 nM rivaroxaban and 50 nM TAFI, clot lysis time is significantly decreased in the presence of the WT variant, but not the T325I variant. A similar pattern can be seen at 400 and 600 nM rivaroxaban, where at 75 nM TAFI, only the T325I variant is still able to significantly prolong clot lysis time, in comparison to no TAFI.
Figure 3.5: The role of rivaroxaban, TAFI levels and the T325I variant on clot characteristics. Clot lysis assays were performed under titrated concentrations of rivaroxaban and both physiological TAFI variants. 0-600 nM rivaroxaban and 0-100 nM TAFI were titrated in the absence of TM. The amount of time required to reach the point of half-maximal turbidity was designated as the clot formation latency (A). Clot formation rate (B) was designated as the slope (absorbance/time) of the line of best fit during the clot formation period, as calculated by the Softmax Pro software. Clot lysis time (C) was designated as the time corresponding to the half-maximum point of.
absorbance during the clot lysis period, minus the clot formation latency time. The data represent means ± standard error of the mean for three independent experiments. Daggers: P<0.05 compared to 0 nM TAFI of the same concentration of rivaroxaban; asterisks: P<0.05 compared to 0 nM rivaroxaban of the same concentration of TAFI.

3.6 Rivaroxaban reduces the TAFI-attributed reduction in plasmin generation

Due to the fact that TAFIa exerts antifibrinolytic effects through the inhibition of plasmin generation, clot lysis assays were performed in the presence of the fluorogenic plasmin substrate, HD-VLK-AMC, in order to assess in a different way the function of TAFIa in the system. The data were then analyzed to calculate the plasmin formation latency (Fig. 3.6 A) and peak plasmin generation (Fig. 3.6 B). It was shown that TAFI did not play a part in the latency of plasmin generation. On the other hand, rivaroxaban was able to significantly prolong latency, which correlates directly to the function of rivaroxaban in delaying thrombin generation and clot formation. Both variants of TAFI were shown to be capable of significantly reducing plasm in generation. This effect was also seen to be greater at higher concentrations of TAFI. However, the introduction of rivaroxaban was shown to abolish the effects of TAFI on plasmin generation. At 200 nM rivaroxaban, it can be seen that only the 100 nM amount of TAFI is still capable of significantly reducing plasmin generation. At 400 nM rivaroxaban, no significant differences in plasmin generation were observed as a function of TAFI concentration, although an apparent trend to small decreases in plasmin generation could be seen. Note that, under these conditions, TAFI at the higher concentrations is capable of eliciting a small but significant increase in lysis time (Fig. 3.5 C). Consistent with the clot lysis time
data, at 200 nM rivaroxaban and 100 nM TAFI, the effects of rivaroxaban seem to be more pronounced on the WT TAFI variant.

A
Figure 3.6: The role of rivaroxaban, TAFI levels and the T325I variant on plasmin generation. Clot lysis assays were performed in the presence of HD-VLK-AMC, a fluorescent plasmin substrate. Raw fluorescence values were monitored over time in order to assess the generation of plasmin. Reactions were performed under titrated concentrations of rivaroxaban and both physiological TAFI variants. 0-400 nM rivaroxaban and 0-100 nM TAFI were titrated in the absence of TM. Plasmin generation latency (A) was designated as the point when fluorescence began to increase significantly from baseline levels. The peak of plasmin generation (B) was designated as the value of the maximum slope of the fluorescence vs. time curve. The data represent means ± standard error of the mean for three independent experiments. Daggers: P<0.05 compared to 0 nM TAFI of same concentration of rivaroxaban; asterisks: P<0.05 compared to 0 nM rivaroxaban of same concentration of TAFI; number signs: P<0.05 significant differences between TAFI variants at that particular combination of rivaroxaban and TAFI concentrations.
3.7 Rivaroxaban does not directly inhibit TAFIa activity

The previous experiments have consistently shown that rivaroxaban is capable of significantly reducing the effects of TAFIa on fibrinolysis. To ensure that this effect was not due to rivaroxaban directly inhibiting the TAFIa enzyme, TAFIa hydrolysis of the Hipp-Arg substrate was performed in the absence and presence of a high concentration of rivaroxaban (Fig. 3.7). These results confirmed that rivaroxaban was not directly inhibiting TAFIa, as no differences in substrate hydrolysis were seen in the presence of rivaroxaban.

![Initial Velocity of Substrate Hydrolysis](image)

**Figure 3.7:** The effects of rivaroxaban on Hipp-Arg hydrolysis by TAFIa. TAFI was activated and added to a reaction containing the Hipp-Arg substrate, in the presence or absence of 800 nM rivaroxaban. Hydrolysis was monitored over time via absorbance. The initial reaction velocities, calculated as the initial slope of absorbance over time, were used to measure TAFIa activity. The data represent means ± standard error of the mean for three independent experiments.
CHAPTER 4: DISCUSSION

One of the main goals of this study was to determine the effects that rivaroxaban had on TAFI activation and the consequences of this relationship on fibrinolysis. This was accomplished through the use of a clot lysis assay. This is a useful method, as plasma can be made deficient of TAFI and controlled amounts of recombinant TAFI can be added back into the system to directly assess the effects of TAFI. With these assays, the clot formation latency, rate of clot formation and the time required for clot lysis can all be measured, thus providing useful information for both coagulation and fibrinolysis. In these experiments, in addition to incorporating rivaroxaban into the system at varying amounts, thrombomodulin (TM) was also titrated. The ability of the thrombin/TM complex to activate TAFI allowed for a setting where the effects of rivaroxaban could be observed under conditions of extensive TAFI activation. Additionally, at higher concentrations, TM can reduce thrombin generation by binding thrombin and hence facilitating the activation of protein C. Moreover, by binding to thrombin, TM inhibits the action of thrombin against procoagulant substrates such as fibrinogen.

The relationship between rivaroxaban and TAFI activation is outlined in Figure 4.1. It was mentioned previously that the primary physiological function of TAFI is to remove carboxyl-terminal lysine residues from fibrin that has been partially degraded by plasmin. These residues are influential to fibrinolysis, as they mediate positive feedback in plasmin generation. By removing these residues, TAFIa can effectively attenuate plasmin generation and, consequently, fibrinolysis. In order for these processes to occur, the TAFI zymogen must be activated by plasmin, the thrombin/TM complex, or
thrombin. The link between fibrinolysis and rivaroxaban reflects the fact that rivaroxaban inhibits FXa, the enzyme primarily responsible for the generation of thrombin.

Ultimately, the rivaroxaban-mediated reduction in thrombin generation could result in less TAFI being activated, allowing for a more rapid clot lysis. This evokes the idea that, in addition to its anticoagulant role, rivaroxaban could have a profibrinolytic effect contributing to its overall antithrombotic function.

Figure 4.1 The link between rivaroxaban and fibrinolysis. Plasmin is the enzyme responsible for the breakdown of a fibrin clot. As it partially degrades fibrin, carboxyl-terminal lysine residues are exposed on the fibrin surface (fibrin”), serving as a cofactor for the promotion of plasmin generation. TAFIa functions by cleaving these residues, thus removing the cofactor ability of fibrin (fibrin”). This results in an attenuation of plasmin generation and clot lysis. In order for TAFIa to accomplish this, it must be formed from TAFI through cleavage by plasmin, the thrombin/TM complex, or thrombin. Rivaroxaban is a direct FXa, the enzyme instrumental to thrombin generation. Due to the fact that thrombin is such an important component of TAFI activation, it is likely that rivaroxaban will decrease TAFI activation, thus promoting a more rapid clot lysis.
When clot formation latency was measured (Fig. 3.1), it was shown that TAFI had no effect (Fig. 3.1 D), which was expected as the relevant effects of TAFIa are on plasmin generation. Conversely, both rivaroxaban and TM were shown to significantly influence clot formation latency. Due to the fact that rivaroxaban inhibits FXa and thus, subsequent thrombin generation, the delay in clot formation is typical. This has been shown before in both plasma and whole blood coagulation models (406, 447).

Rivaroxaban was also shown in this study to decrease the rate of clot formation in a concentration-dependent manner (Fig. 3.2). This also agrees with the decrease in thrombin generation caused by rivaroxaban. To verify that these anticoagulant effects were from the ability of rivaroxaban to reduce thrombin generation, further experiments were conducted using clot lysis assays in the presence of Z-GGR-AMC, a fluorogenic thrombin substrate, in order to monitor thrombin generation under these conditions (Fig. 3.4). It was clearly seen that thrombin generation was both delayed and diminished. The rivaroxaban-dependent delay in clot formation thus correlated directly with the observed delay in thrombin generation. The generation rate of thrombin was also decreased by rivaroxaban (Fig. 3.4 C), thus correlating with the observed reductions in clot formation rate seen when monitoring turbidity.

As TM is known to exert anticoagulant effects at elevated concentrations, varying levels of TM were assessed in this setting as well. TM was found to be capable of exerting effects on coagulation under certain conditions. In the clot lysis experiments it was seen that higher amounts of TM (5 nM) were able to significantly delay clot formation, regardless of the presence of rivaroxaban (Fig. 3.1). Moreover, it was shown
that this effect was enhanced when rivaroxaban was present. The additive effects of rivaroxaban and TM (5 nM) could also be observed in terms of clot formation rate (Fig 3.2). The requirement of 5 nM TM to produce an anticoagulant effect can be related to the biphasic effect characteristic of TM. It has been shown that under low amounts of TM, the thrombin/TM complex favors TAFI activation, whereas at TM concentrations greater than approximately 5 nM, the thrombin/TM complex favors the activation of protein C, thus resulting in anticoagulant effects (227). It is likely that greater amounts of protein C are being activated when 5 nM TM is present in these reactions. In addition to this, TM also has additional anticoagulant functions that could be contributing to this effect. By binding to thrombin, TM has been shown to prevent thrombin from contributing to coagulation and when thrombin and TM are complexed with one another, serine protease inhibitors such as antithrombin III (448) and protein C inhibitor (449) are able to inhibit thrombin over 8 and 140 times faster than free thrombin, respectively. The additive anticoagulation caused by TM in the presence of rivaroxaban is likely due to the fact that rivaroxaban alone is capable of significantly reducing thrombin generation, making the TM anticoagulant effects of TM more apparent at 5 nM. This was supported by the experiments completed in this study where thrombin generation was monitored (Fig. 3.4).

When the presence of either TAFI variant was considered in terms of clot formation rate (Fig 3.2), there seemed to be minor reductions caused by TAFI at greater amounts of rivaroxaban. A potential explanation for this could be that TAFI is acting as a competitive substrate for thrombin. At the elevated rivaroxaban concentrations where this trend is seen, thrombin generation would be very limited. Thus, it is possible that
thrombin is being occupied by TAFI binding and activation, and these effects become apparent only under conditions rivaroxaban has already limited the amount of thrombin available to participate in clot formation. Alternatively, the clot formation characteristics seen throughout these experiments were very dependent on the preparation of PCPS used. That is why additional experiments were completed (Fig. 3.1 D, 3.2D, 3.3 D) where direct comparisons could be made while using the same preparation of vesicles. The quality and performance of these vesicles were very sensitive to factors such as time and although every effort was made to ensure consistency throughout direct comparisons, the differences seen in the presence of TAFI (Fig 3.2 D) could be due to variability in prep quality over time.

The last characteristic monitored during these assays was clot lysis, where the consequences of TAFI activation would be apparent (Fig. 3.3). The clot lysis reactions were first conducted in the absence of any TAFI to determine the effects of rivaroxaban in this context (Fig 3.3 A). These experiments showed that clot lysis time was significantly decreased by rivaroxaban and TM did not have an effect. Due to the fact that TAFI was not a component of these reactions, it is likely that the reduction in thrombin caused by rivaroxaban resulted in the formation of a more porous clot structure with thicker fibers that were more susceptible to fibrinolysis (68, 450). The same conclusion could be made for the rivaroxaban-dependent decreases in clot lysis time in the presence of all TAFI variants, at low TM concentrations where sufficient TAFI activation is not occurring (Fig. 3.3 B-D and Fig 3.5 C). It should be noted that a low concentration of TAFI (10 nM) is used in Figures 3.3 B-D because half-maximal attenuation of clot lysis only requires 1 nM TAFIa and approaches maximal attenuation at 10 nM (204). Although
this low concentration of TAFI required higher concentrations of TM for sufficient activation, in later experiments (Fig 2.5 C), the lowest concentration of TAFI (50 nM) was still capable of eliciting a significant antifibrinolytic effect in the absence of TM.

As TM levels increased in these reactions, a greater extent of TAFI activation was noted by significant increases in the prolongation of clot lysis time. Importantly, the effects of TM in this context are strictly due to TAFI activation, since no effect of TM on lysis time was observed in the absence of added TAFI (Fig 3.3 A, D). In general, even with the greater potential for TAFI activation at greater amounts of TM, significant decreases in clot lysis time could be seen in the presence of rivaroxaban (Fig 3.3 B-D). This contributes evidence to the fact that the reduction in thrombin generation caused by rivaroxaban results in less TAFI activation, thus a more rapid clot lysis. Other studies have supported this result, one of which was completed in a whole blood model of fibrinolysis, providing more physiologically relevant reinforcement to the results obtained in this study. The results of Varin et al. (2013) (450) show that rivaroxaban is responsible for making whole blood clots more porous and more susceptible to fibrinolysis. Additionally, they provide evidence that the effects of TAFI are eliminated by rivaroxaban, even in the presence of TM.

An interesting trend can also be seen at the 5 nM TM level. These data show that in the presence of rivaroxaban, the dose-dependent effect of TM on antifibrinolysis is lost and 5 nM TM no longer causes an increase in clot lysis time via TAFI activation, in comparison to 1 nM TM (Fig 3.3. B, C). As rivaroxaban increases, the clot lysis time at 5 nM TM decreases to a point lower than at 1 nM TM (Fig 3.3 B, C), or equal to 0.5 nM TM (Fig 3.3. D). Multiple factors could be contributing to these effects. As stated
previously, TM has a biphasic effect where it begins to favor protein C activation over TAFI activation at TM levels greater than 5 nM (227). At this point, APC begins to diminish thrombin generation. In addition to this, the ability of TM to reduce the procoagulant activity of thrombin and enhance thrombin inhibition will result in even less thrombin production. Normally, the ability of TM to diminish thrombin generation results in less TAFI activation when TM levels exceed 5 nM. However, due to the fact that rivaroxaban also significantly inhibits thrombin generation, TAFI activation is hindered significantly at 5 nM thrombomodulin in the presence of rivaroxaban, hence the reduced delay in clot lysis (Fig 3.3 B-D). The additional effect on fibrinolysis caused by rivaroxaban is not uncommon in the context of TM levels. Other components that somehow promote the protein C pathway of anticoagulation also reduce the TAFI-attributed delay in clot lysis at lower TM levels. Some examples include increased amounts of EPCR (451) and platelet factor 4 (452), which facilitate greater protein C activation, or increased amounts of protein S (453), which is a cofactor for activated protein C in the inactivation of FVIII.

These results may have implications for the function of rivaroxaban in individuals that present TM-altering disease phenotypes, as certain conditions, such as vascular damage, infections, sepsis and/or inflammation, can cause the levels of TM to rise to almost 5 nM (218-222). Possible evidence for this can be seen for individuals undergoing warfarin treatment (454). It has been shown that high levels of plasma TM is a risk factor for bleeding events in patients receiving warfarin anticoagulation. Furthermore, this could also have implications in the location of a thrombotic event, as TM levels can vary so substantially throughout the vasculature (1-100 nM) (217, 223, 225). The lower
concentrations of TM used in these experiments would represent environments that are characteristic of larger blood vessels such as veins or arteries. Alternatively, the greater TM concentrations could represent the microvasculature where vessel surface area is much greater in relation to blood volume. Again, even though TAFI levels were low in these experiments (10 nM) the effects are still physiologically relevant as half-maximal attenuation of clot lysis only requires 1 nM TAFIa and approaches maximal attenuation at 10 nM (204).

When making direct comparisons between the physiological TAFI variants, significant differences can be noted (Fig 3.3, D). First, the aforementioned effects of rivaroxaban on clot lysis time at 5 nM TM are greater on the wild-type TAFI variant. This can be seen at 600 nM rivaroxaban where only the T325I variant is still able to significantly prolong clot lysis time at 5 nM TM in comparison to 0.5 nM TM. Furthermore, at 300 nM rivaroxaban and 5 nM TM, a significant reduction in clot lysis time, in comparison to 5 nM TM and 0 nM rivaroxaban, is only seen in the wild-type variant. This provides evidence that the T325I substitution in the TAFI protein can allow for more resistance to the effects of rivaroxaban.

This leads to the second major goal of this study; to determine if the profibrinolytic effects of rivaroxaban can be modulated by the T325I variant and greater TAFI levels. As shown in this study, rivaroxaban is capable of exerting a profibrinolytic effect, in addition to its anticoagulant function. Greater levels of TAFI and the stabilizing T325I polymorphism have each been shown to be associated with greater antifibrinolytic potential. Consequently, it was hypothesized that these characteristics could modulate the profibrinolytic effects of rivaroxaban. Further evidence for this hypothesis was sought by
performing clot lysis assays with varying levels of TAFI (Fig 3.5). These were completed in the absence of any additional TM, which is more relevant to the larger veins where thrombotic events are more frequent (226). First, these reactions confirmed that TAFI has no influence on clot formation and that rivaroxaban can both significantly delay and decrease the rate of clot formation (Fig 3.5 A, B). The clot lysis time results of these experiments provided vital evidence to the second goal of this study (Fig 3.5 C). It was shown that the lower amounts of TAFI were more susceptible to the effects of rivaroxaban and higher amounts of TAFI required higher concentrations of rivaroxaban to significantly hinder the increment in lysis time resulting from addition of TAFI. Additionally, the wild-type variant of TAFI was more susceptible to the effects of rivaroxaban than the T325I variant. This was indicated at three separate conditions where only the Ile325 variant was still capable of significantly prolonging clot lysis time in comparison to the no TAFI control. These data were reinforced by the supporting clot lysis assays done in the presence of a fluorogenic plasmin substrate (Fig 3.6). Again, TAFIa inhibits the generation of plasmin, allowing this substrate to act as an indirect measure of TAFIa function. Although controls were completed in the previous experiment without the presence of TAFI, using a plasmin substrate also allowed for a measure of the profibrinolytic effects of rivaroxaban that was separate from the effects induced by the influence of rivaroxaban on clot structure. In these experiments, it was shown that rivaroxaban was able to reduce TAFIa function more readily in lower TAFI concentrations. The plasmin generation results also supported that the function of the Ile325 variant was more resistant to the effects of rivaroxaban than the wild-type TAFI. Taken together, these results show that greater TAFI levels and the Ile325 TAFI variant
are capable of antagonizing the profibrinolytic effects of rivaroxaban. This information provides insight to the pharmacogenomics of rivaroxaban, whose impact would be nontrivial as the allele frequency for the Ile325 polymorphism is approximately 30% within the population. Moreover, plasma TAFI concentrations are normally distributed within the population and range from approximately 75 to 275 nM. Thus, considering the concentrations range of TAFI investigated here (whose upper limit was proscribed by availability of purified protein), it is clear that variation in TAFI levels could materially affect the potency of rivaroxaban, at least as far as its profibrinolytic effects are concerned.

4.1 Future directions

The findings of this study provide potential insight to the pharmacodynamics and pharmacogenetics encompassing the overall antithrombotic function of rivaroxaban. As mentioned previously, in addition to the T325I polymorphism, approximately 25% of the variation in TAFI levels can be attributed to underlying genetic factors. Elevated TAFI levels have been shown to be correlated with a risk for venous thrombosis (455). Additionally, the Ile325 variant has been shown to be an independent factor associated with a poor outcome of stroke treatment, as it is more resistant to thrombolytic therapy (386). Thus, it is reasonable to speculate that high levels and the T325I variant may affect the function of rivaroxaban, as well. This is exactly the conclusion that can be drawn from the current studies. However, it should be noted that previous studies have shown that individuals who express the Ile325 protein are also associated with lower levels of TAFI (166). Thus, future work should be done to assess the independent and cooperative
significance of T325I and TAFI levels in a clinical setting. This could be done by recruiting individuals undergoing rivaroxaban treatment (for example for post-surgical thromboprophylaxis), and measuring TAFI levels and genotyping for the T325I polymorphism. The comparative resistance to the drug attributable to higher TAFI concentration and the Ile325 variant could be reflected in differences in clinical endpoints such as bleeding or thrombosis. For example, individuals with high levels of the Ile325 variant might have less bleeding but a higher residual risk of thrombosis. The influence of TAFI levels and/or CPB2 genotype could necessitate a dosage adjustment.

Several studies have compared the effects of both FXa and thrombin inhibitors in similar contexts (456-461). First, FXa inhibitors have been show to behave differently when TM is present in the context of coagulation. In the presence of TM, although FXa inhibitors maintain their anticoagulant effects, direct thrombin inhibitors conversely present as thrombogenic. Incampo et al. (458) hypothesized a potential mechanism for this effect. Initially, these inhibitors bind thrombin directly, leaving a small amount of free thrombin remaining. Subsequent to this, irreversible thrombin inhibitors such as alpha2-maroglobulin and antithrombin III will slowly bind to the remaining free thrombin, disrupting the equilibrium between thrombin bound to the direct inhibitors and free thrombin. To restore this equilibrium, a small amount of thrombin is released from the direct inhibitors. This process then continues for an extended period of time, until all thrombin is eventually bound to irreversible inhibitors. The amount of thrombin being released over time is small, but still able to sufficiently activate TAFI, due to the presence of thrombomodulin. Although this diminishes the extent of TAFI activation, it has been previously shown that the activation of small amounts of TAFI over a longer period of
time has a much greater antifibrinolytic effect than the short-term activation of a large amount of TAFI (462).

Additionally, separate experiments have acknowledged that FXa inhibitors such as rivaroxaban reduce TAFI activation, resulting in more rapid clot lysis, but have also shown that direct thrombin inhibitors are more effective at reducing TAFI activation. This has been shown in both in vitro and clinical studies (456, 459). Thus, future studies should consider the effects of greater TAFI levels and the T325I variant in the context of direct thrombin inhibition anticoagulation, as this evidence suggests that even more profound effects of TAFI may be seen. Such a possibility could first be addressed by repeating the experiments performed herein, but in the presence of a direct thrombin inhibitor.

Lastly, although the indices explored in this study are strong correlates of TAFIa action, it would also be very beneficial to directly measure TAFI activation throughout the same experimental parameters. Unfortunately, at the present time, this task is difficult and TAFIa production cannot be measured easily over time, as it can for thrombin and plasmin. By utilizing the enzymatic activity of TAFIa, substrate hydrolysis can be used to assess TAFIa production over time with Hipp-Arg or AAFR substrates spectrophotometrically, however, this is only possible in a purified setting and these substrates cannot be used in a plasma setting, as the turbidity associated with fibrin clotting disrupts the absorbance measurements (158, 167, 199). Moreover, other studies have detected hydrolysis of Hipp-Arg in plasma with high sensitivity, however, real-time generation monitoring is not possible as reactions are required to be stopped, centrifuged and processed using HPLC (463). As an alternative to these difficult methods, our group
is currently optimizing a novel fluorescence assay to detect TAFIa activity with high sensitivity, via substrate hydrolysis. The substrate consists of a fluorophore (TAMRA), conjugated to a Gly-Ala-Gly-Arg peptide. In general, during a reaction, the arginine would electrostatically interact with an anionic quencher, consequently, when TAFIa cleaves off the arginine residue, the quencher will be removed from the vicinity of the TAMRA fluorophore, allowing for the production of fluorescence.

4.2 Conclusions

In this study, it was shown that rivaroxaban results in a reduction in thrombin generation, thus exerting its intended anticoagulant effect. Furthermore, by diminishing thrombin generation, rivaroxaban also caused a reduction in TAFI activation, resulting in more rapid fibrinolysis. This supports the idea that rivaroxaban produces antithrombotic effects through both anticoagulant and profibrinolytic tendencies. It was then shown that these effects could be modulated when TAFI levels were elevated, or in the presence of the more stable Ile325 variant. The greater antifibrinolytic potential in such individuals might make them more resistant to the profibrinolytic effects of rivaroxaban, thereby enhancing risk for thrombosis while diminishing risk for bleeding complications. Conversely, individuals with low TAFI levels and/or the Thr325 variant would be at greater risk for bleeding complications while responding better to the antithrombotic effects of the drug. These findings may provide useful insight to the pharmacodynamics and pharmacogenomics of rivaroxaban in the clinical setting.
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APPENDIX

CHAPTER 5: OPTIMIZATION OF THE CHANDLER LOOP ASSAY:
A WHOLE BLOOD MODEL OF FIBRINOLYSIS

5.1 The Chandler loop assay

The primary methodology used to explore the hypotheses of this study was the clot lysis assay. This assay, conducted in pooled human plasma, proved to be an efficient, versatile and sensitive tool. Although this is a well-established assay, some aspects may differ from physiological conditions. Within the vasculature, clots are formed in whole blood and most of the time the clot formation does not occur under complete static conditions. As previously mentioned, the flow of blood can affect the components of clot formation and the resulting clot architecture (280). Whole blood also clots with the contributions of platelets, red blood cells and other types of cells. Due to these differences, it was hoped that the results discovered using the clot lysis assay could be supported using similar conditions in a fibrinolysis assay involving a whole blood clot formed under flow conditions, called the Chandler loop assay (464). Briefly, this model involves using flow conditions to form a clot in whole blood spiked with fluorescent fibrinogen. This clot is then placed in a solution of autologous plasma with added tPA and thus contains the components necessary for fibrinolysis. Fibrinolysis is then monitored by the release of fluorescence over time, produced from the fluorescently labelled fibrin that had been incorporated into the clot. Use of this model has been reported for various applications (465-468). For example, rivaroxaban has been used in
whole blood fibrinolysis assays (469, 470) and TAFI has also been studied using Chandler loop assays (471-473). The most relevant experiments to the applications being explored in the current study were reported by Mutch et al. (2007) (472). The most important result seen by Mutch et al. was that the inhibition of TAFI by PTCI allowed for a greater amount of whole blood clot lysis, as indicated by an increase in fluorescence release (Fig. 5.1).

*Figure 5.1 CPI inhibits TAFIa, resulting in greater lysis of a whole blood clot.* This figure was adapted from (472). The empty circles indicate the extent of lysis occurring over time in the absence of CPI, a specific TAFI inhibitor, as measured by release of fluorescence. The filled circles represent lysis of whole blood clots in the presence of CPI. This shows that CPI is capable of inhibiting the function of TAFIa in a whole blood clot model, resulting in greater clot lysis.
The experiments completed by Mutch et al. were used as a model for developing a system where both the effects of rivaroxaban and TAFI could be assessed simultaneously in a whole blood clot model. The goal was to perform these assays in the presence or absence of both rivaroxaban and PTCI. This would allow for the contributions of each of these components to be evaluated both independently and in combination. Throughout the completion of the research described in this thesis, countless hours of optimization went into attempting to replicate the results seen by Mutch et al., but ultimately the efforts were unsuccessful. This section will describe the scientific rationale for this optimization process.

5.2 Materials and methods

5.2.1 Rivaroxaban

Rivaroxaban was a generous gift from Bayer Pharma AG (Wuppertal, Germany). The powder form of purified rivaroxaban was resuspended in 100% dimethyl sulfoxide (DMSO) to a final concentration of 1 mM and stored at -80°C until use. Quality control NMR studies showed the proper drug composition after one year of storage. All experiments containing rivaroxaban were completed using separate stocks further diluted in 100% DMSO in order to obtain a final value of 1% DMSO in all reactions. All experiments containing 0 nM rivaroxaban included DMSO at 1% of the final reaction volume.
5.2.2 Alexa-488 Labelled Fibrinogen

Human fibrinogen (20 mg/mL) (EMD Millipore) was dialyzed in a buffer containing Na₂CO₃ (0.1 M) and NaCl (0.2 M, pH 8.6), then incubated with 10 mg/mL Alexa-488 dye (succinimidyl ester) (Life Technologies, Eugene, OR, USA) at a 5:1 fibrinogen:dye ratio for 4 hours at 4°C. The reaction was quenched with 0.01 volumes of 1 M TRIS (pH 8.0) and incubated at 4°C for 30 minutes. Free dye was removed by extensive dialysis in HBS. Concentration and degree of labelling were then calculated using the manufacturer’s protocol and the labeled fibrinogen was stored at -80°C until use.

5.2.3 Chandler Loop Assay

This procedure was adapted from (472). Two vials of blood (2 x 8.5 mL) were drawn fresh from a volunteer donor into BD Vacutainer ACD Solution A blood collection tubes (BD, Mississauga, ON, Canada). One was used as a source of whole blood and the other was used as a source of plasma. Plasma was isolated by centrifuging the whole blood at 4000 rpm for 15 minutes at 4°C, then pipetting the top plasma layer into a new conical tube, which was kept on ice until use. Clots were created by combining 0.9 mL of whole blood with a 250 μL mixture of rivaroxaban (0-800 nM, final), CaCl₂ (10.9 mM, final), Alexa-488 labelled fibrinogen (75 μg/mL, final), and PTCI (25 μg/mL, final) as required, brought to volume with HBS containing 0.01% Tween-80. This clotting mixture was then injected into a loop of tubing. The loop consisted of 33 cm long clear PVC tubing (Nalgene) with an inner diameter of 3mm and an outer diameter of 5 mm, connected end-to-end with a 1.5 cm piece of clear PVC.
tubing with an inner diameter of 5 mm. The loop was rotated at 30 rpm at room temperature. To accomplish this, two wooden disks (9.7 cm diameter and 15.5 cm diameter) were attached face-to-face and the axle of a rotating motor passed through their center. The tubing loop was fit around the circumference of the smaller wooden disk and the motor was started. After 90 minutes, the clot was poured from the tubing, washed with 0.9% NaCl and carefully blotted dry with filter paper. The clot was weighed and added to a lysis solution (150 µL fresh plasma, rivaroxaban (0-800 nM), CaCl$_2$ (25 mM), t-PA (15 nM), PTCI (25 µg/mL) as required, and made up to 500 µL with HBST). Clots were lysed at 37°C in this solution for 240 min total. 5 µL samples were taken from this solution, diluted 1/50 in HBST, and added to a fluorescence microplate to measure fluorescence (495 excitation, 519 emission) with a SpectraMax Plus M5e microplate reader (Molecular Devices).

5.3 Optimization of Conditions

Initially, the Chandler loop experiments were performed via the aforementioned procedure, in accordance with the Mutch et al. studies. However, when the experiment was completed, the fluorescence values obtained from the samples at each time-point were very erratic (Fig. 5.2), contrary to the consistent increasing trend of released fluorescence over time seen by Mutch et al. During the incubation period at 37°C, it was noted that the waterbath being used showed variability in the temperature. It was thought that this variation in temperature could contribute to changes in clot lysis rate. To correct this temperature instability, the incubation period was then performed on a heating block. In addition to this, the clots were also weighed before they were put into the lysis
solution. The fluorescence value obtained for each clot at each time point was then divided by this weight, in order to control for the proportion of fibrin weight that is capable of being released over time. Neither the heat block nor normalization to the clot weight assisted in reducing this variability (as seen in Fig5.2), so it was suspected that the variability was caused by the fluorescence values being close to the detection limit of the microplate reader, as this would account for the lack of precision in the fluorescence measurements. After this, the time-point samples were diluted 1/20, rather than 1/50, and throughout lysis, the increases in fluorescence seemed to be more consistent over time.

Consequently, a trial was attempted in both the presence and absence of PTCI. Here (Fig. 5.3), it was seen that fluorescence increased with the PTCI treatment, agreeing with the concept that PTCI is inhibiting TAFIa activity, thus promoting clot lysis.

![Fluorescence Release (RFU)](image)

**Figure 5.2** The initial replication of Mutch et al. showed erratic results. When the experiment were initially performed, the conditions of Mutch et al. were replicated, but the results were not similar. Rather than displaying a consistent trend, the release of fluorescence over time in these experiments was extremely erratic.
Figure 5.3 Chandler loop experiments showed an increase in lysis when TAFIa was inhibited by PTCI. Florescence release was shown over time as lysis occurred for whole blood clots created in the Chandler loop. The introduction of PTCI showed an increase in lysis, correlating with an inhibition of TAFIa, thus replicating what was demonstrated by Mutch et al.

Subsequently, an attempt was made to introduce rivaroxaban at 50 and 100 nM concentrations. These results showed that greater amounts of rivaroxaban led to a greater extent of clot lysis (Fig. 5.4). This correlates with the study showing that rivaroxaban caused whole blood clots to be more porous and more susceptible to clot lysis (450). The same results were seen in experimental repeats.
Figure 5.4 Rivaroxaban increases clot lysis. Fluorescence release from the whole blood clot is shown over time. Greater amounts of rivaroxaban are shown to increase clot lysis. This agrees with the effects of rivaroxaban on whole blood clots seen in (450).

With the apparatus constructed for the Chandler loop assay, it was possible to perform ten separate clots at one time, in one day (one trial), but the timing of each was staggered so all time intervals were equal between samples. As the experiments continued, it was realized that the sample arrangement was the same every time the experiment was completed. In the trial including ± PTCI treatment, the reactions with PTCI were completed after those without PTCI. In the trials containing rivaroxaban, the higher concentrations of rivaroxaban were tested in later samples. In order to see if the results being obtained were an artifact of the assay, the samples were organized in reverse order. Unfortunately, the exact same pattern was seen as before, proving that the results that had been shown in the previously completed experiments were, in fact, an artifact of the way the assay was being performed. At this point, the half-hour intervals between
each sampling time point were maintained between each sample, however, once the 5 µL sample was taken and diluted in HBST buffer, it remained in an amber tube for the rest of the sampling period. Once all samples were taken at all time points, each were then loaded into individual wells of a fluorescence microplate and read. Due to the fact that the samples taken later on in this assay developed greater amounts of fluorescence, it was hypothesized that fluorescence of the diluted sample was being quenched over the period of time preceding the microplate reading point. To solve this issue, each time a sample was taken, it was diluted and immediately measured by fluorescence.

When experiments were continued with this real-time sample measurement procedure, the results remained the same as before, proving that fluorescence quenching was not the issue at hand. This led to the belief that there may be a problem with the fluorescence plate being used. To test this hypothesis, various fluorescence readings were completed with different plate types, the presence of buffer, different dilutions of pure fluorescent fibrinogen, and different volumes of liquids. It was then determined that when the well volumes fell below 200 µL, the fluorescence readings became erratic, as opposed to a consistent increase, and the same trend in fluorescence seen in each of the preceding experiments was visible in the presence of buffer only.

The next experiments then included dilutions of the 5 µL samples taken at each time point into a final volume of 200 µL with buffer. To test the proper functionality of the assay under these conditions, two different concentrations of tPA (15 nM and 20 nM) were used in combination with two different time-point sample volumes (5 µL and 10 µL). These conditions were also arranged in a random order to remove any experimental setup bias. This trial showed the expected results as the greater sample volume yielded
greater fluorescence values and the greater amount of tPA was shown to slightly induce more lysis, as indicated by greater fluorescence values (Fig 5.5).

![Graph showing release of fluorescence over time from whole blood clots undergoing fibrinolysis.](image)

**Figure 5.5 Confirmation of the expected fluorescence readout.** This graph shows the release of fluorescence over time from whole blood clots undergoing fibrinolysis. Due to the fact that an increase in sample volume and an increase in tPA concentration showed greater amounts of fluorescence, these findings show that the fluorescence readout is no longer an artifact of the assay.

The assay was then performed in the presence of 0, 400 or 800 nM rivaroxaban, in order to complement the concentrations used within the clot lysis assays. The findings from these experiments initially showed somewhat promising results. Greater rivaroxaban concentrations seemed to yield greater amounts of lysis, as expected based on previous studies (Fig. 5.6 A, B). However, these effects were not consistent between
different individuals (Fig. 5.6 A compared to D), or even the same individual on different
days (Fig 5.6 B compared to C).
**Figure 5.6 Inter- and intra-individual variability.** Release of fluorescence is shown over time for a whole blood clot undergoing fibrinolysis. These clots were lysed in the presence of 0, 400 or 800 nM rivaroxaban. A and D are different individuals and C and D are the same individual on different days.

After this variation was seen between different individuals and the same individual on different days, it was concluded that all conditions being tested must be performed in the same day, in the same trial. The next experiment then included 0 or 400 nM rivaroxaban in combination with 0 or 25 ug/mL PTCI (Fig. 5.7). The results of this trial were inconclusive, as slightly lower levels of lysis were seen in the samples containing rivaroxaban and the samples containing PTCI did not show significantly
greater amounts of lysis. Due to the fact that no significant differences were seen between treatments, it was then presumed that improving the resolution between samples would be necessary.

Figure 5.7 Direct comparison of rivaroxaban and PTCI effects showed inconclusive results. This graph represents the release of fluorescence over time from whole blood clots undergoing fibrinolysis. Here, combinations of 0 or 400 nM rivaroxaban and 0 or 25 ug/mL PTCI were included. The different conditions of this experiment showed unpredictable results that were deemed inconclusive.

Over the next group of experiments, several strategies were implemented independently of one another, in order to attempt to increase the differences in
fluorescence seen between the different experimental conditions. First, the amount of fluorescent fibrinogen added to the forming clot was increased from 75 µg/mL to 100 µg/mL and 150 µg/mL, with the intention to increase the amounts fluorescence being released during lysis, thus creating larger differences between experimental conditions. Additionally, the amount of PTCI added was increased from 25 µg/mL to 35 µg/mL, to ensure that all TAFIa activity was being inhibited since a clear effect of TAFIa inhibition was not seen. This was followed by an attempt to increase the proportion of plasma present in the lysis solution from 30% to 50%, with the idea that this would increase the amount of available components such as plasminogen and TAFI. With greater amounts of plasminogen, the effects of TAFIa on decreasing clot lysis should be more apparent and with greater amounts of TAFI present, the reactions containing PTCI should show a more dramatic increase in lysis. Another experiment was completed with increasing plasma in the lysis reactions, except the amount of tPA was increased from 15 nM to 20 nM. This should have allowed the effects of TAFIa, or the lack thereof to be more apparent in the context of lysis. The length of sampling time was also increased from 180 minutes to 240 minutes in order to allow a sufficient amount of time for lysis rates to differ from one another between experimental conditions. At one point, an unsuccessful attempt excluded rivaroxaban from the clot formation solution, only including it in the lysis buffer. This was completed to rule out the possibility that the presence of rivaroxaban yielded a less substantial clot, causing less fluorescent fibrinogen to be present in the clot and ultimately resulted in a fluorescence release unrepresentative of the lysis that was actually occurring. In one last attempt to replicate the results seen by Mutch et al., an unsuccessful trial was completed in the presence and absence of PTCI, but using TBST buffer instead
of HBST. Although this should not make a difference based on scientific rationale, it was one of the only remaining differences between our experiments and those of Mutch et al.

Each of these attempts were unsuccessful at increasing the differences seen between the different conditions of PTCI and rivaroxaban addition in these experiments. Some results showed the expected effects of both PTCI and rivaroxaban, but others showed the opposite. Most showed no significant differences between different conditions. Most of the hypothesized explanations for the inability to reiterate the results of Mutch et al. were explored, thus it is difficult to explain these findings. Although the procedure provided in the paper was followed precisely, it is still possible that there may be certain techniques involved that were not included. Two other theories could be tested. First, greater effects of TAFI and TAFI inactivation by PTCI may be seen if additional Glu-plasminogen is added to the reaction. This would contribute to a greater increase in clot lysis when TAFIa is inhibited by PTCI, as TAFIa would normally inhibit the activation of Glu-plasminogen, effectively. Lastly, there may have been an issue with the fluorescently labelled fibrinogen. Although Mutch et al. used fibrinogen labelled with a FITC fluorophore, other studies exploring fibrinogen activation and fibrin aggregation have used Alexa-488 labelled fibrinogen (ex. (474)), and both dyes are reactive towards amino groups. Studies have shown that this fluorophore should actually be superior to FITC, in terms of the stability of the fluorescent signal. There still may be a possibility however that an unappreciated anomaly occurred during the labelling procedure. The labelled fibrinogen product was subjected to SDS-PAGE without any indication of degradation. Heterogeneity or dysfunction may have arisen due to the propensity of the fluorescent fibrinogen stocks to aggregate, before, during, and after the labelling process.
The manufacturer provided indications to heat the fibrinogen to reduce aggregation, which was done in these studies, but may have been less necessary if the fluorescently labelled fibrinogen was stored in more dilute stocks. Although this is not a likely cause, a new preparation of fluorescent fibrinogen could be made to test this hypothesis.

To conclude, the Chandler loop experiments presented in this study were unsuccessful at replicating the results seen by Mutch et al., thus discounting their use in supporting the previously described clot lysis assay results.
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