The use of antiplasmin-specific Affimer as a tool to modulate fibrin clot properties and thrombosis risk

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Submitted in accordance with the requirements for the degree of Doctor of Philosophy
April 2020

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Acknowledgement

First and foremost, praises and thanks to God almighty, for the blessings throughout my research work I am extremely grateful to my late parents, God bless their souls, for their love, prayers, caring and sacrifices for educating and preparing me for my future. Also, to my wife and family for their understanding and continuing support to complete this work. I would like to express my deep and sincere gratitude to my research supervisor, Professor Ramzi Ajjan, for giving me the opportunity to do research and providing invaluable guidance throughout my work especially lately in the time of COVID 19. His dynamism, vision, sincerity and motivation have deeply inspired me. I am extremely grateful for what he has offered me. Also, I am indebted to my other supervisors, Dr. Christian Tiede for teaching me various laboratory techniques and guiding me throughout my work and Dr. Darren Tomlinson for introducing to the world of Affimers and facilitating the conduction of my studies. I also thank my department, university and all people who have supported.

Abstract

Introduction: Vascular thrombosis represents one of the most common causes of mortality in the western world. The obstructive thrombus is composed of a mesh of fibrin fibres with blood cells trapped in these networks. Fibrin clot lysis, that limits thrombus formation and helps to prevent extensive vascular occlusion, is controlled by a number of factors including incorporation of anti-fibrinolytic proteins into the clot, most importantly plasmin inhibitor (PI).

Aim: The aim of the study is to identify the role of artificial binding proteins (also termed Affimers) in modifying the effects of PI on fibrin clot lysis.

Method: A large library of Affimers was screened for PI binding using a phage display system. High affinity PI-binding Affimers were screened for modulation of fibrin clot lysis. Validated turbidimetric assays were employed to assess the role of PI-binding Affimers on fibrin clot structure/lysis, using both purified and plasma systems. In order to investigate mechanistic pathways, a number of techniques were employed including confocal and electron microscopy, immunoblotting, pull-down assays, mutagenesis work, mass spectometry and molecular modelling.

Result: Following three rounds of phage panning of two independent screening tests, a total of 167 high affinity PI-binding Affimers were isolated. Out of these 167 Affimers 24 had different sequences and these were subcloned and expressed *E. coli*. One Affimer (A68) consistently inhibited the prolongation of PI-induced clot lysis in a purified system and also reduced plasma clot lysis from 650±23 to 420±21 sec ($p<0.001$). The effects of the Affimer were maintained in whole blood systems and a number of different clinical conditions indicating consistency of action. Mechanistic work indicates that Loop 1 of Affimer A68 is responsible for PI inhibition with possibly multiple areas on the protein responsible for interaction with the Affimer.

Conclusion: The work so far provides proof of concept that PI-binding Affimers represent a viable tool to modulate fibrin clot lysis and may help to identify novel therapeutic targets. Future animal work is warranted to understand whether Affimer A68 reduces thrombosis in vivo, with the potential to offer a new therapeutic agent to decrease the risk of vascular occlusion.

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List of Abbreviations

ADP	Adenosine diphosphate
Ala	Alanine
α2M	α2 macroglobulin
°C	Degrees Celsius
2-MEA	2- mercaptoethylamine
3D	3 dimensional
aa	Amino acids
APCE	Antiplasmin cleaving enzyme
Arg	Arginine
Asn	Asparagine
BLAST	Basic local alignment search tool
Bp	Base pairs
$CaCl_2$	Calcium chloride
Carb	Carbenicillin
CT	Clotting time
CVD	Cardiovascular disease
Da	Daltons
ddH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	Glutamic acid
E. coli	*Escherichia coli*
EDTA.	Ethylenediaminetetraacetic acid
ELISA	linked immunosorbent assay
F	Phenylalanine
FDP	Fibrin (or fibrinogen) degradation products
FpA	Fibrinopeptide A
FpB	Fibrinopeptide B
FV	Factor five
FVII	Factor seven
FVIII	Factor eight
FIX	Factor Nine
FX	Factor ten
FXI	Factor eleven
FXII	Factor twelve
FXIII	Factor thirteen
g	G force/relative centrifugal force
GFD	Growth factor domain
Glu	Glutamic acid
Gly	Glycine
H	Histidine
H_2O	Water
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HER	Human epidermal growth factor receptor
His	Histidine

HMW	High molecular weight hr Hour
HRP	Horseradish peroxidase
I	Isoleucine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K	Lysine
K_a	Association rate constant
K_d	Dissociation rate constant
K_D	Equilibrium dissociation constant
kDa	Kilodaltons
L	Leucine
LB	Lysogeny broth
LC- MS/MS	Liquid chromatography tandem mass spectrometry
LSCM.	Laser scanning confocal microscopy
LDS	Lithium dodecyl sulphate
M	Molar
MCF	Maximum clot firmness
MES	2-(*N*-morpholino) ethanesulfonic acid
Met	Methionine
mg	Milligram
mg/l	Milligram per litre
mg/ml	Milligram per millilitre
min	Minutes
ML	Maximum lysis
mM	Millimolar
mm^2	Millimetres squared
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MW	Molecular weight
Na	Sodium
Na_2HPO_4	Sodium phosphate dibasic
NaCl	Sodium chloride
NaH_2PO_4	Sodium phosphate monobasic
NETs	Neutrophil extracellular traps
ng	Nanograms
ng/ml	Nanograms per millilitre
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanometre
nM	Nanomolar
NP	Normal pool
NTP	N-terminal peptide
OD	Optical density
OPD	O-phenylenediamine dihydrochloride
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor- 2
PB	Permeation buffer
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween-20

PCR	Polymerase chain reaction
PDB	Protein database
PD-L1	Programmed death ligand-1
PEG	Polyethylene glycol
PI	Plasmin inhibitor
Plg	Plasminogen
Pro	Proline
PSMA	Prostate specific membrane antigen
PVDF	Polyvinylidene difluoride
R	Arginine
ROTEM	Rotational thromboelastometry
rpm	Revolutions per minute
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
Sec	Seconds
SEM	Scanning electron microscopy
Serpin	Serine protease inhibitor
SH2	Src-Homology 2
SPR	Surface plasmon resonance
SQT	Stefin A quadruple mutant-Tracy
T&L	Turbidity and lysis
TAFI	Thrombin activatable fibrinolysis inhibitor
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween-20
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
Thr	Thrombin
TNC	Tenascin C
tPA	Tissue plasminogen activator
TRAP	Thrombin receptor activating peptide
TTP	Thrombotic thrombocytopenic purpura
TXA	Tranexamic acid
U/ml	Units per millilitre
µg/ml	Microgram per millilitre
µM	Micromolar
uPA	Urokinase type plasminogen activator
V	Volts
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor
WB	Wash buffer

Note: (a) after the coagulation factor indicates the activated form of that coagulation factor.

Chapter 1 : Introduction

1.1 Cardiovascular Disease

Cardiovascular Disease (CVD) is the leading cause of mortality worldwide [1]. According to data compiled by WHO, CVD claimed 17.9 million lives in 2016, representing 31% of global deaths, of which 85% were due to a heart attacks and strokes [1]. BHF statistics in the UK showed that 170,000 deaths/year occur as a result of CVD, representing 28% of all-cause mortality keeping with international trends [2]. CVD affects a diverse group of population making it an important and active area of medical research.

Individuals with diabetes are at a particular risk of CVD, which remains the main cause of mortality in this population [3, 4]. While both type 1 and type 2 diabetes show elevated plasma glucose levels, the aetiology of the two conditions is very different. Type 1 diabetes is an autoimmune condition by which the body destroys its insulin producing β-cells of the pancreas. In contrast, the main pathophysiological abnormality in type 2 diabetes is insulin resistance, which the pancreas responds to by increasing insulin production. However, it reaches a point where it cannot keep up with the demand to compensate for insulin resistance, leading to elevated glucose levels [5, 6]. High blood glucose level increases the risk of atherosclerosis and later atherothrombosis, consequently leading to vessel occlusion and potentially end organ damage [7].

Several factors contribute to the development of CVD and these can be broadly classified into modifiable and non-modifiable risk factors [8]. The former group includes smoking, dyslipidaemia, hypertension, diabetes and obesity, which can be controlled and play a key part in the strategies aiming to prevent vascular damage. Indeed, smoking cessation, management of dyslipidaemia, control of hypertension and reduction of glucose levels in diabetes have all shown to improve cardiovascular outcome. Many of the above risk factors have an additive effect and can significantly alter disease progression and predisposition to complications. Non-modifiable risk factors include age, gender and family history. For example, post-menopausal women have a higher incidence of CVD in comparison to pre-menopausal women who are less prone due to the protective effect of female hormones [9]. Although regarded as non-modifiable risk factors, the “adverse effects” of genetic factors and age can be controlled by more intensive cardiovascular preventative strategy. It should be pointed

out that the importance of various risk factors appear to be in constant change with diabetes and obesity taking a central stage [8, 9].

1.2 Atherosclerosis and atherothrombosis

The pathophysiology of cardiovascular disease is complex and starts with the slow progression of atherosclerosis and ending with plaque rupture that initiates blood clotting resulting in the formation of a vascular obstructive thrombus [10]. This is turn results in restriction of blood supply to a particular part of the body, culminating in end organ damage and consequently causing mortality or long-term morbidity. Atherosclerosis, a disease of the large arteries, is the primary cause of heart disease and stroke, and, as alluded to earlier, is responsible for around a third of all deaths [11]. The atherosclerotic process is driven by oxidative stress and inflammation in the arterial wall [10], and is characterised by endothelial cell dysfunction, lipid deposition, smooth muscle proliferation and intimal hyperplasia, eventually leading to plaque formation [12]. The formation of discrete plaques represents the hallmark of atherosclerosis, which contain different elements. The fibrous cap, representing the luminal area in contact with blood cells, is composed of smooth muscle cells and fibroblasts covered by endothelial cells. Other components of the atherosclerotic plaque include lipid particles and inflammatory cells.

Some plaques are more likely to rupture than others. Important characteristics of a weak plaque include a thin fibrous cover, lipid-rich and hypocellular core, the presence of white blood cells that produce metalloproteinases (MMPs) and other factors that lead to extracellular matrix degradation and apoptosis. The rupture of the fibrous cap, or simply plaque disintegration, exposes a prothrombotic core that starts the process of coagulation culminating in the formation of the intravascular thrombus [12]. The different stages of the atherosclerotic plaque formation are depicted in Figure1.

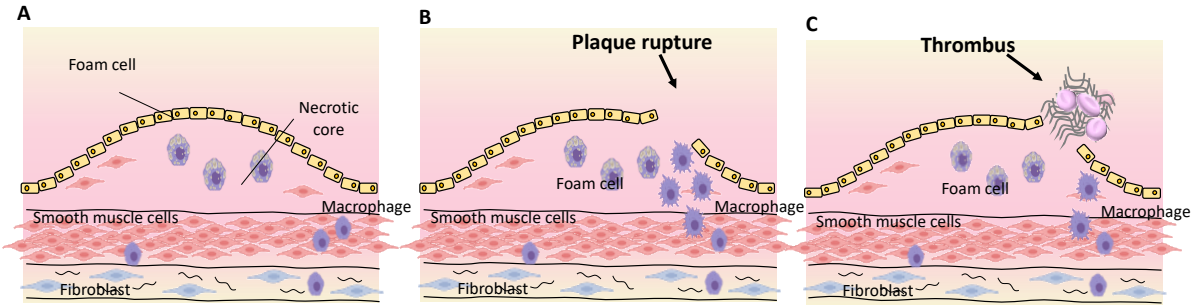

Figure 1. Atherosclerotic plaque rupture and thrombosis
A. Coronary atherosclerotic plaque with thin-walled fibrous cap and thrombogenic necrotic core. B. Plaque rupture exposes a prothrombtic core that initiates blood clot formation. C. Thrombotic response. There is no obstruction to blood flow when thrombus formation is not extensive, which may not even cause clinical symptoms. However, if thrombus formation is extensive, blood flow is obstructed, and blood flow to the organ is compromised. In case the obstruction of the coronary arteries, acute coronary syndrome develops.

The structure of a normal artery is shown in Figure 2. The initial lesions of atherosclerosis mainly comprise sub-endothelial gathering of cholesterol-engorged macrophages, which are referred to as ‘foam cells. Collection of foam cells forms the ‘fatty streak’, which is the earliest abnormality in the atherosclerotic process and can be seen in the first ten years of life in the aorta while the coronary arteries can be affected in the second decade, and the cerebral arteries in the third and fourth decades. Due to differences in blood flow dynamics, there are preferred sites of lesion formation within the arteries [10], with atheromas having a tendency to form in areas of blood turbulence.

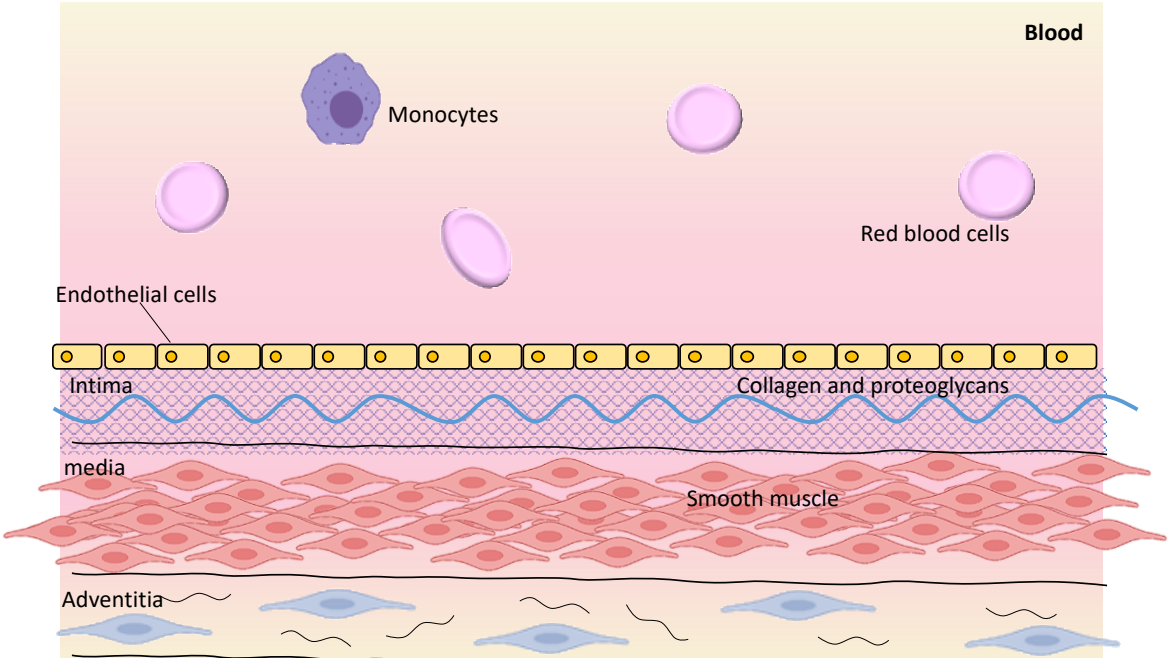

Figure 2 Structure of a normal large artery.
A large artery comprises three morphologically separate coating. The intima, the first and the innermost layer, is formed by a monolayer of endothelial cells on the luminal side and a sheet of elastic fibres, the internal elastic lamina on the peripheral side. The normal intima is a thin region and includes extracellular connective tissue matrix, primarily proteoglycans and collagen.

Unstable plaques can lead to sudden deterioration of blood supply to an organ, through thrombus formation and vascular occlusion, and therefore require prompt medical intervention [13]. Recent studies have revealed that coronary luminal thrombus formation mainly arises from plaque rupture (55–65%), followed by plaque erosion (30–35%) and less frequently from calcified nodules (2–7%) [14].

Features of the atheromatous plaque can determine risk of atherothrombosis, including lesion size and whether the plaque is prone to rupture [15]. The stable plaque has a preponderance of SMCs embedded in a dense matrix of collagen whereas the unstable plaque is composed of a large core of inflammatory cells and cell debris with a fragile cap that is predisposed to rupture [10].

Atherothrombosis is a term which describes atherosclerotic plaque disruption with superimposed thrombosis highlighting the interdependence of these two important events [16]. Thrombosis happens due the “unstable” atherosclerotic plaque and is the main mechanism underlying acute coronary disorders, and vascular research has focused mostly on this model. Atherosclerotic plaques can also cause ischaemic

stroke with most recent evidence indicating the main mechanism of thrombus formation in carotid arteries is almost the same to the coronary model.

Once the plaque ruptures, or in cases, the erosion is severe enough to expose the vascular wall, both the cellular and acellular arms of coagulation are activated resulting in thrombus formation, which is further detailed below.

1.3 The coagulation Cascade and its role in thrombus formation

The coagulation system comprises a cellular component represented by platelets and a fluid phase that includes a number of plasma coagulation proteins [17]. There are important interactions between the cellular components and coagulation factors that ensure the formation of a stable blood clot. Effective thrombus formation is key to prevent blood loss following vascular injury, secondary to trauma, but in case of intravascular injury (i.e. plaque rupture) this can become a harmful process leading to unwanted vascular occlusion.

Platelets adhere to the damaged endothelial wall, forming a platelet aggregate by spreading over the surface, via specific platelet receptors. The platelets bind to von Willebrand factor (vWF) and collagen via glycoprotein (GP)Ib-V-IX and GPVI respectively. Platelets activation occurs after adherence to extracellular matrix components and interaction with soluble agonists such as thrombin. Activated platelets change shape, express integrins and release autocrine agents which cause further platelet adhesion and activation. The cell-based thrombin generation happens due to exposure of phosphatidylserine on the membrane surface following platelet activation. Platelets interact with fibrinogen via receptor GPIIb/IIIa, causing platelets to aggregate via fibrinogen bridges, and producing fibrin networks through the action of thrombin, which form the skeleton or backbone of the blood clot [18].

Activation of the coagulation factors occurs simultaneously with platelet activation. The coagulation cascade has been traditionally divided into two pathways: an intrinsic and an extrinsic [19]. The intrinsic pathway is activated by contact of factor XII with collagen, whereas the extrinsic pathway is initiated by tissue factor (TF). Through a series of reactions with various coagulation proteins including factors XI, IX, V and VIII, the common coagulation pathway is propagated culminating in the production of

thrombin that mediates the conversion of soluble fibrinogen into insoluble fibrin networks (Figure 3) [20].

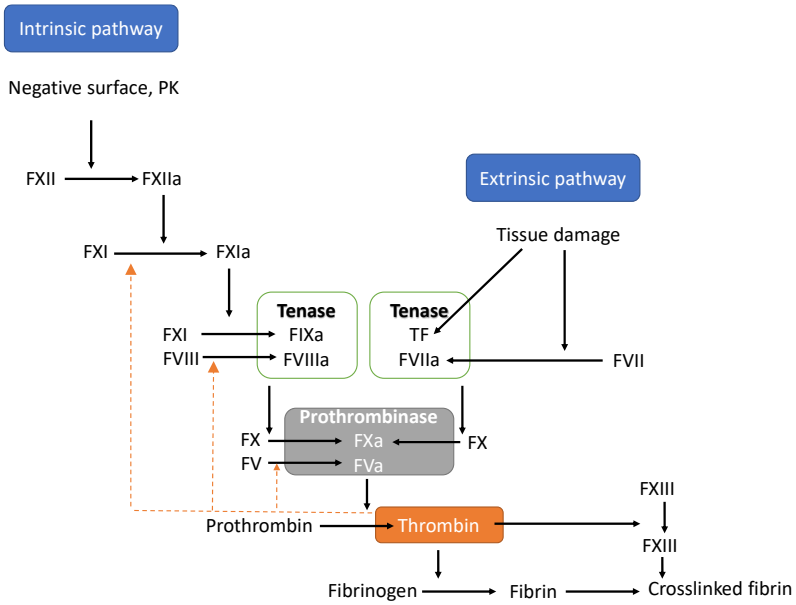

Figure 3. Simplified diagram of the coagulation proteins and their pathways.
The two pathways, intrinsic and extrinsic, culminate in the generation of thrombin, which mediates the conversion of soluble fibrinogen into insoluble fibrin networks. The intrinsic pathway is started after the activation of factor (F) XII by contact with surface that is negatively charged and involves prekaillikrein (PK). The extrinsic pathway is started after damage of tissue and the release of tissue factor (TF). Both of these pathways join at the start point of FX to FXa and the conversion of prothrombin to thrombin. Thrombin cleaves soluble fibrinogen to form insoluble fibrin, and also activates FXIII which introduces the crosslink into fibrin fibres and also incorporates antifibrinolytic proteins to stabilise the clot and prevent breakdown.

While this division into extrinsic and intrinsic pathway works well in laboratory environment, it is less convincing as an in vivo model, in which the two pathways are less clearly defined. For example, if the intrinsic and extrinsic systems were operating independently in vivo, individuals with haemophilia (due to factor VIII deficiency) should not have significant bleeding following trauma as the “extrinsic pathway” is theoretically intact. Therefore, a more realistic pathway has been proposed that incorporates the two pathways into one as follows: After vascular external injury (or plaque rupture), tissue factor (released by vascular endothelial cells) serves as the

primary initiator of the coagulation cascade [21]. This latest model of coagulation depicts the process of coagulation into three phases; initiation, amplification and propagation.

Initiation phase

After damage to the vessel wall (external injury or plaque rupture), platelets adhere to site of injury, and they become activated. Exposed TF binds FVII and activates this coagulation factor forming FVIIa. The complex of TF and FVIIa activates FIX and FX that result in the formation of FIXa and FXa with the latter activating FV. Cofactor FVa associates with FXa in order to form a prothrombinase complex on TF expressing cells, and this complex converts prothrombin into thrombin.

Amplification phase

This occurs through accumulation of thrombin generated during initiation phase. Thrombin then activates more platelets at site of injury and releases activated vWF and also converts platelet-derived FV to FVa, thus intensifying the activity of prothrombinase. Furthermore, thrombin activates FVIII to FVIIIa that serves as a cofactor to FIXa on the surface of activated platelets, enhancing FXa generation. Moreover, FXI is converted into FXIa by thrombin.

Propagation phase

While the initiation stage occurs at the location of vessel injury, the propagation phase takes place on procoagulant phospholipid surface, such as activated platelets. FXIa converts FIX into FIXa which is then associated with thrombin cleaved FVIII. In a similar fashion to initial TF release, Xa is formed following FIXa/VIIIa complex formation, which is further complexed with Va. This complex helps to produce large amounts of thrombin, particularly if sufficient platelets are recruited to the site of injury. The transglutaminase FXIII, activated by thrombin, then cross-links fibrin fibres and also cross-links antifibrinolytic proteins into the fibrin network, collectively helping to stabilize the clot [21-23]. Figure 4 summarises the proposed in vivo model of coagulation, involving both platelets and coagulation factors.

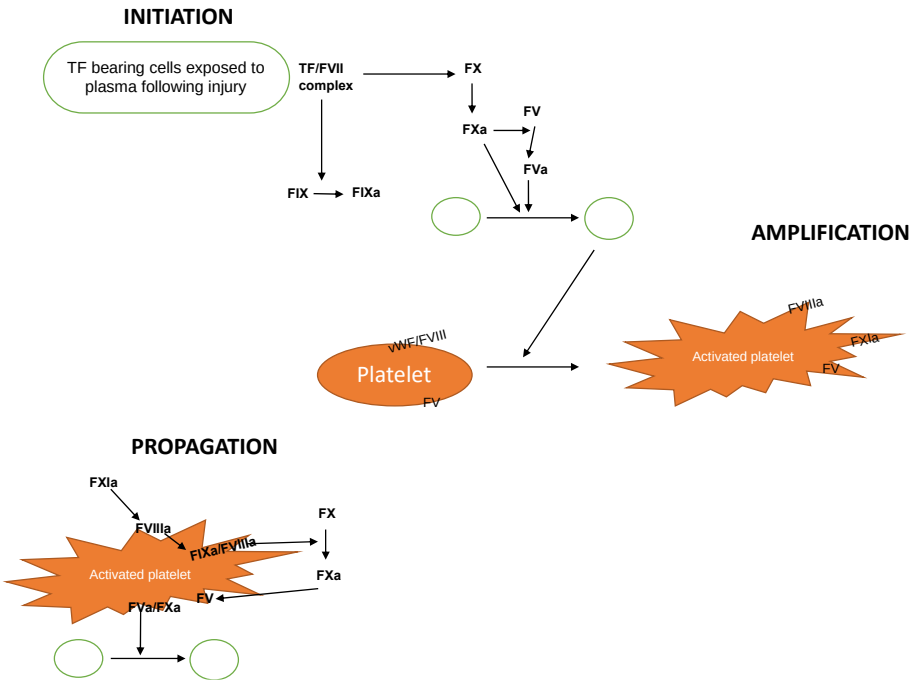

Figure 4. The coagulation cascade in relation to atherothrombosis.
A break in the vessel wall brings the plasma into contact with tissue factor (TF) bearing cells. Platelets adhere to the site of injury and become activated. In the initiation phase, factor (F) VII binds to TF and is activated. FVII/TF complexes activate FIX and FX and the latter activates FV. Activated FX and FV cleave prothrombin to generate thrombin. Collagen-bound, partly activated platelets become further activated by the limited amount of thrombin generated in this phase. In the propagation phase, fully activated platelets release FV, and this in turn gets activated by both FX and thrombin. In addition, thrombin cleaves FVIII releasing it from vWf and activates FXI bound to platelet surface. FX is recruited to activated platelets and is activated by FIXa/VIIIa complex. Platelet surface FXa/Va complexes result in the generation of sufficient thrombin from prothrombin to convert fibrinogen into fibrin and activate FXIII, which further ensures the formation of a stable haemostatic plug.

1.4 Fibrin clot formation and stabilisation

The fibrin network is formed from fibrinogen through two essential steps that involve FpA and FpB release. When thrombin binds to the central E region of the molecule, it removes FpA from the α-chain of fibrinogen first, generating a new N-terminal site (called A site). This is subsequently followed by the formation of a non-covalent bond between A on the α-chain and “a” pocket on the chain of a neighbouring molecule. This produces a half staggered chain of double stranded protofibrils thus elongating

the fibrin chains. In a second step, FpB is released from the N-terminus of the beta-chain of fibrinogen, resulting in the formation of site B. This then binds with a “b” pocket located in the D-region of another fibrinogen beta-chain, which promotes lateral aggregation making the fibres thicker. In short, FpA release helps making longer fibrin fibres while FpB release is essential for rendering the fibres thicker [24].

Once fibrinogen is converted to fibrin, a number of steps take place to ensure the formation of a robust clot that is resistant to lysis. The crosslinking of neighbouring fibrin fibres, by activated FXIII, results in branched fibrin clot structure that increases its stability [24]. Moreover, thrombin activated FXIII crosslinks antifibrinolytic proteins into the clot which further stabilises the fibrin network (detailed below). In normal physiology, a balance is maintained between clot formation and lysis in order to prevent widespread vascular occlusion or excessive bleeding. Shifting the balance to either side can result in a wide array of pathologies. Just like the coagulation cascade, the process of fibrin breakdown (fibrinolysis) is also carefully regulated by a number of factors [25]. Plasmin, generated from plasminogen, is the primary enzyme responsible for fibrin breakdown. The conversion of plasminogen to plasmin is mediated by serine protease tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). The former is synthesized by endothelial cells and binds to fibrin, whereas uPA [26] does not require fibrin as a cofactor and is produced by monocytes, macrophages and urothelium [27].

1.4.1 Inhibition of fibrinolysis and maintaining physiological haemostasis

Similar to activators, inhibitors are also important in preventing excess and unregulated plasmin and plasminogen activator activity [28]. These are serine protease inhibitors also known as serpins which covalently bind to their target molecules. Inhibitors are essential to maintain haemostasis as deficiencies can lead to unstable clot formation and increased tendency to bleed [27]. The two serpins crucial to fibrinolysis are 1) plasminogen activator inhibitor-1 (PAI-1), 2) Plasmin inhibitor (PI), also referred to as α2-antiplasmin (PI). PAI-1 binds tPA, interfering with its ability to convert plasminogen to plasmin. Plasmin inhibitor (PI), which is crosslinked into fibrin networks by FXIII, acts as a potent inhibitor of plasmin [27]. Apart from serpins, a non-serpin fibrinolysis inhibitor, thrombin activated fibrinolysis inhibitor

(TAFI) also plays a role by cleaving C-terminal lysine and arginine residues on fibrin, thereby decreasing the number of available plasminogen binding sites, consequently slowing plasmin generation, thus stabilising the clots [29].

Interestingly, proteins that are not regarded as coagulation factors can also act as fibrinolysis inhibitors. For example, the inflammatory protein complement C3 has been shown to delay fibrin clot lysis through incorporation into the clot, which compromises the fibrinolytic efficiency of plasmin [30]. The mechanisms behind the antifibrinolytic effects of C3 include “exhausting” the effects of plasmin (given C3 is a substrate for this enzyme) and possibly mechanical inhibition of lysis secondary to binding and cross-linking of this protein into the clot.

1.4.2 Plasmin inhibitor (PI): A principal regulator of fibrinolysis

Plasmin inhibitor (PI), a key regulator of fibrinolysis, is a glycoprotein of 63-67 kDa molecular weight [31]. It circulates in plasma at concentrations of 69±6µg/ml [32] and is produced by liver parenchymal cells; consequently plasma levels are reduced in liver disease [33]. There are other PI producing organs, as both the kidney and the brain have been implicated, but the liver remains the main organ synthesising the protein [34]. The mean absolute synthetic rate of PI has been assessed at 2.1 mg/kg per day [34]. The in vivo half-life of PI is about 2.6 days but plasmin-antiplasmin complexes (PAP) are cleared much faster from the circulation with a half-life of approximately 0.5 day [35].

The main role of PI is inhibition of plasmin-mediated fibrinolysis [36, 37]. The inherited deficiency of PI often results in a severe bleeding disorder [38, 39], while high levels of PI have been linked to an increased risk of myocardial infarction (MI) [40]. This serpin mediates its action through three different pathways: (i) forming a complex with plasmin; (ii) inhibiting adsorption of plasminogen to fibrin; and (iii) modulation of tPA and urokinase activity [41, 42]. Two forms of PI circulate in human plasma: plasminogen binding PI (PB-AP), a 464-residue protein with methionine as the amino-terminus (Met-PI) and non-plasminogen binding PI (NPB-AP), a protein with N-terminally shortened 452-residue form with asparagine as the amino-terminus (Asn-PI). Human plasma contains both forms at around 30% Met-PI and 70% Asn-PI. The latter is a result of the action Met-PI antiplasmin cleaving enzyme (APCE) and this form is more rapidly crosslinked into fibrin by FXIIIa. Around 30-40% of circulating PI

is also reduced at the C-terminus which is able to slowly bind plasminogen [43, 44]. Even though free PI is capable to inhibit plasmin, the complex of fibrin-bound PI has a more significant effect on clot lysis [45, 46].

Early studies, in which special monoclonal antibody inhibitors were used, demonstrated that PI was a strong agent that controlled fibrinolysis. PI-specific antibodies have shown that inhibition of PI resulted in quicker clot lysis and this even occurred without the use of exogenous plasminogen activators [47]. Inhibition of PI caused spontaneous lysis of human plasma clots without even adding exogenous plasminogen activators [48]. Moreover, to enhance thrombus dissolution in experimental venous thrombosis, specific inhibition of fibrin-bound PI was sufficient to allow enhanced fibrinolysis [49].

On the other hand, PI deficiency is directly linked to haemophilia-like bleeding symptoms, related to increased fibrinolysis, which typically occurs after initial haemostasis as a result of premature dissolution of fibrin network that holds the blood clot [38]. People who are suffering from advanced liver disease can have reduced synthesis of PI, explaining their increased risk of bleeding that can be often difficult to control [50]. In cases of disseminated intravascular coagulation disorder, such as that occurring with blood malignancies, PI is one coagulation factor that becomes depleted, adding to the risk of bleeding [50].

1.4.3 Mechanism of plasmin inhibition by PI

The major physiological role of PI is the quick inhibition of plasmin, which occurs when PI forms a 1:1 stable complex with plasmin, either in the circulation or on the fibrin surface[51]. The reaction proceeds via a 2-step mechanism. (Figure 5). At the start, in a reversible second-order reaction, the C-terminal end of PI, which contains 6 lysine residues, binds noncovalently to the lysine binding sites (LBSs) of plasminogen[52]. This step can be competitively inhibited by plasminogen fragments that contain LBSs or by lysine analogues such as e-amino caproic acid [52]. The newly crystallized structure of natural plasminogen showed that in Glu-plasminogen (closed conformation), the initial binding of PI would be primarily LBS independent, whereas in Lys-plasminogen (open conformation), the LBS of kringle 2 is mainly involved in this binding [53]. Previous studies have shown that kringle 4 is crucial in the interaction of

plasmin with PI, but that kringles 1, 2, 3, and 5 are also involved [54]. In the second step, the first irreversible reaction, the arginine residue at position 376 of PI (numbering according to methionine at position 1) in the interactive media loop (RCL) forms a covalent bond with the active site serine of plasma. This PAP results in complete loss of plasmin activity and division of the PI series peptide bond [55].

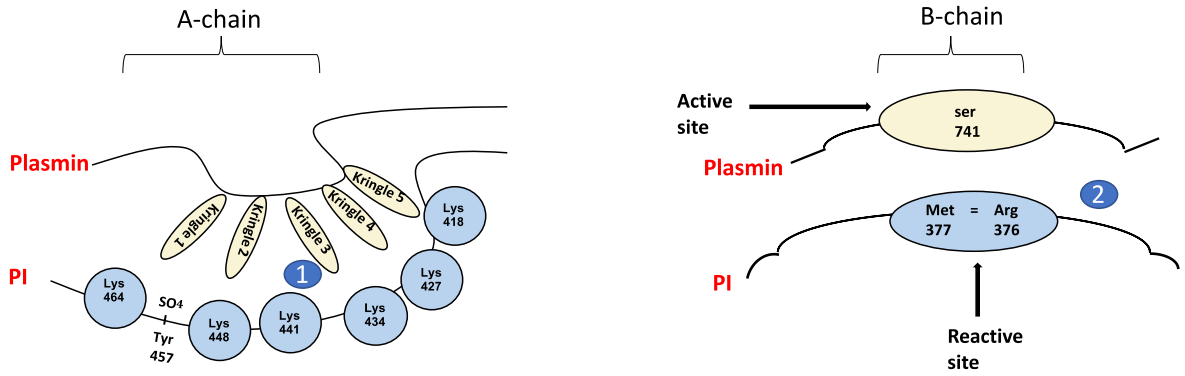

Figure 5. Schematic illustration of plasmin inhibition by plasmin inhibitor (PI).

PI inhibits plasmin by making a 1: 1 complex with protein. The reaction continues more in a two-step mechanism. The C-terminal of PI, which contains 6 residues of lysine, is covalently bound to LBSs present in the kringle domains of plasmin. The second step is an irreversible first-degree reaction whereby the arginine residue at position 376 of the PI makes a covalent bond with the active site serene at position 741 of plasmin.

1.4.3.1 N-terminal variation

After expression, the full-length PI protein is modified, leading to a variety of circular PI molecules (Figure 6). As mentioned earlier, the N-terminus of PI is cleaved between the proline residue at position 12 and the asparagine residue at position 13, resulting in Asn-PI, which represents 70% of the protein in the circulation [56]. This results in the formation of a PI molecule with an asparagine (Asn) residue at the N terminus (Asn-PI) [57, 58]. The remaining 30% of the protein circulates in plasma with a methionine (Met) residue at the N terminus (Met-PI). This N-terminal cleavage affects the ability of PI to become crosslinked to fibrin by FXIIIa. It has been shown, in

recombinant studies, that the amount of crosslinked recombinant Met-PI at 5 minutes incubation was less than one-third that of plasma PI [45], and it was suggested that the crosslinking site (the glutamine at position 2 in Asn-PI, which corresponds to position 14 in Met-PI) could be delayed by the additional 12 amino acids present in Met-PI. In line with this, Holmes et al [59] found that recombinant Asn-PI with an extension of 3 extra N-terminal amino acids were not cross-linked into fibrin. In more recent studies, these findings were verified and extended in further studies by Lee et al [56], who demonstrated that during the initial 10 minutes of clot formation, plasma-purified Asn-PI became cross-linked to fibrin about 13 times faster than plasma purified Met-PI. In addition to this, they demonstrated that urokinase-induced plasma clot lysis rates were delayed with direct proportion to the ratio of Asn-PI to Met-PI in human plasma, with an extremely delayed clot lysis when only Asn-PI was available [56, 60]. These studies collectively indicated that, due to the greater efficiency of Asn-PI incorporation into fibrin, Asn-PI hinders plasmin-mediated fibrin digestion more effectively as compared to Met-PI. While these are laboratory findings, they may have important clinical applications as described below. Further studies examined the importance of the N-terminal sequence of PI on the interaction with FXIIIa. They presented that both Asn1 in Asn-PI (corresponding to Asn13 in Met-PI) and amino acids 7 through 12 in Asn-PI (corresponding to amino acids 19-24 in Met-PI) as a secondary binding site that are pivotal for an effective enzyme-substrate interaction [61, 62].

1.4.3.2 C-terminal variation

The C-terminus of PI is post-translationally adapted and the region of C-terminal PI is unique as it contains almost 55 additional amino acid residues compared to the C termini of other serpins that are present in the haemostatic system [63, 64]. This extension of C-terminal is preserved in various species, which are 67% identical between human and bovine and 61% identical comparing human and mouse protein [65]. Further research demonstrated that 2 molecular species of PI are present in plasma of which only 1 can bind to LBS of plasminogen [66]. The form that can stick to plasminogen was called plasminogen-binding PI (PB-PI) and it comprises around 65% of circulating PI. The form which is unable to bind plasminogen was called non-

plasminogen-binding PI (NPB-PI) and that comprised the remaining 35% of circulating PI [66, 67]. Kluft et al [44, 68] presented the occurrence of the 2 varieties in plasma and in serum by modified cross immune-electrophoresis and shown that PB-PI was generated by the liver. They also demonstrated that NPB-PI was formed in the circulation and these 2 forms of PI were also present in rat PI [69]. Various studies investigating the mechanism and kinetics of the reaction between PI and plasmin have shown that the very fast-acting inhibitor of plasmin was originally PB-PI; and in all such studies PI was purified using a column with plasminogen or plasminogen kringles 1-3, consequently only PB-PI was purified [70]. It was also found that the portion of PI that had not absorbed onto plasminogen LBS (ie, NPB-PI) was still active as it could make a complex with plasmin even though it was a very slow reaction [66, 71]. The cleavage of C-terminal PI may result in significant clinical consequences, due to the removal of this portion of PI C-terminus, which alters protein anti-fibrinolytic activity. It has been shown, in a previous study analysing C-terminal variation in PI, that the highest PI activity is detected with higher abundance of PB-PI [72]. Approximately 38% of all circulating PI is cleaved at its C-terminus with a wide inter-individual range from 10% to 60%. On the other hand, no difference was detected in the percentage PI C-terminal cleavage comparing male myocardial infarction patients with healthy controls, casting doubts on the clinical significance of C-terminal variants [72]. However, numbers were relatively small to draw definitive conclusions. Almost all in vitro studies of PB-PI emphasize the value of the PI C-terminus in inhibiting the interaction with plasmin(ogen), which happens via the lysine (K) residues that are present in the C-terminus (K^{418}, K^{427}, K^{434}, K^{441}, K^{448}, and K^{464}; Figure 5) with the LBS in the kringle domains of plasmin(ogen), as mentioned above [73]. Different studies have examined the role of these lysine residues in the interaction with the LBS of plasminogen, even though the results have not been consistent. Early studies analysed the association of PI with plasmin in the presence of synthetic peptides imitating the PI C-terminus, with K^{464} and K^{448} being main areas of interaction with plasmin [74, 75]. Further peptide studies have shown that the PI C-terminus is flexible and the most important role for K^{464} is binding of the C-terminus to plasminogen kringles. Moreover, the internal K residues may support this binding in a sequential zipper-like way [76]. On the other hand, using recombinant PI, Wang et al have shown that K^{448}, not K^{464}, played important roles in the LBS-mediated interaction between

intact plasminogen or kringles 1 to 3 and PI. Their data support the notion that K^{464} may be more involved in interactions with smaller molecules but may be less involved with the whole plasmin molecule [77]. In a more recent study, Lu et al conducted comprehensive experiments in which they systematically and sequentially altered the 5 most conserved C-terminal lysines (K^{427}, K^{434}, K^{441}, K^{448}, and K^{464}) in recombinant human PI. They examined plasmin inhibition rate and the binding affinity of full-length recombinant PI and plasmin and demonstrated that each conserved K residue participated in the binding and inhibition of plasmin, with K^{464} being the most important lysine residue. They further proposed the additional interactions may happen between the serpin core domain of PI and plasmin [78]. There are other possibilities of electrostatic interactions that could exist between the C terminus of PI and plasminogen kringle domains. In physiological pH, the kringle domains have a positive charge and the C-terminus of PI has a net negative charge, with 9 aspartic acid and glutamic acid residues as well as a sulfated tyrosine residue (Figure 1) [54, 79]. Other than the lysine residues, the C-terminal region of human PI also contains an arginine-glycine-aspartic acid (RGD) sequence (Figure 6), a sequence crucial for cell recognition and adhesion via integrins [80]. The functionality of this RGD sequence in PI has not been widely studied. However, it has been shown that a hybrid peptide (RGDF coupled with the 26 C-terminal amino acids of PI) could inhibit platelet activation and accelerate in vitro fibrinolysis at the same time [81]. The very same peptide had been used to show that the binding of plasminogen to platelets with integrin $\alpha 2b\beta 3$ is improved in the presence of the peptide [82]. Additionally, one study has shown that PI C-terminal peptide could bind to human umbilical vein endothelial cells with the RGD sequence, and that was facilitated mainly by integrins $\alpha 5\beta 1$ and $\alpha\upsilon\beta 3$ [83]. It is quite interesting to speculate that the interaction of PI with integrins at cell surfaces have a role in controlling cell-mediated fibrinolysis (eg, by regulating local plasmin activity on cell surfaces and resisting plasmin-induced migration of endothelial cells) [84]. Taken together, these data suggest that the RGD sequence in PI gives the protein other distinct functions, which are yet to be fully determined. Interestingly, the RGD sequence is only seen in human and cow PI and is not conserved across other species such as rats, mice, and rabbits [73].

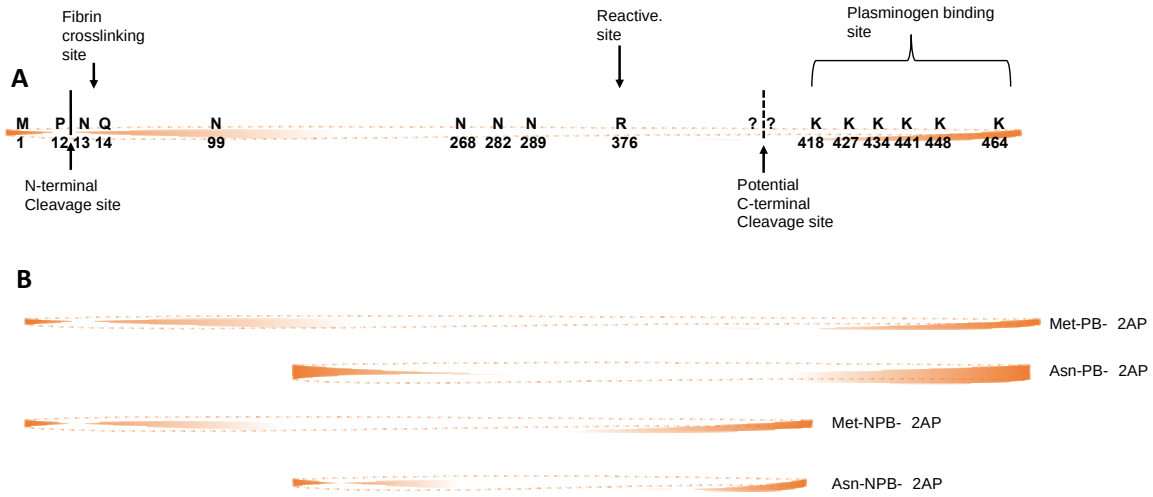

Figure 6. Schematic representation of the PI protein and the variety of molecular forms.
(A) Originally expressed PI and amino acid residues with their respective areas in the PI protein. N-terminal cleavage which results in the adaptation of Met-PI to Asn-PI: this occurs between proline at position 12 and asparagine at position 13. The glutamine residue is at position 14 (position 2 in Asn- PI) and it is at the fibrin crosslinking site. The dashed line shows the potential C-terminal cleavage site. The question marks indicate that the actual C-terminus cleavage site is unknown. The plasminogen-binding site of PI is situated at the C-terminal end. (B) The different molecular forms of PI, 2 plasminogen binding (PB) forms, and 2 non-plasminogen-binding forms.

1.5 Antifibrinolytic proteins

Many proteins inhibit plasmin or plasminogen activator activity, and thus prevent excessive degradation of clots, these are summarized in the table 1. And figure 7.

1.5.1 Role of PI in cardiovascular diseases

Venous thrombosis and pulmonary embolism, common causes of morbidity and mortality, are conditions where the thrombus is rich in fibrin [85]. An experimental model where acute PE was induced in mice deficient in PI showed reduced mortality rate when compared with wild type mice [86]. This suggests that inhibition of PI along with thrombolytic therapy may offer advantages over thrombolytic therapy alone [87]. However, it is important to understand that normal function of PI is imperative for

maintenance of hemostasis and absence of PI can lead to severe hemorrhagic and life threatening bleeding as seen in rare cases of congenital protein deficiency [88].

In addition to thromboembolic disease, PI appears to have a role in high pressure arterial systems. The work of Nagai et al in animal models of stroke, using mice deficient in α_2-antiplasmin, has revealed that inhibition of PI with antibodies or microplasmin reduces areas of focal cerebral injury [89, 90]. Apart from animal model of mice, only a single human based work has been published so far to investigate the safety of neutralizing PI activity by microplasmin. The study included 60 subjects, distributed in 10 groups (6 subjects in each group), who were randomised to receive microplasmin (0.1, 0.5, 1, 1.5, or 2 mg/kg over 15 minutes) or placebo The study was conducted in 3 parts: in the first part, volunteers received a single bolus of microplasmin or placebo, in the second part they received a fixed bolus of microplasmin followed by an infusion of ascending doses over 60 mins and in the third part, individuals aged >55 years received a fixed dose bolus followed by a fixed dose infusion. All these experiments were placebo controlled. The primary end point was a change in PI activity, measured at different time points and up to 4 days following microplasmin administration. Adverse events were monitored up to 21 days following microplasmin administration to ensure safety of this approach [91].

In part 1, all those receiving microplasmin showed a dose-dependent inhibition of PI ranging from 12-84%, compared with 7% following placebo administration. In part 2, maximal inhibition of PI ranged from 75-99%, while in part 3 the inhibition was 70%. A total of 3 subjects treated with the highest dose of microplasmin developed an urticarial reaction. This study shows that PI can be inhibited in vivo and that this is safe in the short term and dose not increase the risk of complications. It should be noted, however, that the study was conducted in healthy volunteers and it is unclear whether extreme PI inhibition in those with co-morbidities, which is often the case in individuals with CVD, is associated with increased bleeding risk.

A crucial observation is the concentration-dependent action of PI suggesting that only partial inhibition is required to avoid unwanted thrombosis thus ensuring that bleeding risk is controlled. Furthermore, with reference to secondary microvascular thrombosis and infarct size, PI has been shown to have profound dose-related effects. It has a role in ischemic brain injury, swelling, haemorrhage, and survival after cerebral thromboembolism. When compared to tissue plasminogen activator (tPA), the protective effects of PI deficiency or inactivation seem to be mediated through

reduction in microvascular thrombosis [92]. It should be noted, however, that uncontrolled inhibition of PI may have detrimental effects as this protein regulates vascular endothelial growth factor (VEGF) expression [93]. In an experimental model of mice deficient in PI, animals had a higher mortality rate 24 hrs post-acute myocardial infarction compared with wild type counterpart. The mechanism postulated is that local deficiency of PI induces VEGF release, which in turn increases vascular permeability leading to pulmonary oedema. Additionally, the lack of PI enhances activation of plasmin resulting in haemorrhagic lesions within the heart muscle causing cardiac failure [93, 94]. Collectively, these studies suggest that PI represents a credible therapeutic target, although caution should be exercised at achieving therapeutic inhibition of action without causing unwanted side effects.

1.5.2 α2-macroglobulin

α2-macroglobulin (α2M) is a non-serine protease that acts to inhibit plasmin, synthesised by the endothelial cells and macrophages which are present in platelet α-granules [95] . This large (725 kDa) tertrameric glycoprotein makes non-covalent complexes with plasmin but it is relatively weak inhibitor of the protein, limiting its activity to about 10% of the efficiency of PI [96]. Other than plasmin, α2M is also able to inhibit virtually any protease, including trypsin, α-chymotrypsin, which makes it difficult to recommend as an antithrombotic therapeutic target. α2M 's unique mechanism of action, whereby target protein enzymes are locked rather than deactivated, thus preventing the substrates from reaching the active protease site [95].

1.5.3 Thrombin activatable fibrinolysis inhibitor (TAFI)

TAFI is a 60 kDa protein that is non-serpin inhibitor of fibrinolysis, produced by the liver and megakaryocytes [97, 98]. TAFI circulates in the plasma at a concentration of 4-15 μg/ml, and about 0.1% of the total TAFI protein can be detected in platelets [98, 99]. The presence of TAFI in platelet granules represents one of the links between the cellular and protein arms of coagulation. TAFI circulates in an inactive zymogen form and it gets activated by thrombin or the thrombin-thrombomodulin complex, which cleave TAFI to release its activation peptide. The thrombin-thrombomodulin complex

cleaves TAFI with a 1250-fold higher catalytic efficiency than thrombin alone [100]. Plasmin often has also been shown to activate TAFI thus ensuring that TAFI is produced near the site of fibrin formation to stop premature and uncontrolled fibrinolysis [101]. There is an additional method for the localisation of TAFI through crosslinking by FXIII to the fibrin clot [102]. The effects of TAFI (TAFIa) on inhibition of fibrinolysis are indirect as the activated protein chops off C-terminal lysine residues from partly degraded fibrin, which are crucial for the binding of plasminogen and as a result, plasmin generation is reduced, as a consequence of reduced plasminogen binding to the fibrin network [103, 104]. The removal of C-terminal lysine by TAFIa also removes plasmin binding sites and increases degradation of this protein [105]. Furthermore, at high concentration, TAFIa can directly inhibit plasmin [106]. High TAFI plasma levels lead to an increase in hypofibrinolysis associated with an increased risk of thrombosis [107]. High plasma levels of TAFI have been linked to increased risk of cardiovascular disease [108].

There are studies suggesting that the antifibrinolytic effects of TAFI are enhanced in diabetes and that contributes to hypofibrinolysis in this condition. Moreover, TAFI levels show correlation with HbA1c, directly implicating glucose control in modulating plasma protein levels in individuals with diabetes [109, 110]. Compared with healthy controls, plasma levels of TAFI are increased in T2DM patients, particularly in the presence of obesity, implicating insulin resistance as an additional factor [110]. Therefore, raised plasma levels of TAFI in diabetes appear to be related to both hyperglycaemia and insulin resistance. Moreover, raised TAFI levels have been linked in T2DM to the presence of microvascular complications [111]. On the other hand, Chudý et al. did not find significant variations in TAFI levels when normoalbuminuric T2DM patients were compared with controls [112]. Moreover, Yener and his co-workers have shown that TAFI levels do not increase in normotensive T2DM subjects without diabetic complications [113]. To further complicate matters, others found TAFI levels to be decreased in non-obese T2DM individuals [114]. Taken together, these data reflect the inconsistencies in the relationship between TAFI and diabetes complications, which is perhaps relate to the heterogeneity observed in these patients. Therefore, such studies require a large number of patients with diabetes, which will allow appropriate subgroup analysis and which will help to understand the role of TAFI in those with deranged glucose metabolism [109].

1.5.4 PAI-1

Plasminogen activator inhibitor (PAI-1) is a single chain glycoprotein of 52 kDa that is synthesised by endothelial cells, monocytes, macrophages, hepatocytes, adipocytes and can also be released by platelets. PAI-1 acts in a quick way to inhibit tPA and uPA just like other serpins, forming a steady 1:1 complex that inhibit these proteases. PAI-1 is able to inhibit other serine proteases, including plasmin and APC. PAI-1 is produced in an active form but can be converted into a non-active form, which is seen under normal physiological conditions [115]. PAI-1 is frequently associated in plasma with vitronectin (originally based on plasma and other extracellular matrix), which helps to stabilise PAI-1 given the short lifespan of ~1 hour of the protein [116]. Circulating plasma levels of PAI-1 range from 2-20~ ng/ml but higher levels have been recorded in individuals at high vascular risk such as those with insulin resistance and type 2 diabetes. While circulating PAI-1 levels are relatively low, concentration of the protein at site of vascular injury is likely to be higher given the release from activated platelets and synthesis by endothelial cells [117]. It has been repeatedly shown that PAI-1 levels are increased in conditions that are associated with vascular inflammation including insulin resistant states [118].

PAI-1 appears to have a role in cardiovascular disease (CVD), metabolic disorders and cancer. High plasma levels of PAI-1 have been linked with arterial disease, including myocardial infarction and also non-arterial disease such as venous thromboembolism [46]. In addition to macrovascular disease, PAI-1 appears to have a role in microvascular disease. In patients with type 2 diabetes, impaired fibrinolysis and elevated PAI1 levels have shown associations with the development of diabetic retinopathy (DR)[119]. The matrix accumulation of PAI-1 is linked with the development of retinal changes in the transgenic mice, directly implicating this protein in the pathophysiological changes observed [119].

1.5.5 PAI-2

PAI-2 is also a member of serpin family and acts as an inhibitor of fibrinolysis. Mainly found in human plasma during pregnancy, this protein is produced by epithelial cells, monocytes, macrophages and keratinocytes. PAI-2 is present as both a 60 kDa and a

47 kDa: a secreted form and an intracellular form respectively. It circulates in plasma at low concentrations of <0.01 µg/ml [115]. PAI-2 is able to prevent uPA, and to a lesser extent tPA activity [118]. PAI-2 is cross-linked into the fibrin clot by FXIIIa through a number of lysine residues in the α-chain of fibrinogen [120].

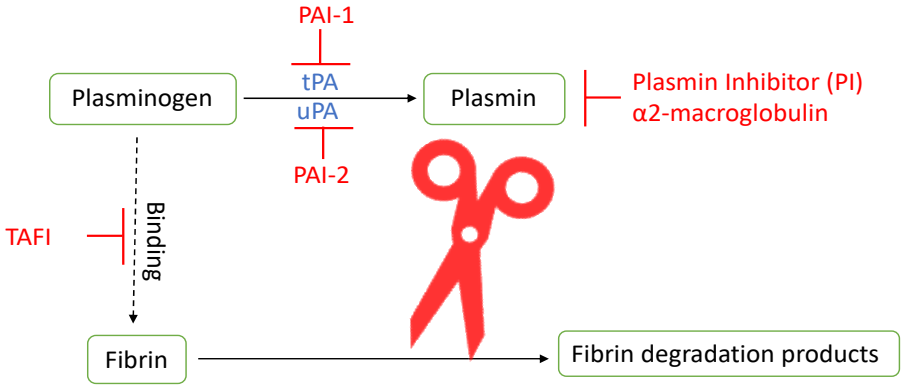

Figure 7. The fibrinolytic system.
Plasmin is formed by cleavage of plasminogen, through the action of tissue plasminogen activator (tPA) or urokinase type plasminogen activator (uPA) and is responsible for fibrin clot breakdown. Plasmin digestion of fibrin is inhibited directly by plasmin inhibitor (PI) or to a lesser extent by α2-macroglobulin. Plasminogen activators are inhibited by plasminogen activator inhibitor 1 (PAI-1), or by thrombin activatable fibrinolysis inhibitor (TAFI) which is able to cleave C-terminal lysine residues in partially digested fibrin that are needed for plasminogen binding and activation.

inhibitors of fibrinolysis	**Molecular Weight**	**Circulating plasma concentration**	**Primary protease target**
PAI-1	52 kDa	2-20 ng/ml [3]	tPa and uPA
PAI-2	60 kDa (secreted form)	<0.01 µg/ml [115]	As above, but to a lesser degree
Plasmin inhibitor	70 kDa	70 µg/ml [121]	Plasmin
TAFI	60 kDa	4-15 µg/ml [98]	Reduces activation of plasminogen
α2-Macroglobulin	725 kDa	1.2 mg/ml [96]	Variety of proteases, including plasmin

Table 1 Summary of inhibitors of fibrinolysis.

1.6 Fibrin clot lysis and vascular disease

After immunoglobulins and albumin, fibrinogen is the third most abundant of all human plasma proteins [122]. In normal conditions, fibrinogen circulates in the plasma at 1.5–4.0 mg/ml with higher levels observed in those at increased vascular risk. Supported by a set of 29 disulphide bonds, it is a sizeable glycoprotein at 340 kDa. Fibrinogen is composed of two identical subsets, each including three polypeptide chains (*Aα, Bβ- and γ-chains).* Every single chain congregates at the innermost part of the molecule (N-terminus) in an area called the E-region, where the cleavage sites for thrombin are positioned. The Aα- Bβ- and γ-chains interlock into two D-regions and form alpha-helical coil structures. The connecting pockets that are fundamental in polymerisation are located here. The three nodules (one E and two D regions) are held together by two coiled-coil regions. The Aα-chain obtrudes from the D-region creating a long and stretchy αC region of more than 350 amino acid residues, while both the Bβ- and γ-chains end in the D-region without any extension. Fibrinogen is primarily secreted by the liver, where the chains are amassed rapidly in the endoplasmic reticulum, producing a complete molecule in as little as 5 minutes.[122].

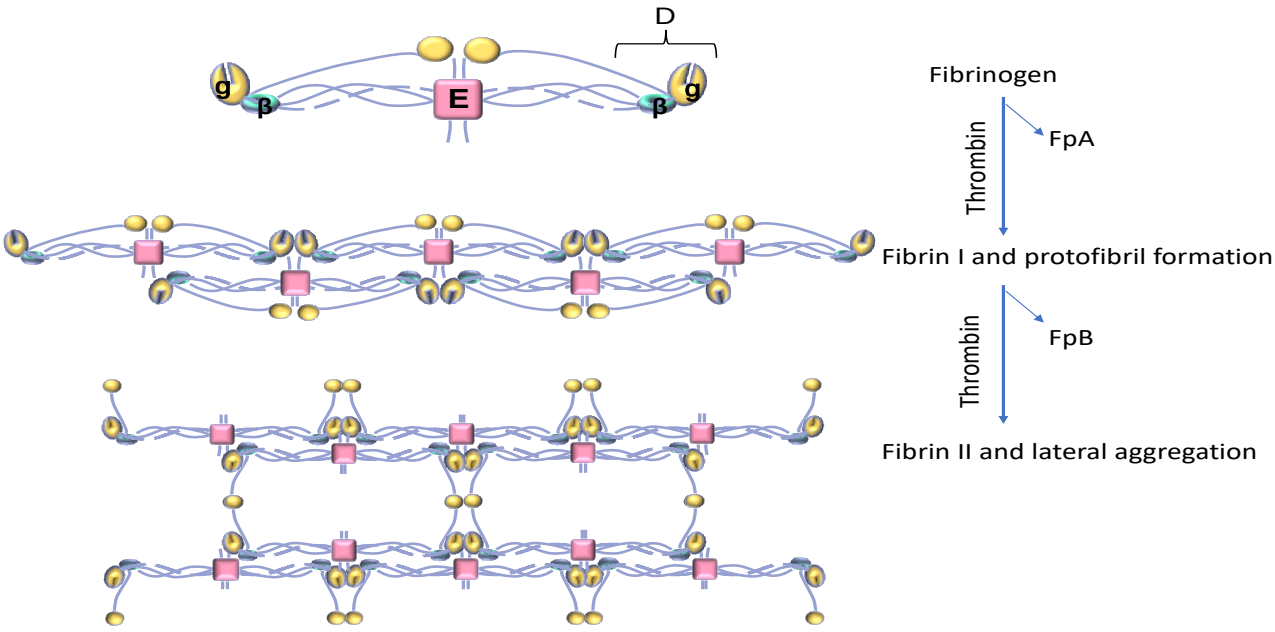

Figure 8. Fibrinogen and fibrin formation.
Fibrinogen consists of 2 sets of 3 chains 2Aα, 2Bβ and 2γ arranged in binary symmetry. The middle region contains N-termini for all six series. Zone D consists of the C-terminus of the β and γ chains, while the C-terminus of the α-chain is extended and folded to interact with the E region. FpA (red) and FpB (green) are cleaved by thrombin, which starts the process of fibrin polymerisation [123].

Fibrin network structure plays a critical part in determining prediction to atherothrombotic events because clots with small pores and thin fibees have been traditionally associated with premature and more significant cardiovascular disease [124].

The fibrinogen molecule can undergo both quantitative and qualitative changes; plasma levels of this protein can vary dramatically as it forms part of the inflammatory response and this has an effect on the final structure of the fibrin network. Qualitative changes are related to post-translational modifications in the protein that can occur in various pathological conditions that can also affect the structure of the fibrin clot. A relationship between elevated plasma levels of fibrinogen and the risk of CVD has been repeatedly documented in a number of studies [125]. The evidence gathered to date suggests that high plasma fibrinogen levels affect the structure of the clot due to modulation of network density, leading to changes in fibrin fibre thickness with an increased number of branch points. This kind of structure results in stiffer clots that are more resistant to lysis, thus conferring higher risk of thrombosis [126].

Post translational modifications in fibrinogen include nitration [127], phosphorylation [128, 129], glycation [5] , oxidation [130] and homocysteinylation [131]. The changes in the fibrinogen are often linked to the production of fibrin clots that are more resistance to fibrinolysis states. However, not all post-translational modifications result in the formation of more compact clots as fibrinogen acetylation results in fibrin network with thicker fibres that are easier to lyse, which has been observed after aspirin administration [132].

1.6.1 Fibrinogen variants and vascular disease

In addition to qualitative and quantitative changes in fibrinogen, this molecule has a splicing variant that can affect the final structure of the fibrin network. Fibrinogen-γ' is generated through splicing of γ chain mRNA that lead to a new replacement of the C-terminal 4 amino acid (aa) residues the γA chain with a 20 aa stretch [133]. The variation exists in ~10% of total fibrinogen molecules and is mostly found as the

heterodimer form γA/γ'. The C-terminal extension in γ' often carries a negative charge that contains a binding site for thrombin and FXIII [134, 135]. Cooper et al have demonstrated, using high-performance liquid chromatography (HPLC), that the rate of FpB release from γA/γ' fibrinogen is reduced, which in turn results in delayed lateral aggregation of protofibrils and the formation of thinner fibres with increased number of branch points, leading in the process to compact clots with reduced pore size [136]. Other studies have also shown that clots formed with γA/γ' are mechanically stiffer and tighter and they show increased resistance to fibrinolysis [137, 138]. Fibrinogen γ' has been linked to the thrombotic disease, but it is not entirely clear whether γ' is a marker of CVD or an independent risk factor having a direct input into the pathology of vascular occlusive disease [139, 140].

There is another variant of Fibrinogen that is 420 (Fib420), produced secondary to another splicing in mRNA transcription. This Fib420 represents 1-2% of total fibrinogen concentration [141] and has a higher molecular weight when it is compared to the most abundant form of fibrinogen (420 kDa vs 340 kDa) [142]. This extra molecular weight arises from 236 amino acid extensions on the fibrinogen Aα chains that are homologous to the C-termini of the Bβ and γ chains, forming an additional globular domain (αE domains). These extra globular domains comprise calcium binding sites that protect fibrinogen from proteolysis by plasmin [143]. The αE globular domains do not have additional fibrin binding FXIII crosslinking sites [144].

A very common fibrinogen polymorphism is an arginine to lysine substitution at residue 448 in the Bβ chain C-terminus having a frequency of 15-20% in the Caucasian population [145]. The clots composed of Lys448 fibrinogen have thinner fibres and smaller pores with increased toughness and are more resilient to lysis [146]. BβArg448Lys has been linked to thrombotic and coronary artery disease [147, 148]. Interestingly, the effects of this polymorphism on clot structure are still apparent in complex clinical conditions that are known to alter fibrin network characteristics, such as diabetes[149].

1.6.2 Hypofibrinolysis and Arterial Disease

It has been repeatedly shown that denser clots that are more difficult to breakdown are associated with enhanced risk of atherothrombotic disease. Clot permeability below the 10th percentile of control has been demonstrated in 50% of patients with established CAD [150]. Another study found that patients with complex CAD especially those who are 60 years or older had Increased clot permeability and lysis times. The presence of fibrin does not only facilitate thrombotic vessel occlusion but may also promote plaque growth thus contributing to the atherosclerotic process [150]. Collet et al (2006) has shown that clots from young survivors of MI were more compact with thinner fibres. Moreover, clots from patients having an acute MI showed them to be more compact compared with clots from patients with stable angina [150]. Stent thrombosis and no-reflow phenomenon are two life threatening conditions, where fibrin network structure is altered displaying a more thrombotic phenotype with tightly packed fibrin fibres [151]. Therefore, clot structure does not only correlate with risk of vascular disease but also severity of the ischaemic episode.

While previous work has consistently shown a relationship between hypofibrinolysis and arterial thrombotic disease, longitudinal studies have been lacking, until recently [152]. The work by Sumaya et al in over 4000 patients with acute coronary syndrome (ACS) [153] has shown that fibrinolysis is an independent predictor of cardiovascular mortality in individuals managed using modern treatment strategies and strong antiplatelet agents. The predictive value of fibrin clot lysis was evident after correcting not only for traditional risk factors but also a variety of prognostic biomarkers [153]. Data from this work indicate that doubling of lysis time is associated with over 70% increased risk of cardiovascular mortality. This raises the exciting possibility that outcome in a subgroup of ACS individuals with prolonged lysis time can be improved by targeting this abnormality [153].

Interestingly, the findings by Undas and Ariëns (2011) indicate that apart from other factors associated with stent thrombosis (including the procedure itself, patient and lesion characteristics, stent design, and premature cessation of antiplatelet drugs), fibrin-related factors might contribute not only to late thrombosis but also acute and subacute stent thrombosis, in particular when stent malposition or under-expansion

are excluded. In the same way, patients with a record of no-reflow can have abnormal fibrin structure [151].

In addition to coronary artery disease, peripheral arterial disease (PAD) has also been shown to be associated with abnormal fibrin structure and compromised fibrinolysis [151]. Of note, individuals with PAD have a 6-fold increase in cardiovascular mortality and presence of this vascular pathology usually indicates involvement of multiple vascular beds.

1.6.3 Hypofibrinolysis and Venous Disease

Extensive studies have proven that patients with venous thromboembolic (VTE) disease have altered fibrin clot structure just like arterial disease. An important aspect of venous disease is the reduced role of platelets compared with atherothrombotic conditions and therefore the role of the fibrin network is likely to be more important. Compared with controls, clots that are less porous, denser and have compromised lysis time ex vivo are typically found in patients with VTE. There is also a tendency to inherit predisposition to venous obstruction, just like in arterial disease [155]. Pathophysiologically, venous diseases are linked to venous reflux or obstruction, or both. Venous valves allow antegrade and antigravity flow via the veins. Inflammation and oedema can be experienced when venous outflow obstruct or venous reflux cause ambulatory venous hypertension. At least 1–2% of the aged population is affected by venous ulcers that adds considerable burden on quality of life and health-care costs. It has been estimated that in the United States annual costs of care associated with chronic venous disease go beyond $3 billion and the toll it takes on quality of life and morbidity is much large than perceived [154]. Patients with pulmonary embolism, a more serious condition that can result in death, is a form of venous obstructive disease, which is also associated with changes in fibrin network characteristics. These findings support the concept of similar pathophysiology involving changes in the structure of fibrin in both arterial and venous thrombosis [123, 155]. Curnow and colleagues (2007) documented reduced fibrinolysis and increased fibrin generation in hypercoagulable patients with VTE or arterial thrombosis, autoimmune diseases or pregnancy complications [156]. These observations support the idea of similar pathophysiological processes involving modifications of fibrin structure in arterial and

venous thrombosis [151]. However, it remains unknown whether VTE patients with transient risk factors like trauma or surgery present altered fibrin properties. It is established that fibrin clots produced ex vivo from plasma samples of patients with prior thromboembolic events are denser and less susceptible to lysis. A prothrombotic fibrin clot phenotype like this is seen as a new risk factor for venous thromboembolism. To evaluate if the abnormal clot properties can predict recurrent deep-vein thrombosis (DVT), Cieslik (2017) studied 320 successive patients (aged 18 to 70 years) following first-ever DVT. They evaluated plasma clot after 3 months of anticoagulant treatment since the index event. The average duration of anticoagulation was 10 months (range: 4-20). Recurring DVT was observed in 77 patients (25% or6.6% per year) during an average follow-up of 44 months. The main strengths of this study is the reasonable number of patients recruited, small loss to follow up, tests performed in a centralised laboratory and relatively long follow up This study has demonstrated that a prothrombotic fibrin clot phenotype with lower permeability and prolonged clot lysis time is associated with recurrent DVT. This has given new insights into the recurrence of DVT, which may have practical implications in the future. However, there was some overlap creating a “grey area” and therefore the techniques need to be further refined before fibrin clot properties can be used to decide the clinical management of individuals with VTE. Therefore, further longitudinal studies are required to confirm findings in different population of patients and refine the criteria used to assess clot structure parameters [157]. Identifying those at risk of recurrent DVT will help to tailor the length of anticoagulant therapy thus increasing both efficacy and safety.

1.6.4 Mechanisms of Hypofibrinolysis

As alluded to earlier, fibrin clot lysis predicts cardiovascular mortality in individuals with acute coronary syndrome. Therefore, targeting prolonged clot lysis may be an option to improve outcome in people with CAD following an acute event. The main mechanisms for impaired fibrin clot lysis include: i) formation of compact clots, ii) increased incorporation of anti-fibrinolytic proteins into the clot and iii) derangement in the fibrinolytic system.

1.6.4.1 Compact fibrin networks

Compact fibrin networks are tightly packed with thin fibres and are associated with risk of cardiovascular diseases [158]. The association may be due to reduced permeation of fibrinolytic enzymes into compact clots, directly implicating fibrin clot structure in the pathophysiology of the atherothrombotic process [109]. While single thick fibrin fibres are lysed at a slower rate than thin fibres, clots made up of increased number of densely packed thin fibres are usually slower to lyse [124]. Additionally, these thinner fibrin fibres also support a slower rate of tPA-mediated plasmin generation as compared to thick fibres, explaining the observed slower rate of fibrin digestion by plasmin [159, 160]. It should also be noted that increased incorporation of antifibrinolytic proteins into compact clots further contributes to their resistance to breakdown and therefore the relationship between structure and lysis is more complex than initially thought [146].

1.6.4.2 Increased incorporation of anti-fibrinolytic proteins

A number of antifibrinolytic proteins contribute to clot stability but these can also become pathological by making the fibrin network abnormally resistant to lysis thus facilitating clot formation and vascular occlusion.

1.6.4.2.1 *Complement C3*

Complement C3 had been considered as an inflammatory protein until relatively recently with studies demonstrating that it plays a role in fibrin clot resistance to lysis. By using a proteomics approach, two researches have been able to show the presence of C3 and its metabolites in plasma clots [30, 161]. C3 binding/cross-linking into the fibrin network via FXIII, increases resistance of the clot to lysis [30]. Importantly, presence of C3 in the clot compromises fibrinolysis, thought to be related to decreased efficiency of plasmin (given that C3 is a substrate for this enzyme) and possibly through interference with binding of fibrinolytic proteins (steric hindrance).

1.6.4.2.2. Plasmin inhibitor (PI)

Congenital deficiency of PI leads to serious bleeding diseases. On the other hand, the high concentration of PI is also linked with an enhanced risk of first MI. Agren et al. have shown enhanced integration of PI into fibrin networks in people with T1DM [162]. A previous study conducted by Dunn et al, has demonstrated increased cross-linking of PI into fibrin networks in T2DM and this enhanced the resistance to lysis when compared with controls. These observations suggest that PI represents a diabetes-specific therapeutic target to reduce the hypofibrinolytic environment, typically found in this condition [163].

1.6.4.3 Derangement in the fibrinolytic system

Diabetes is a condition characterised by hypofibrinolysis and increased risk of both arterial and venous thrombotic events. As discussed above, increased incorporation of antifibrinolytic proteins (PI and C3 into diabetes clots has been reported but there are additional mechanisms responsible for impaired fibrin clot lysis [163, 164]. Plasma levels of PAI-1 are higher in diabetes and insulin-resistant states, compromising the fibrinolytic process by limiting the generation of plasmin [3]. Moreover, increased glycation of plasminogen reduces conversion to plasmin and modulates protein activity [165]. The various mechanisms operating in diabetes ensure the presence of a hypofibrinolytic environment that in turn increases predisposition to vascular occlusive events.

1.6.4.4 Modulation of clot structure/lysis by non-anticoagulant agents

Statin treatment, routinely used in the management of cardiovascular disease, is associated with altered fibrin network characteristics. Both simvastatin and atorvastatin are able to increase fibrin permeability and enhance lysis time within a 4-week treatment [151]. The mechanism for this is unclear but likely to be indirect related to a reduction in the inflammatory environment by statin therapy. Aspirin, a classical antiplatelet, has been shown to alter fibrin network structure and facilitate fibrinolysis, giving this agent an additional non-platelet mode of action for the reduction of thrombosis [151].

Hypoglycaemic agents have also shown to affect clot structure, particularly metformin, which may alter FVII and PAI-I levels. The latter effect is likely to be responsible, at

least in part, for enhanced fibrinolysis with metformin, although a direct effect for this agent on the fibrin network has also been suggested (Standeven et al) [166] [167]. Thiazolidinediones (TZD) are peroxisome proliferator- activated receptor (PPAR) stimulators and they have antithrombotic properties. Their use is associated with lower FVII, PAI-I and fibrinogen levels [168]. TZDs are able to delay intra-arterial thrombus formation, which may be related to stimulation of thrombomodulin expression in the vessel wall [169].

The renin-angiotensin system plays a role in blood clotting by increasing the expression of TF and stimulating the synthesis of PAI-1. Angiotensin converting enzyme inhibitor (ACEI) and angiotensin receptor blocker (ARB) treatment is associated with a reduction in fibrinogen levels by mechanisms that are unclear [170].

1.7 Pathological relevance of altered lysis

While hypofibrinolysis increases thrombosis risk, excessive clot breakdown can also have unwanted effects. Hyperfibrinolysis can occur in genetic disorders such as haemophilia, which are not necessarily associated with a derangement in the fibrinolytic proteins per se. There are 3 types of haemophilia: A, B and C secondary to deficiency of coagulation factors VIII, IX and XI, respectively. Bleeding episodes would be common in individuals with haemophilia, some of those are highly spontaneous and unprovoked. The factors that take part in coagulation deficient in haemophilia are actually involved in the positive feedback mechanisms of the coagulation cascade mediated by the intrinsic Xase complex [171]. Due to FVIII deficiency, there is a delay in thrombin generation and the propagation phase of coagulation, while the start of thrombin generation is not significantly affected [172]. In individuals with haemophilia, sufficient fibrin for clot formation is generated at the start of clotting, suggesting that bleeding is due to clot volatility rather than fibrin deficiency [171], a theory reinforced by the high levels of D-dimer in the circulation of patients with haemophilia [173, 174]. In keeping with the effects of thrombin concentration on the structure, the reduced production of thrombin in individuals with haemophilia results in the formation of loosely woven thicker fibres rendering the clots more permeable, thus increasing vulnerability to lysis [175, 176]. The reduced thrombin production in individuals with haemophilia also delays TAFI generation as well as the activation of FXIII, further reducing clot stability [177, 178]. Another example that leads

to increased fibrinolysis is FXIII deficiency [179, 180]. This condition can be missed, or diagnosis delayed given it is a rare condition. The key function of FXIIIa is to covalently join fibrin fibres to stabilise the fibrin clot. In addition to the stabilising function, it cross-links fibrinolytic inhibitors into the fibrin network thus reducing fibrinolysis. The most recent studies have shown that FXIII performs different functions not only in the coagulation reactions, but also in different vital biological processes that include angiogenesis, wound healing, maintenance of pregnancy and vascular permeability [181]. The linkage of fibrin chains by FXIIIa leads to increased resistance to lysis through the formation of γ-chain dimers and high molecular weight α-polymers that help to enhance the strength of the clot and further prevent it from shear stress in the circulation [182]. FXIIIa also takes a key role in incorporating PI into the clot, which stops the premature clot dissolution by the fibrinolytic system [183]. The lack of FXIII is quite a rare hereditary bleeding disorder and symptoms range from mild skin bleeding to life threatening haemorrhagic events [184] with the degree of FXIII deficiency correlating with severity of bleeding [185]. The European Network of Rare Bleeding Disorders (EN-RBD) study, performed different studies from 2007 to 2010, and presented a strong link between bleeding severity and FXIII activity in plasma of patients with FXIII deficiency [185].

Therefore, it is important to acknowledge that while targeting hypofibrinolysis represents a credible strategy to reduce thrombosis risk, caution must be exercised to keep this in check, perhaps by aiming at specific pathways that address the pathophysiological changes in coagulation while keeping the physiological response largely intact.

1.8 Clinical targeting of the fibrin clot to modulate fibrinolysis

1.8.1 Fibrinolysis and thrombosis

Myocardial infarction, stroke and ischaemic damage are the result of the formation of obstructive thrombus. The basic purpose of the treatment is to initiate reperfusion and get the blood flowing again in the affected vessel. In case of an acute thrombotic event, there are different surgical approaches that can be applied, including angioplasty and thrombectomy. On the other hand, anti-thrombotic and fibrinolytic drugs are used as additional tools to help vessel reperfusion and prevent further occlusion [186].

1.8.2 Thrombolytic drugs in current use

A variety of thrombolytic drugs are available which mainly function by enhancing the generation of plasmin and these include streptokinase, uPA and tPA. Streptokinase is the pioneer enzyme that was used as a thrombolytic agent and is still being used in some countries because of its low cost. However, it is not fibrin-selective, so it is associated with generalised proteolysis [187], potentially leading to uncontrolled plasmin generation [188].

On the other hand, tPA is fibrin specific (in that its activity increases up to 1000-fold in the presence of fibrin) but the continuous infusion of the protein administered to compensate for protein's short half-life (4 minutes) removes its fibrin-specificity. Therefore, bleeding complications can still occur in a significant number of individuals [188]. The development of latest thrombolytic agents with adjustments to half-life, activity, and fibrin specificity has succeeded in improving both efficacy and safety [188]. For example, tenecteplase is a mutated variant of tPA that has been able to extend protein half-life and enhance the fibrin specific mode of action [189]. As a result of the Tenecteplase has 14-fold relative fibrin specificity when compared to tPA localising plasmin production to thrombus site and limiting systemic plasmin generation [190].

There are other approaches that help achieve fibrin specificity of plasminogen activators and, these approaches have shown success in *in vitro* assays, but none

have proceeded to be tested in human clinical studies thus far. The latest and newer delivery methods for thrombolytic drugs have been developed to ensure fibrin specificity are the focus of modern research [191]. Of the many novel approaches, one option involves fusion of plasminogen activator proteins with anti-fibrin antibodies [192]. Antibody-conjugated thrombolytics have yet to make it to clinical use, due to the complexity involved in their commercial and industrial manufacturing and concerns in relation to long-term stability of such agents [188]. Other approaches include the encapsulation of tPA in liposomes [193], fibrinolytic-bearing nanoparticles and fibrinolytic-bearing erythrocytes [194]. Introduction of drug delivery systems, such as liposomes, microspheres consisted of biodegradable polymers, emulsions and cyclodextrins [193]. Interestingly, tPA-loaded with liposomes have appropriate physical features to work as drug carrier for parenteral administration in terms of particle size, polydispersity index and zeta potential [193]. The already prepared liposomes are moderately stable when stored for 45 days at 4°C. From these *in-vivo* studies, it could be determined that encapsulation into liposomes is a practical way to enhance the circulating half-life of tPA in the blood stream. This will allow the use of lower doses of tPA, potentially with reduced risk of haemorrhagic complications [193].

1.9 Different techniques used to make agents that enhance fibrinolysis

A universal problem with traditional thrombolytic agents such as streptokinase and tPA, is the high risk of bleeding. The reason is that these are very powerful agents and therefore cause complete dissolution of the clot rather than limiting clot formation. While this may be helpful in the acute stage, it is less useful for chronic use. A more specific approach will enable targeting a particular pathway that is responsible for fibrin clot lysis. This will potentially reduce the risk of thrombosis while limiting the risk of bleeding and thus giving a more favourable risk:benefit ratio. Therefore, a number of studies have investigated targeting one or more antifibrinolytic factor as a mean of reducing thrombotic events while minimising bleeding risk. The main antifibrinolytic proteins of the coagulation system are TAFI, PAI-1 and PI have been suggested as potential targets to facilitate clot lysis.

1.9.1 TAFI

The inhibition of TAFI has been carefully considered as a strategy in thrombotic conditions and TAFI inhibitors have even made it into clinical studies. However, only a limited number of patients have been tested in clinical trials and unfortunately these agents were then discontinued presumably due to unfavourable side effect profile or limited efficacy [195, 196]. Buelens et al tried another approach by developing a panel of TAFI-inhibiting nanobodies that were effective against the various modes of TAFI activation and activity [197].

1.9.2 PAI-1

Inhibiting PAI-1 is also a therapeutic target that has anti-thrombotic potential and use of nanobodies to inhibit this coagulation protein has been investigated [198]. These nanobodies showed profibrinolytic properties in an *in vitro* clot lysis evaluation [198], however further *in vitro* and *in vivo* characterisation are awaited. Various attempts have been made to inhibit PAI-1 with antibodies [195, 199]. The latest research work in mouse models of thrombotic stroke has shown that the simultaneous inhibition of PAI-1 and TAFI with a bi-specific antibody results in substantial improvement of fibrinolysis, without significant increase in bleeding risk [200]. The short half-life of the PAI-1 of less than 1 hour under physiological conditions [116] may limit its use for chronic conditions. On the other hand, the counterargument is that the short half-life makes it an appropriate target for acute vascular thrombosis. In spite of the several studies of different PAI-1 inhibitors *in vivo* and *in vitro* to date, clinical PAI-1 inhibitors are not available [201]. An inherent difficulty in developing a reliable inhibitor of PAI-1 may be related to the structure of the protein. The crystallographic data of PAI-1 in complex with vitronectin is not yet complete so it hinders rational design of small molecules to be able to bind and inactivate PAI-1 that is bound to vitronectin. In addition, the mechanism by which active PAI-1 evolves to its latent form are not fully understood and studied. Although frequent PAI-1 inhibitory compounds have been studied and tested, studies have suffered from a lack of mechanistic insight into the action of these inhibitory molecules, further compromising translation of these findings into the clinical arena.

1.9.3 PI

Certain efforts to target plasmin inhibitors have been made and they include the use of antibodies and production of mutant forms of the protein. The incorporation of PI into fibrin clots increases resistance of the clot to lysis as alluded to earlier [183]. Given the established functional sites of PI, one group targeted residue Arg364 in the active site of PI [202], and formed an Arg-Ala mutant [203]. These modified PI variants kept their ability to be cross-linked to the fibrin network by FXIIIa, but they lost their ability to inhibit plasmin, at least partly, which in turn enhanced fibrinolysis.

Given the importance of the N-terminus of PI for cross-linking into fibrinogen [204, 205], Kimura et al formed a 12-residue synthetic N-terminal peptide of PI that was able to reduce the incorporation of native PI into fibrin networks by FXIIIa *in vitro*. This cross-linking of the synthetic peptide to fibrin accelerated spontaneous, as well as tPA-induced fibrinolysis [206]. While these preliminary data were promising, none of these approaches, related to PI therapeutics have been clinically adopted. However, studies are currently ongoing for the use of antibodies against PI to aid in the treatment of pulmonary embolic disease and these are yet to report their results. Taken the studies of antifibrinolytic inhibitors together, it appears that translation of *in vitro* and even animal *in vivo* findings into human studies can be difficult and more complex than initially envisaged.

1.10 Antibodies for modulation of thrombosis risk

First developed in 1978 by Kohler and Milstein, monoclonal antibodies have today come of age as both diagnostics and therapeutics with many monoclonal antibodies in current clinical use. These form a promising group of drugs with potentially impressive therapeutic outcomes. Monoclonal antibodies are being delivered in the treatment of several major diseases including autoimmunity, cardiovascular disease and cancer [207, 208].

However, there are certain limitations to antibodies including the complexity of developing target-specific antibodies, sensitivity to high temperatures and relatively high cost of production. They are generally large proteins that require glycosylation and formation of disulphide bonds for activity and stability. Therefore, the production is usually limited to eukaryotic systems that are more costly than bacterial systems.

Also, the presence of glycosylation can introduce batch to batch variations. An alternative to antibodies in protein scaffolds and there are new 50 novel non-antibody protein scaffolds which have come into existence. These include Anticalins [209], Affibodies [210], Ankyrins [211], engineered Kunitz domains [212], Nanobodies [213] and Affimers [214].

1.10.1 Modulation of fibrinolysis using PI-specific antibodies

With respect to the fibrinolytic system, several antibodies have been created that target different inhibitors of fibrinolysis. A monoclonal antibody was created by Sakate and colleagues in 1989 which targeted PI by interfering with formation of plasmin-PI complexes and increased the effectiveness of tPA-mediated clot lysis [215]. Similarly, an experiment was performed on rabbit jugular vein thrombosis to evaluate the efficacy of monoclonal antibody (MAb RWR) against PI. This *in vivo* experiment suggested that a combination of a PI inhibitor and a plasminogen activator could prove to be a more potent thrombolytic strategy [216, 217]. Kumada *et al* also reported a reduction in level of circulating PI levels in rats by repeated injection of polyclonal anti-PI $F(ab')_2$ fragments. This experiment led to an acceleration of thrombolysis by enhanced fibrinolytic activity mediated by urokinase-induced plasma clot lysis [218].
These results on animal models are promising but clinical application in man is still lacking. Reasons for this may be related to a different effect in humans or fears over safety, although a phase I/II study in individuals with PE was explored but unfortunately withdrawn this year for reasons that remain unclear (ClinicalTrials.gov; ***NCT03316729***). It should be mentioned that the high cost of monoclonal antibodies is another major limiting factor making widespread use problematic.

1.11 Alternative approaches to antibodies

Small engineered protein platforms have some common advantages over antibodies [219]. Numerous non-antibody scaffolds do not have cysteine residues that allow easier large-scale production of the proteins and also allow the accumulation of a free thiol to the scaffold for site-specific modification. Generally, the engineered protein scaffolds are characterised by high thermal and chemical stability that make them

preferable to antibodies in applications involving exposure to harsh chemical or environmental conditions [219]. Non-antibody protein scaffolds also have some other advantages over antibodies in certain applications such as *in vivo* imaging [220]. The main features of engineered protein scaffolds are summarised in Table 2.

1.11.1 Anticalins

These are derived from human lipocalins that are a family of naturally associated protein. Anticalin proteins are used instead of antibodies, but they are about eight times smaller, as they are composed of 180 amino acid and a mass of about 20 kDa. Anticalins are a new type of biopharmaceuticals which demonstrate promising features as an imaging agent. For example, anticalin PRS-110 has been developed to target the oncogene MET with high affinity and specificity, for use as imaging agents in positron emission tomography (PET)[221].

1.11.2 Affibodies

These are small molecules of 6.5 kDa, which display a three-helix bundle domain structure. Since their introduction 20 years ago as a substitute to antibodies for biotechnological uses, the first therapeutic affibody molecules has now entered clinical development for in vivo tumour imaging and for investigating inflammatory disorders [222].

1.11.3 Designed ankyrin repeat proteins (DARPins)

These are genetically engineered antibody mimetic proteins. A single ankyrin domain is 3.5 kDa, so DARPins vary from 14-21 kDa depending on the number of repeats they contain**.** DARPins are able to recognize targets with specificities and similarities that are equal to those of antibodies, however due to their robustness and extreme stability, they allow a more advanced formats and applications. These molecules have been taken as intracellular, real-time sensors of protein conformations and as crystallization chaperones [223]. For future therapies, DARPins have been established by advanced,

structure-based protein engineering to selectively induce apoptosis in tumours by opening surface receptors from their signalling cascades. They have been used effectively for targeting viruses as well. In recent clinical trials, DARPins have been able to show good safety and efficacy in macular degeneration disease. All these developments eventually exploit the high stability, solubility, and aggregation resistance of these molecules, allowing a wide range of conjugates and fusions to be produced and utilised [223].

1.11.4 Engineered Kunitz domains

Kunitz domains are areas of proteins that hinder protein degrading enzymes or, more specifically, domains of Kunitz-type are protease inhibitor. They are relatively small in size with a length of about 50 to 60 amino acids and a molecular weight of 6 kDa. Examples of Kunitz-type protease inhibitors are aprotinin (bovine pancreatic trypsin inhibitor, BPTI), Alzheimer's amyloid precursor protein (APP), and tissue factor pathway inhibitor (TFPI) [224]. While these can be effective, they usually lack specificity making clinical applicability uncertain.

1.11.5 Nanobodies

Nanobodies have various integral, beneficial properties, for instance, their low molecular mass (15kDa), low immunogenicity, high affinity, high solubility and stability, and the most important is the easy production of the recombinant VHH in bacteria or yeast. The hypervariable regions (i.e. complementary determining regions (CDR)) of nanobodies are longer on average than those of conventional antibodies, most probably to compensate for the loss of the binding regions on the light chain. Nanobodies are mostly strong and powerful enzyme inhibitors, because of their long CDR3 domain, which is often used to make an extended loop and therefore it can penetrate into the catalytic cleft of enzymes[197].

A major development in relation to antibodies took place when Hamers et al. (1993) mentioned that camels produce, in addition to the well-characterized antibodies that contained a heavy and a light chain, an additional and distinct species of antibodies

containing only heavy chains. The binding affinity and selectivity of these single-chain antibodies turned out to be comparable to those of classical antibodies, and the high-affinity antigen-recognizing region could be taken away from the single heavy chain and expressed as a single polypeptide chain. These tiny antibody fragments were named VHH (from variable domain of heavy chain antibodies, also referred to as nanobodies) and they had vividly changed the way antibodies have been used in developmental biology and in the research field [223]. A summary of “antibody substitutes” and their potential use is provided in Table 2 while Table 3 highlights the main advantages and disadvantages of each.

Engineered protein scaffold	**Size**	**Scaffold based on**	**Example of target proteins**
Anticalins	~20 kDa	Lipocalins	• Oncogene MET, for use as imaging agents in positron emission tomography (PET) [221] • Prostate specific membrane antigen (PMSA) [225] • Vascular endothelial growth factor A (VEGF-A) [226]
Affibodies	6-7 kDa	Z-domain of staphylococcal protein A	• Human epidermal growth factor receptor-2 (HER-2) [227] • Tumour necrosis factor-α (TNF-α) Affibodies have reached clinical development for *in vivo* tumour imaging and for inflammatory disorders [222]
Affimers	~13 kDa	Type I Affimer scaffold based on human Stefin A Type II Affimer scaffold based on phytocystatin consensus sequence	• VEGF-2, ~40 – 240 nM [228] • Tenascin C (TNC) [228] • Ubiquitin [229] Avacta has a pre-clinical stage biopharmaceutical pipeline of Affimer therapeutic candidates, with a focus on oncology target programmed death ligand 1 (PD-L1)[230]

DARPins (designed ankyrin repeat proteins)	14-21 kDa	DARPins consist of multiple connected ankyrin molecules	• Epithelial cell adhesion molecule [231] • VEGF-A for treatment of macular degeneration (good clinical safety and efficacy profile in early studies) [223]
Engineered Kunitz domains	~7 kDa	Disulphide crosslinked serine protease inhibitor, typically of human origin e.g. Kunitz domain of TFPI	• Plasma kallikrein, 10 pM Ecallantide (inhibits kallikrein) received FDA approval in 2009 for the treatment of hereditary angioedema [224]
Nanobodies	~15 kDa	A single variable domain derived from a heavy chain antibody, contains a disulphide bond	• Interleukin 6 (IL-6) [232] • -Lysozyme [233] Ablynx currently has 8 Nanobodies in clinical development, and caplacizumab (anti- vWF Nanobody) has been approved for the treatment of acquired thrombotic thrombocytopenic purpura (TTP) [234]

Table 2 Summary of antibody substitutes for research purposes and potential clinical use for diagnosis and treatment

Engineered protein scaffold	**Advantages**	**Disadvantage**
Anticalins	• Anticalin proteins prevent undesired agonistic receptor cross-connecting movement with the help of their monovalent and monomeric structure.	• No clear disadvantage known
Affibodies	• Can be used for diagnostic imaging • Can be used to test the viability of targeted recognition of tumor cells • Have the capability to bind the protein targets by way of high similarity and selectivity	• Difficult to modulate properties (such as half-life extension)

Affimers (discussed further below)	• Small size and stability even at higher temperature • Simple to produce • Easily modifiable (half-life extension, other)	• Can aggregate • In vivo safety studies are lacking
DARPins (designed ankyrin repeat proteins)	• Synthesised quickly and cheaply • Easily modifiable	• As binding modules include their curving in the binding surface, stiffness/lack of flexibility, and incomplete randomization of amino acid residues in changeable places/locations, which could possibly limit the range of targets.
Engineered Kunitz domains	• Have the capability to identify exact structures of protein • Kunitz domains work as viable protease inhibitors within their free system	• Kunitz type domains have the restriction of binding merely to proteases
Nanobodies	• Can penetrate hard tumours, bind to a bigger range of areas on a target particle • Adaptability and stability Viable tools for cell-specific delivery of toxic substances (i.e. treatment of cancer cells)	• Generally low affinity • The lack of an Fc tail or a lower affinity in Nanobodies can occasionally make them less potent that full length antibody

Table 3 Summary of Advantages and disadvantages of engineered protein scaffold

1.11.6 Affimers: Engineered Protein Scaffolds

Affimer proteins are relatively new affinity reagents which mimic the molecular recognition of antibodies, but they can be isolated rapidly and formed recombinantly. They are synthetic in nature and are derived from either a human cystatin A (type I) or a plant-derived phytocystatin consensus structure (type II) [235]. Affimers proteins are very small (11-15kDa, 3nm in diameter), highly stable (70°C < Tm < 100 °C), monomeric proteins and they do not have disulphide bonds. Their construction based on a single α-helix and four β-strands, with the molecular recognition sites being positioned within two variable loops, each up to nine amino acids in length. Their

advantages include stability, even at high temperature, and the fact that the production process is both simple and inexpensive. Huge phage display libraries (~1.3×10^{10} variants) have been created and screened against hundreds of target proteins, peptides and small compounds. A wide range of Affimer binders have now been identified from these phage libraries to different targets and have been implemented for applications as diverse molecular research tools [235]. These Affimers are capable of recognizing binding hot spots which lie on the target protein and that are amenable to interaction with peptides sequences. Affimers may be used to study protein function and identify areas that can be targeted to modulate activity. Modulation of protein function can then be achieved by using Affimers directly or synthesising suitable small molecules based on the knowledge provided by analysing Affimer protein-target protein interactions. Of the non-antibody approach to modulate protein function, my work focused on the use of Affimers and therefore further details on these small proteins are provided. The Affimer scaffold is a consensus sequence of plant-derived phytocystatins (cysteine protease inhibitors) that constrain two 9 amino acid variable regions. The inhibitory sequences within the Val Val Ala Gly and Pro Trp Glu loops of the consensus phytocystatin were substituted with nine randomised amino acid positions in each loop [214].

Affimers have been developed as versatile and high stability proteins with the potential to use in a variety of clinical and research applications.

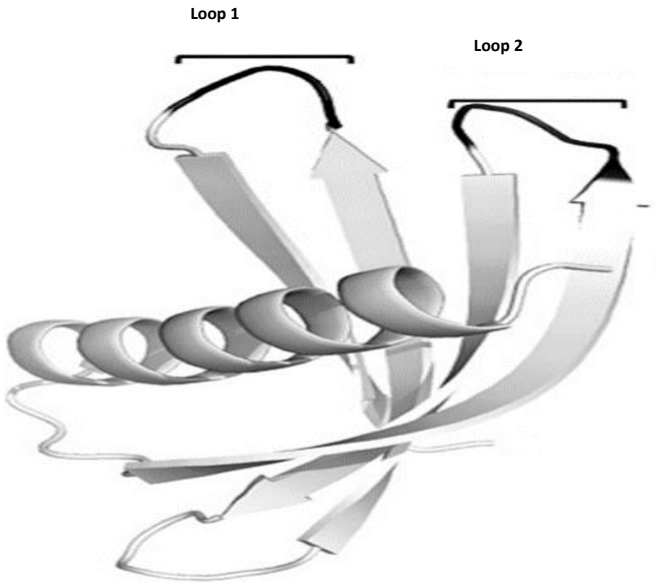

Figure 9. X-ray crystal structure of artificial binding proteins (Affimers; PDB ID no.4N6T)

The protein scaffold and the two loops of 9 amino acids each (the variable regions) are presented.

1.11.6.1 Current uses of Affimer proteins

As discussed above, Affimers, like many other small engineered protein scaffolds, can replace antibodies, and they are used for understanding protein-protein interactions [236, 237] and in a range of sensor technologies [238, 239]. Affimer proteins have been employed in a broad range of applications to date with a large number of target proteins, including vascular endothelial growth factor proteins (VEGF), ion channels and human epidermal growth factor receptor 4 (HER-4), among others [228]. Our group described Affimers that bind fibrinogen and stabilise the fibrin network, which may be useful for bleeding conditions [240].

Affimer proteins have been used selectively to target members of closely associated protein families. Src-Homology 2 (SH2) domains are protein domains that combine specially to phosphorylated tyrosine residues. Tiede et al examined the ability of the Affimer phage panning process to separate high affinity Affimer proteins against specific SH2 containing proteins with little or no cross reactivity against closely associated family members [228].

TNC (Tenascin C), a protein that is frequently controlled in tissues of cancer and related to metastasis, is an extracellular matrix protein. Therefore, TNC proteins signify a potential marker for tumours in imaging applications and therapeutic targets in vivo [241]. The large size of antibodies is an advantage in therapeutic applications, as it convenes longer blood circulation times. However, longer circulation time of antibodies can often result in high levels of background signal and poor imaging resolution because of the time taken for the unbound antibody to be cleared out [220]. Effective imaging agents are generally small, and they can penetrate into tissues easily and cleared rapidly out of the body that allow greater resolution in a short span of time [219]. Tiede et al have developed a high affinity (KD 5.7±2.8 nM) TNC-binding Affimer that was C-terminally labelled with fluorescent molecule Rhodamine red for imagining of TNC in organs from cancer-bearing mice. Anti-TNC Affimers showed localisation to tumours and demonstrated benefits over TNC antibodies for cancer cell imaging due to rapid clearance and limited background interference [228].

In addition to stability, ease of production in large quantities [228], an important feature of Affimers is their comparatively easy alteration to permit half-life extension or other modifications that increases their usability. When taken together, Affimers are promising substitutes to antibodies in different applications, including research setting and clinical diagnostic and therapy areas.

1.11.6.2 Rational for using Affimers to target the plasmin inhibitor (PI)

The present trends to prevent arterial thrombus formation are focused the inhibition of platelet function so the fibrin network is not usually targeted. However, several pieces of evidence suggest that modulation of fibrin network formation is also effective to reduce thrombotic risk [242]. Previous studies have indicated that warfarin has promising effects but this high bleeding risk and narrow therapeutic window prevented routine clinical use [243]. Coagulation factors such as X inhibitors have shown more promising and consistent effects but these agents are still associated with significant bleeding risk when used with antiplatelets, limiting widespread adoption of such an antithrombotic strategy [244].

One option is to target more specific pathways in coagulation that can reduce thrombosis without significantly increasing bleeding risk, which would be particularly effective if the pathway in question is affected by a pathological process. For example,

PI has shown increased incorporation into fibrin networks from patients of diabetes [162, 163]. Therefore, PI inhibitors may be particularly effective in those with diabetes as they target a pathological pathway.
The Bioscreening Technology group and its associates at the University of Leeds have developed that Affimer technology and given the advantages of these small proteins (described above), identification of PI-specific Affimers that modulate protein function may be an important step to develop new antithrombotic therapeutic agents.

1.11.7 Selection of Affimer reagents by phage display

To validate novel therapeutic target and drug design, the usage of phage display technology is on the rise in translational research. This technology has multi usages, from studying protein to protein interactions, enzyme specificity, antibody antigen interactions and epitope mapping [245].
Phage display is a technique that has been available for four decades that allows easy production of different proteins. The approach was first presented by George Smith in 1985 and continued to develp finally leading to the construction of phage libraries for both biotechnological and medical applications [246].

1.11.7.1 M13 Phage

The filamentous bacteriophages are viruses that contain a circular single-stranded DNA genome inside a long, thin protein capsid cylinder. Among the phage which specifically infect *E. coli*, the F pilus-specific phage (M13, fd and f1) have been the most studied until now. As their names indicate, they use the tip of the F conjugative pilus as a receptor and they are special only for *E. coli* cells containing the F episome [247].

The phage genome is encapsulated in a flexible protein cylinder, which is based on approximately 2700 molecules of the major coat proteins pVIII. Moreover, each end of the phage is based on two different pairs of proteins: approximately five molecules each of pIII and pVI proteins, and on the other end there are five molecules each of pVII and pIX proteins. This end has the packaging signal and is the first part of the particle to be assembled [248].

The most common bacteriophages, which are used in phage display are M13 and fd filamentous phages though T4, and T7 phages have also been used [246]. The M13 phage which has high capacity for duplication and is able to accommodate larger foreign DNAs, thus making it the most used phage display vector. It is a non-lytic phage that consists of a limited number of structural proteins assembled around a single-stranded circular genome DNA (ssDNA) encoding the phage proteins. The infection of host bacteria is supported by the attachment of phage pIII to the F′-pilus of *E. coli*. The ssDNA first enters into the bacteria where it is converted by the host enzymes into double-stranded DNA that then generates ssDNA and phage proteins. The assembly of phage occurs in or near the inner membrane of host bacteria and in the periplasm. Each particle of M13 has 3–5 copies of the pIII protein, as opposed to the most abundant coat protein pVIII with 2700 copies. These two coat proteins are often used for expression of peptides, and high-affinity binders are selected from both systems. Usually the antibody fragments are merged with N-terminus of pIII protein [246].

1.11.7.2 Affimer library generation

In order to get the binding reagents for a desired target, the process of screening is required, and it is done on the large and diverse libraries to make sure the selection of binders having the optimal binding specificity and affinity. Oligonucleotide-directed mutagenesis is a time tested method in order to randomise a residue at a defined location completely. Degenerated oligonucleotides that join the mixtures of the four natural bases to code randomly for amino acids are available for the generation of diverse libraries. On the other hand, for randomisation introduced in this manner, it is very difficult to control diversity, which leads to bias where one combination is often more or less represented than another [249]. Furthermore, the merger of undesired amino acids or stop codons are impossible to control. Bias is also introduced because of the incongruity among the numbers of coding sequences per amino acid; for example, arginine is represented with six codons, however histidine is only represented by two codons. To overcome this bias, trinucleotide phosphoramidites were generated which had utilised the codon mixtures rather than nucleotide mixtures

[250]. Moreover, this method is used for the removal of codon bias and this allows for randomisation with all twenty amino acids.

All of the primers which are utilized in order to generate libraries in this work were produced by Ella Biotech GmbH. Ella Biotech had used the scheme which was developed by Kayushin et al., 1996 to produce 20 trinucleotide phosphoramidites and each correspond to one of the 20 amino acids (excluding stop codons). These 20 trimers are selected from a possible 64 combinations in the randomisation scheme NNN and are chosen as they are major codons in E.coli and will not cause lower transcription. Quality in terms of rate of dimer/tetramer directly affects the frameshift rate, so is extremely important to create high diversity. Reaction factors for all of the trimers are considered and used to calculate the ratio which is needed to ensure equal incorporation into the primers. It is also highly likely to request specific subsets of trimers and therefore we have requested the exclusion of cysteines to reduce the possibility of non-specific crosslinking via disulphide bonds and allowing for the addition of a cysteine at a later date for site specific labelling, if that is required at any stage later. A lack of disulphide bonds also helps in making Affimers better tolerated for using in cellular reducing environments.

1.11.7.3 Screening Affimer phage display library

Binding peptides are generally taken from recombinant-display libraries by affinity selection experiments in which a phage is taken, by its displayed peptide, to a target protein. This process is called screening or panning. The target protein-phage complex is stationary in wells or to beads in order to allow for the removal of non-binding phage just by washing. The target-bound phage can then be eluted and amplifiedby infection and growth in *E coli*. The resulting phage pool is enriched for phage that bind to the desired target and this is reflected in the increased phage yield in subsequent panning rounds. Clones are chosen from affinity-enriched pools on the basis of a high yield that is compared to a negative control and can then be tested in an ELISA to confirm binding to the target. DNA sequencing also reveals the amino acid sequences and classifies any consensus sequences between clones [251].

Almost thirty years after its discovery, phage displays remain one of the most widely used *in vitro* selection methods. Originally developed to revolutionise the generation

of therapeutic antibodies, phage display is now the first choice for screening artificial binding proteins. Using the phage display, Affimer reagents are isolated against a range of diverse molecular targets have been described [228].

1.12 Hypothesis and Aims

Prevention of intravascular thrombus formation and/or facilitation of breakdown has important future clinical applications. It is well accepted now that hypofibrinolysis is associated with increased risk of adverse vascular outcome with significant morbidity and mortality. A long-standing difficulty with enhancing fibrinolysis is the increased risk of bleeding events. Moreover, employing aggressive clot dissolution techniques can be justified in the acute stage of vascular occlusion, given the favourable benefit to risk ratio, but this can be more difficult in chronic disease where a more balanced approach is required. Therefore, targeting specific pathways in fibrinolysis may be the best way forward in order to restore physiological fibrinolysis without increasing bleeding risk.

Given the key role of PI in controlling the fibrinolytic process and the potential for Affimers to modulate function of proteins, I hypothesise that PI-specific Affimers represent a tool to facilitate fibrin clot lysis without interfering with the physiological structure of the fibrin network. This, in turn, will help to reduce the hypofibrinolytic environment in pathological conditions while keeping the coagulation process close to normal physiology, thus limiting bleeding risk.

Therefore, my project has three distinct aims:

1. Isolate a set of high affinity PI-binding Affimers, with the capability to modulate protein function and fibrin clot lysis
2. Test the consistency of action in various blood components and different individuals at increased risk of cardiovascular disease.
3. Understand the molecular mechanisms for modulation of clot lysis by Affimers to help identify new therapeutic targets.

Chapter 2 Experimental design, materials and methods

2.1 Materials

Material	Source/composition
PBS	Tablets, 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4; Sigma-Aldrich, Dorset, UK
PBST	PBS with the addition of 0.1% v/v Tween-20
PEG	Polyethylene glycol, Sigma-Aldrich, Dorset, UK
Plasmin Inhibitor	Sigma-Aldrich, Dorset, UK
Skimmed milk powder	Marvel foods
Sodium azide	Sigma-Aldrich, Dorset, UK
Tetracycline	Sigma-Aldrich, Dorset, UK
Thrombin	Merck Millipore, Watford, UK
tPA	Technoclone, Pathway Diagnostics; Dorking, UK
Tris-base	Thermo Fisher Scientific, Loughborough, UK
Tris-HCl	Thermo Fisher Scientific, Loughborough, UK
Tryptone	Sigma-Aldrich, Dorset, UK
Tween-20	Thermo Fisher Scientific, Loughborough, UK
Yeast extract	Sigma-Aldrich, Dorset, UK
LB media	Per 1 L: 10 g tryptone, 5 g yeast extract, 10 g NaCl
LB agar	LB media with the addition of 20 g agar per litre
LB carb	LB media or LB agar with the addition of 100 µg/ml carbenicillin
Methanol	Thermo Fisher Scientific, Loughborough, UK
Multiskan Go plate reader	Thermo Fisher Scientific, Loughborough, UK
Na2HPO4	Sigma-Aldrich, Dorset, UK
NaCl	Thermo Fisher Scientific, Loughborough, UK
NaH2PO4	Sigma-Aldrich, Dorset, UK
Nanodrop	Nanodrop, Wilmington, USA
Normal pool plasma	First Link UK Ltd., Birmingham, England
Permeation buffer, PB	50mM Tris, 100mM NaCl, pH 7.4
2TY media	For 1L: 10 g yeast extract; 16 g tryptone; 5 g NaCl
2TY carb	2TY media with the addition of 100 µg/ml carbenicillin
10X casein blocking buffer	Sigma-Aldrich, Dorset, UK
2X casein blocking buffer	10X casein blocking buffer diluted in PBS
Agar	Sigma-Aldrich, Dorset, UK
Acetone	Sigma-Aldrich, Dorset, UK
CaCl2	Sigma-Aldrich, Dorset, UK
Cacodylate acid	Thermo Fisher Scientific, Loughborough, UK
Carbenicillin	Thermo Fisher Scientific, Loughborough, UK
DMSO	Sigma-Aldrich, Dorset, UK
EDTA	Sigma-Aldrich, Dorset, UK
Glu-plasminogen	Enzyme Research Laboratories, Swansea, UK
Glutaraldehyde	Sigma-Aldrich, Dorset, UK
Glycerol	Sigma-Aldrich, Dorset, UK
Glycine	Thermo Fisher Scientific, Loughborough, UK
H2SO4	Sigma-Aldrich, Dorset, UK

HCl	Sigma-Aldrich, Dorset, UK
Imidazole	Sigma-Aldrich, Dorset, UK
EZ-Link NHS-SS Biotin	(Thermo Fisher Scientific)
EX-tem reagent	(Tem International GmBH)
star-tem reagent	(Tem International GmBH)
Mini Trans-Blot western tank	(Bio-Rad Laboratories Ltd., Watford, UK)
sponge	(Bio-Rad Laboratories Ltd., Watford, UK)
Filter paper	(Bio-Rad Laboratories Ltd., Watford, UK)
Polyvinylidene difluoride (PVDF) membrane	(Thermo Fisher Scientific, Loughborough, UK)
G:Box Chemi XT4 imaging system	(Syngene, Cambridge, UK)
GelCode Blue Safe Protein Stain	(Thermo Fisher Scientific, Loughborough, UK)
MOPS buffer	(Precision Plus protein standard, Bio-Rad Laboratories Ltd., Watford, UK)
MES buffer	(Precision Plus protein standard, Bio-Rad Laboratories Ltd., Watford, UK)
Glutaraldehyde	Sigma-Aldrich, Dorset, UK
Alexa Fluor-488 Microscale Protein Labelling Kit	(Invitrogen, Paisley, UK)
mouse, Bovine, Rat and Pig plasma	(Seralab, Bio-IVT)
Fibrinogen	Calbiochem, Merck Millipore, Watford, UK)
Gelcode blue stain reagent	(Pierce, Rockport, IL, USA)
Ethanesulfonic acid (MES) buffer	Invitrogen, Thermo Fisher Scientific, Loughborough, UK)
NuPAGE 3-(N-morpholino) propanesulfonic acid (MOPS)	(Invitrogen, Thermo Fisher Scientific, Loughborough, UK),
XCell SureLock electrophoresis system	(Invitrogen, Thermo Fisher Scientific, Loughborough, UK)
NuPAGE sample reducing agent	(Invitrogen, Thermo Fisher Scientific, Loughborough, UK)
NuPAGE lithium dodecyl sulphate (LDS) sample buffer	(Invitrogen, Thermo Fisher Scientific, Loughborough, UK),
10% Bis-Tris gel	(Invitrogen, Thermo Fisher Scientific, Loughborough, UK)
4-12% Bis-Tris gel	(Invitrogen, Thermo Fisher Scientific, Loughborough, UK)
10X reducing buffer	(Invitrogen)
LDS sample buffer	(Invitrogen, Carlshad, CA, USA)

10x6mm cellulose membrane tubing	(Sigma-Aldrich)
Concentration tube	(0.1M NaCl, 0.05M Tris, pH of 7.4)
Ni-NTA	(Amintra, Expedeon Ltd, Cambridge, UK)
Triton X-100	(Sigma-Aldrich, Dorset, UK)
Halt Protease Inhibitor	(Thermo Fisher Scientific, Loughborough, UK)
Lysozyme	(Thermo Fisher Scientific, Loughborough, UK)
Benzonase	(Merck Millipore, Watford, UK)
IPTG	(Sigma-Aldrich, Dorset, UK)
SOC medium	(Thermo Fisher Scientific, Loughborough, UK)
XL1-Blue supercompetent cells	(Agilent Technologies; Stockport, UK)
T4 DNA ligase	(New England Biolabs, Ipswich, US)
QIAprep Spin Miniprep Kit	(Qiagen; Sollentuna, Sweden)
QIAquick PCR purification kit	(Qiagen, Manchester, UK)
DpnI	(New England Biolabs, Ipswich, US)
NotI	(New England Biolabs, Ipswich, US)
***Nhe*I**	(New England Biolabs, Ipswich, US)
BL21 Star™ (DE3) cells	(Life Technologies, Paisley, UK)
pET11 vectors	BSTG
Anti-Fd-Bacteriophage-HRP	(Seramun Diagnostica GmbH)
kanamycin stock	Thermo
96 deep-well plates	Thermo
M13K07 helper phage	(New England Biolabs, Ipswich, US)
ER2738 cells	(Lucigen)
streptavidin	(Thermo Fisher Scientific)
TMB	(SeramunBlau fast TMB/substrate solution, Seramun)
Nunc-Immuno MaxiSorp	
Washing buffer	50mM NaH_2PO_4, 500mM NaCl, 10% glycerol, 20mM Imidazole and having a pH of 7.4
Elution buffer	50mM NaH_2PO_4, 500mM NaCl, 300mM Imidazole, 10% glycerol and having a pH of 7.4
Dialysing buffer	(0.1M NaCl, 0.05M Tris, pH of 7.4).
sodium cacodylate buffer	0.078M Cacodylic acid (Sigma), pH7.4
GelCode Blue Safe Protein Stain	(Thermo Fisher Scientific, Loughborough, UK)
2% Glutaraldehyde	(Sigma)
Biacore 3000	(GE Healthcare, Little Chalfont, UK)
IBIDI slide	(Thistle Scientific, UK)
Parafilm	(Sigma-Aldrich, Dorset, UK)

2.2 Phage Display to isolate PI-specific Affimer

As described earlier, Affimers are small proteins consisting of a scaffold which constrains two variable 9 amino acid regions. However, single loop libraries have also been developed which may have the advantage to quicker identification of interaction sites allowing, theoretically quicker development of therapeutic targets. To isolate PI-specific Affimers, two phage libraries were screened against PI, one with two variable regions and one with a single variable region [252].

2.2.1 Biotinylation of PI

For the purpose of the study, PI was biotinylated using EZ-Link NHS-SS-Biotin (Thermo Fisher Scientific), as per the manufacturer's instruction. PI was incubated with NHS-SS-Biotin in DMSO for an hour at room temperature followed by treatment with Zeba spin desalting columns to remove the non-reactive biotin. Biotinylated PI was mixed with an equal amount of 80% glycerol and stored at -20ºC.

2.2.2 ELISA to confirm biotinylation of PI

Three wells (A, B and C) of Nunc-Immuno MaxiSorp strip were chosen with wells A & B containing 1 & 0.1µl of biotinylated PI while the third acting as the control containing just phosphate buffered saline (PBS; Sigma). These wells were left to incubate overnight at 4ºC. 10X blocking buffer (Sigma) was added to each of these three wells and left for 3 hours at 37ºC. Wells were then washed with PBS-tween three times using a plate washer. High Sensitivity Streptavidin-HRP was diluted at a ratio of 1:1000 with a 2X blocking buffer/PBS. Fifty µl of this dilution was added to all the wells and left to incubate at room temperature for about an hour, after placing the wells on a vibrating platform shaker. Wells were washed using a 6X PBS-tween on a plate washer, incubated with 50µl TMB (SeramunBlau fast TMB/substrate solution, Seramun) and allowed to develop.

2.2.3 Phage Panning

The Affimer phage display library has a diversity of up to 2.3 $X10^{10}$ different peptide sequences in the variable regions. Three panning rounds of phage display was performed to identify the Affimers that bind to PI. Biotinylated PI was added to streptavidin coated wells (Thermo Fisher Scientific) for 1 hour and after a washing step, the Affimer phage display library was added. Affimers were incubated with biotinylated PI for 2 hours, after which the wells were washed 6 times and the bound phage was eluted and used to infect *E. coli* ER2738 cells (Lucigen). Colonies of the ER2738 cells were grown on LB-carb plates overnight after inoculation with M13K07 helper phage (New England Biolabs, Ipswich, US) in order to generate a new library for the second-round panning (Figure 10). To refine the phage pool and select high PI binders, competitive elution with PI was applied in the third panning round [252]. The panning procedure was mainly performed by one of my supervisors, Dr. Tiede.

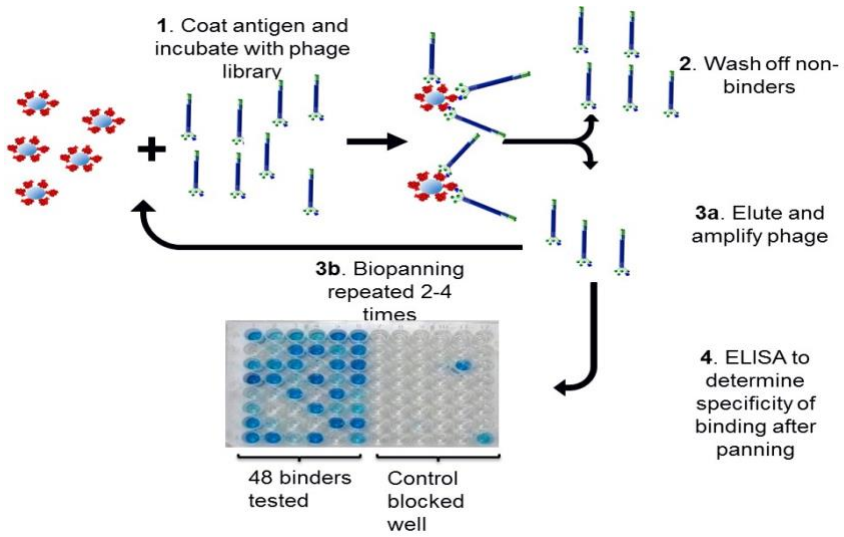

Figure 10. Details of the panning process.
Biotinylated PI is immobilised on streptavidin plates followed by the addition of Affimers. After extensive washing, PI-bound Affimers are eluted and the process is repeated to enrich for high affinity PI-binding Affimers (Figure has been kindly provided by my supervisors, Ajjan/Tomlinson)

2.3 Phage ELISAs to identify specificity of PI-specific Affimers

2.3.1 Phage ELISA, preparation of phage

From the three panning phases, individual colonies were selected and introduced into a 96 deep-well plates, incubated in 200µl 2TY carb media (2TY with 100 µg/ml carbenicillin) and left overnight. A new 96 deep-well plate containing 200µl 2TY carb was incubated with 10µl aliquot culture for one hour. The helper phage M13K07 was incubated in freshly grown cultures and kept for 30 minutes at room temperature. A total of 25mg/ml of the kanamycin stock (Thermo Fisher Scientific, Loughborough, UK) was diluted at a ratio of 1:20 in the 2TY Carb and 10µl of the dilution was added into each well and left to incubate overnight. The cultures that were infected with the phage were centrifuged for 10 minutes at 3500xg. The obtained supernatant contained the phage and was transferred into the ELISA plate, to test for binding to my target protein, PI.

2.3.2 Phage ELISA

The 96 well plates coated with streptavidin were blocked with 2X blocking buffer at room temperature overnight. The first 6 columns of the streptavidin plates had biotinylated PI added with remaining 6 columns having blocking buffer added to act as a negative control. Of the wells where PI was added, three columns were used to screen double looped Affimers and three to screen single looped Affimers. The plate was then incubated for a period of one hour at room temperature. After washing once with 300µl PBST, they were blocked with 10µl blocking agent (milk), followed by the addition of 40µl of the phage containing supernatant solution and incubated for one hour; this allowed the phage to be tested against PI as well as the negative control. The plates were then washed again, and phage was detected by a dilution of 1:1000 of Anti-Fd-Bacteriophage-HRP (Seramun Diagnostica GmbH) for one hour, followed by visualization with tetramethylbenzidine (TMB) and measurement at wavelength 620nm.

2.4 Affimer expression and purification

2.4.1 Affimer protein production

2.4.1.1 Sub-cloning of phage display derived Affimer proteins

In order to produce in large quantities, the Affimers of interest were subcloned into the pET11 vectors and were expressed in *E. coli* BL21 Star™ (DE3) cells (Life Technologies, Paisley, UK). DNA coding sequences of Affimers were amplified by PCR, digested using *NheI* (New England BioLabs) and *NotI* (New England BioLabs) and cloned into pET11. From the phagemid vector (Figure 2) using the following primers:

Forward Primer 5' – ATGGCTAGCGGTAACGAAAACTCCCTG

Reverse Primer 5' – TACCCTAGTGGTGATGATGGTGATGC

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	98°C	30 seconds	1
Denaturation, Annealing & Extension	98°C, 54°C & 72°C	20 seconds 20 seconds 20 seconds	30
Final Extension Hold	72°C then 4°C	10 minutes then Hold	

DpnI (New England Biolabs, Ipswich, US) was added into each of the PCR reaction tubes in order to remove *dam* methylated template DNA. PCR products were filtered and cleaned using the QIAquick PCR purification kit (Qiagen, Manchester, UK) according to manufacturer's instructions. PCR-amplified Affimer sequences and pET11 plasmid were subjected to restriction digest with NheI and NotI restriction enzymes (both from New England Biolabs, Ipswich, US). Ligation of the NheI-NotI digested Affimer DNA was added into the pET11 vector by incubating 75 ng of pET11 DNA with 25 ng of insert DNA with T4 DNA ligase (New England Biolabs, Ipswich, US) overnight at room temperature. XL1-Blue supercompetent cells (Agilent Technologies; Stockport, UK) were then transformed with ligated Affimer/pET11 construct. One µl of

ligation mix was then incubated with 10µl of competent cells on ice for half hour. Samples were subjected to heat shock by placing them in a water tub at 42°C for 45 seconds, before incubating on ice for 120 seconds. A total of 180µl of pre-heated SOC medium (Thermo Fisher Scientific, Loughborough, UK) was then added and incubated at 37°C for 60 minutes with shaking at 250 rpm. A total of 100µl of each transformation mixture was then plated onto LB-carb plates and incubated overnight at 37°C. Single colonies were chosen and transferred into 5 ml of LB-carb media (LB media containing 100 µg/ml carbenicillin). They were left to grow overnight at 37°C with shaking at 230 rpm, and plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen; Sollentuna, Sweden) as per manufacturer's protocol. The purified plasmid DNA was further sent for sequencing to confirm correct Affimer DNA sequence in the pET11 vector.

2.4.1.2 Affimer protein expression and purification

BL-21 cells (Life Technologies, Paisley, UK) were transformed with the Affimer-pET11 construct using the transformation process described above. After the transformation within the BL-21, 0.1mM Isopropyl β-D-1- thiogalactopyranoside (IPTG, Sigma-Aldrich, Dorset, UK) was introduced into 200ml of the culture on the LB medium. The cells that were grown overnight, harvested and lysed using a total of 1ml lysis buffer containing Benzonase Nuclease (Merck Millipore, Watford, UK), Lysozyme (Thermo Fisher Scientific, Loughborough, UK), EDTA-Free (100X) Halt Protease Inhibitor (Thermo Fisher Scientific, Loughborough, UK) and Triton X-100 (Sigma-Aldrich, Dorset, UK).

After incubation at 50 °C denatured non-specific proteins and lysates were centrifuged at 4800 xg for 20 min followed by isolation of the supernatant and a further centrifugation step at 12,000 xg for 20 min to get clean supernatant. The next step was the addition of the clear supernatant to 300µl Ni-NTA (Amintra, Expedeon Ltd, Cambridge, UK) slurry with the mixture kept for one hour. The mixture was then washed using a washing buffer containing 50mM NaH_2PO_4, 500mM NaCl, 10% glycerol, 20mM Imidazole and having a pH of 7.4. Until Nanodrop readings at 280 nm consistently read <0.09 An elution buffer containing 50mM NaH_2PO_4, 500mM NaCl, 300mM Imidazole, 10% glycerol and having a pH of 7.4, was used to elute the mixture

prior to dialysing overnight in permeation buffer (0.1M NaCl, 0.05M Tris, pH of 7.4). The phagemid vector map is shown in Figure 11.

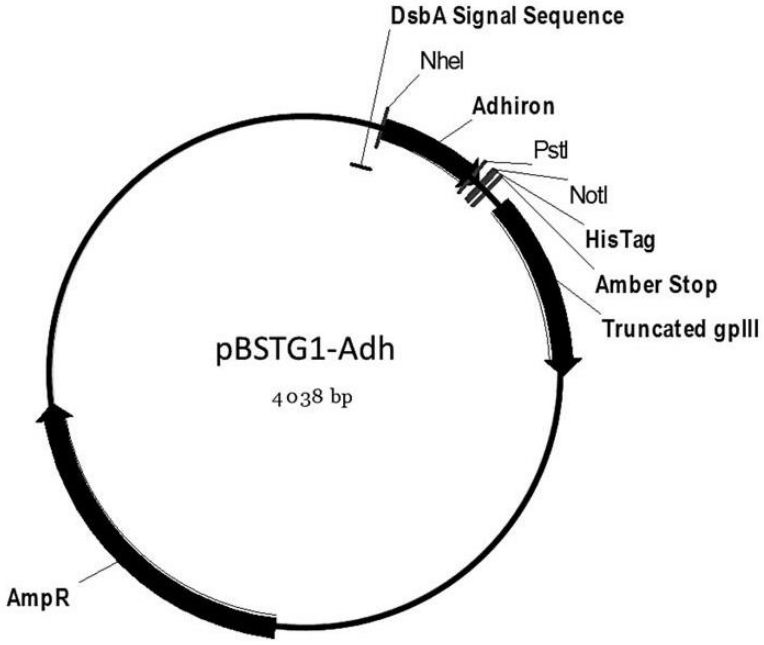

Figure 11. Phagemid vector pBSTG1 phagemid vector containing the coding region for Affimer 92(Adhiron92). Schematic drawing representation of the pBSTG1-Affimer phagemid vector. Related features of the construct and the coding area for the Affimer are highlighted. The Affimer sequence is in the middle of NheI and NotI restriction places/locations followed by a 6-histidine tag.

2.4.2 Concentration & dialysis

Using a concentration tube (100,000 MWCO vivaspin20), pooled fractions were added and centrifuged at 4000 x g at 23^{o}C so that a final volume of the sample of 1ml was attained. These concentrated samples were dialysed against permeation buffer overnight. The dialysis was performed using 10x6mm cellulose membrane tubing (Sigma-Aldrich). To this solution, a freshly prepared dialysis buffer was introduced after a period of an hour on two occasions and then left at a temperature of 4^{o}C

overnight. Concentration of all Affimers proteins was measured on a Nanodrop Spectrophotometer (details of manufacturer).

2.4.3 SDS-PAGE gel

In order to assess the integrity and the purity of samples, SDS-PAGE gel electrophoresis was performed. Seven µl LDS sample buffer (Invitrogen, Carlshad, CA, USA) and 3 µl 10X reducing buffer (Invitrogen) were mixed with 21 µl of each of the Affimer samples. The prepared mixture was centrifuged and incubated for 10 mins at 95^{o}C. This was then introduced into 4-12% Bis-Tris gradient gel using 20X MES SDS running buffer 25 ml MES running buffer (20X) up to 500ml with dH2O and run at 160V for a period of 1hour. Gelcode blue stain reagent (Pierce, Rockport, IL, USA) was used to stain the gels for 1 hour and later the gels were left overnight to de-stain using distilled water.

2.5 Turbidimetric assays

Turbidimetric assays were used in purified (fibrinogen and PI only) as well as plasma systems to analyse modulation of clot structure and/or fibrinolysis. All experiments were done in duplicates and repeated at least 3 times, and all Affimer experiments contained two negative controls (buffer and scaffold only Affimer).

The Multiscan Go Microplate reader (Thermo Fisher Scientific) was used to analyse clot formation and lysis for at least 60 mins. Readings were taken at 12 second intervals using 320 nm wavelength. Clot lysis was calculated by measuring time from full clot formation to 50% lysis, whereas clot maximum absorbance was measured as clot final turbidity (Figure 12).

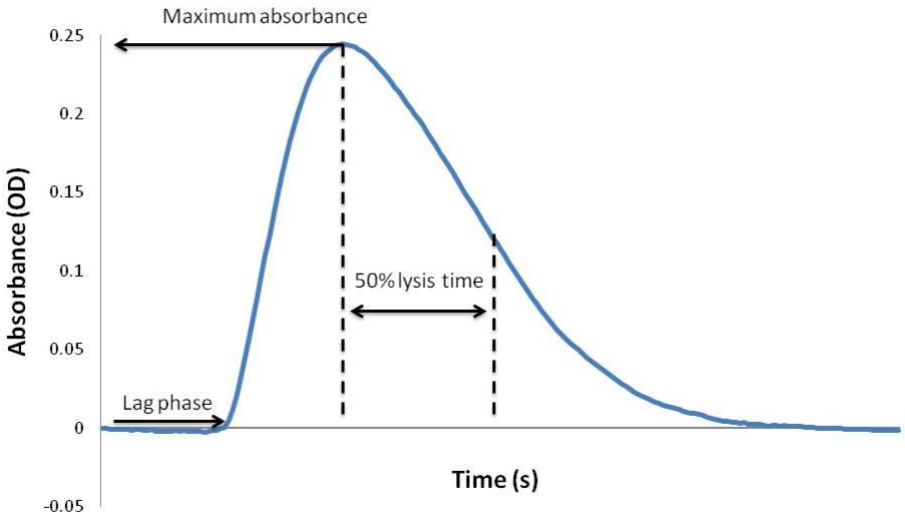

Figure 12. Details of the measurements conducted in the turbidimetric assay.
Maximum absorbance (clot final turbidity) analyses fibre thickness and clot compactness whereas time to 50% lysis is an indicator of fibrinolysis potential.

2.5.1 Plasma system

To the 96 well plate, 25µl of the plasma was applied and incubated for a period of 30 minutes at room temperature with 25µl of the Affimer on a shaker. The concentration selected was based on the molar concentration of Affimer:PI ratio of approximately 5:1 to 20:1 (given PI concentration in plasma is around 70µg/ml). After the incubation process was completed, 50µl of lysis mix containing tissue plasminogen activator at 0.83µg/ml and 50µl activation mix containing $CaCl_2$ at 7.5mM and thrombin at 0.03U/ml was added into each well.

2.5.2 Purified Experiment

Affimers at 35µg/ml and PI at 35µg/ml were incubated in 2.5 ml eppendorf for 30 minutes at room temperature then added to 96 well plate containing 0.5mg/ml of fibrinogen and incubated at room temperature for 30 mins followed by the addition of 11µg/ml FXIII into each well. The lysis mix containing (0.03µg/ml) of tPA and (25µg/ml) plasminogen was added with the help of a multichannel pipette to each well followed

by an activation mix composed of (2.5mM) $CaCl_2$ and thrombin at 0.5U/ml. With the help of a Multiscan Go Microplate reader using 340 nm wavelength, changes in optical density were recorded every 12 seconds at 37°C.

2.5.3 Mouse, Bovine, Rat, and Pig plasma

Turbidimetric experiments were also used with mouse (Seralab, Bio-IVT), Bovine, Rat and Pig plasma (Seralab, Bio-IVT). These experiments were done similarly to those in human plasma. Affimer protein was incubated with 25 µl individual plasma in PB for 30 minutes at room temperature, prior to the addition of lysis mix(LM) including tPA (225ng/ml) and followed by activation mix (AM) including thrombin (0.03 U/ml) and CaCl2 (7.5 mM). Measurements of the optical density at 340 nm were taken at 12 second intervals on a Multiskan Go plate reader at 37°C.

2.5.4 Turbidimetric assays with Affimer variable region peptides

Four linear peptides of the same sequence as the variable regions of Affimer proteins A68 were created by Thermo Fisher Scientific with peptide purity >95%. These peptides were then reconstituted in PB (1mg/ml) and tested for an effect on the clot formation and lysis with the help of the plasma turbidimetric protocol using 10:1 ratio.

2.5.5 Purified turbidimetric assay, final (optimised) protocol

To conduct the turbidimetric experiments in a purified system, 0.5 mg/ml fibrinogen (Calbiochem, Merck Millipore, Watford, UK) was added to the wells of a 96-well plate and optimisation experiments were conducted using different concentration of PI starting 0 µg/ml, 17.5 µg/ml,35 µg/ml, 70 µg/ml, and 140 µg/ml in a total volume of 50 µl PB. After half an hour the incubation at room temperature on a shaker, a lysis mixture that contained 3 µg/ml plasminogen and 39 ng/ml tPA was added to each well in a volume of 50 µl and that was followed by 50µl of activation and that mixture contained 0.05 U/ml thrombin and 2.5 mM CaCl2. Measurements for the optical density at 340 nm were taken at 12 second intervals on a Multiskan Go plate reader at the temperature of 37°C.

2.6 Confocal microscopy and scanning electron microscopy

Confocal microscopy and SEM were employed to analyse the effects of the Affimers on the structure of the fibrin network.

2.6.1 confocal microscopy

Confocal microscopy was employed to analyse whether the Affimers were affecting the structure of the hydrated fibrin network. A total of 7.5µl pooled human plasma was incubated with 0.03 mg/ml Alexa Fluor-488 labelled fibrinogen (Invitrogen, Paisley, UK) and Affimer protein for thirty minutes in a total volume of 30µl at room temperature. After thirty minutes, followed by the addition of an activation mix containing 5 mM CaCl2 and 0.05 U/ml thrombin in 5µl was added to each sample, and mixed three times to form the clot and then transferring to an IBIDI slide (Thistle Scientific, UK) to be viewed. Different confocal settings were used in order to visualise the two separate colours. The master gain was kept at 967 for all images, detector gain at 1.02 and pinhole 92µm. The image was split into green (PI) and red (fibrinogen) channels which were then superimposed onto each other to allow the fluorescence intensity from each pixel to be determined. Samples were prepared in duplicate. To ensure full clot formation samples were stored overnight in a humidity chamber in the dark. Fibrin networks were visualised on a LSM880 microscope (Carl Zeiss, Welwyn Gardens City, Hertfordshire, UK) using a 40x 1.4 oil objective lens. Z stacks of clots were imaged - 30 slices at 0.7µm intervals (clot thickness 20.3µm). Fibre count was determined using an in-house generated macro for ImageJ (National Institutes of Health, Bethesda, US). This macro calculates the number of fibres crossing 10 horizontal and 10 vertical lines drawn on top of a confocal image of a clot. From this the average number of fibres/100µm can be calculated.

2.6.2 Scanning electron microscopy

Clots were prepared for viewing with a scanning electron microscope which provides additional information on clot ultrastructure. For purified experiments, a total of 50µl of control purified PI (1mg/ml) was incubated with 50µl Affimer (0.1mg/ml). The PI:Affimer ratio was thus maintained 1:2 for purified experiments as per the turbidimetric assays. All clots were produced in duplicate. To determine the mean fibrin

fibre diameter of each clot, 40 fibres were measured from at least one areas of clot for each sample and mean fibre thickness was calculated.

2.6.2.1 Fixing and dehydration

When the Parafilm (Sigma-Aldrich, Dorset, UK) was removed, the clots were washed by placing the lids that contained the clots in a beaker of sodium cacodylate buffer {0.078M Cacodylic acid (Sigma), pH7.4} for a duration of 10 minutes. The buffer was replaced with a fresh preparation and left for another 10 minutes. These clots were then fixed by transferring the lids to 2% Glutaraldehyde (Sigma) in sodium cacodylate buffer for a duration of 30 minutes and they were washed by the same process as mentioned above. Once they were fixed, these clots were dehydrated by placing them in acetone at increasing concentrations, 30%, 50%, 70%, 80%, 90% and 95% for 10 minutes each, after this process they were treated with 100% acetone 3 times, for 10 minutes each.

2.6.2.2 Critical point drying (CPD)

CPD was performed by Martin Fuller at the University of Leeds, using E3000 critical point dryer (Quorum Technologies Ltd, UK). It is important to keep in mind that the surface of the fibrin network is not disturbed during the drying process as EM depends on analysing the surface morphology of biological specimens. Therefore, the process of air drying is unsuitable because it may lead to deformation and collapse of protein structures as water has a high surface tension to air. The surface tension is reduced in CPD by the replacement of water in the sample with liquid CO_2, and the surface tension between the liquid in the sample and surrounding air is therefore gradually decreased rather than going through abrupt changes. Liquid CO_2 transforms to gas state thus avoiding damage to the surface of the sample.

2.6.2.3 Sputter coating

The samples were covered and protected by a thin layer of conducting metal for the purpose of stopping the clots from getting a charge while directly under the electron beam. These clots were then kept on aluminium specimens and they were covered in

a carbon film. The clots were then coated with a 7nm thick layer of platinum palladium which was applied in a 208HR high resolution sputter coater (Cressington, UK).

2.6.2.4 Clot imaging

The samples were imaged and photographed in a Quanta 200F FEGESEM field-emission scanning electron microscope (FEI, Oregon, USA). Three different areas of each clot were visualised at magnifications of x5000, x1000 and x30000. Fibre diameters of all clots were detected with image analysis software package ImageJ, 23y (National Institutes of Health, Bethesda, MD, USA).

2.7 Biacore surface plasmon resonance (SPR) experiments

SPR experiments were conducted by Dr Nikoletta Pechlivani with the help of Dr Iain Manfield at the Centre for Biomolecular Interactions technology facility. Purified human PI (5 µg/ml in 0.1 M sodium acetate buffer, pH 5.6) was kept immobilized to 2000 RUs by amine-coupling using an NHS/EDC-activated CM5 chip that was followed by deactivation with ethanolamine/HCl using a Biacore 3000 (GE Healthcare, Little Chalfont, UK). A reference surface was made and prepared by activating and deactivating the dextran without protein. The running buffer for binding assays was of different denominations e.g. 100 mM NaCl, 50 mM Tris, 2mM CaCl2, 0.1% (v/v) Tween-20, pH 7.4. Affimer proteins were diluted in running buffer for the purpose of experiments and they were injected for 120 seconds at 50 µl/min in three replicate experiments (Affimer concentrations 6.125–800 nM). The surface was then redeveloped by flowing running buffer for 15 minutes that was sufficient enough to return signal to baseline. The data were managed by subtraction of sensorgrams from the reference flow cell and a buffer injection over the derivatised surface. The PI surface was washed by injecting 1M NaCl, 50 mM Tris, pH 7.4 at 30µl/min.

2.8 Molecular Modelling

Given that human PI crystal structure is yet to be described, the published crystal structure of mouse α2-antiplasmin (PDB ID 2R9Y) was used to make a homology model of human alpha-2-antiplasmin using I-TASSER [253] A homology model of Affimer A68 was created using I-TASSER and the Maestro graphical user interface to

check the validity of the model produced [253]. The published crystal structure of the Affimer scaffold (PDB ID: 4N6T) was used as a template to create a model of Affimer A68 [252]. Docking of this A68 model to the homology model of human alpha-2-antiplasmin was carried out using AutoDock 4.2 [254]. A total of 100 docking iterations were calculated for each predicted site, using a Lamarckian Genetic Algorithm. The resulting poses were clustered, based on a 2 Å root mean squared deviation. The cluster with the lowest energy conformation, and also the most populated cluster pose, were further examined using PyMOL [255]. The screening using AutoDock was carried out by Dr Katie Simmons, LICAMM, University of Leeds.

2.9 Cross linking analysis using mass spectrometry (MS)

Chemical cross-linking (XL) happens with the addition of a reactive substance to a native protein to covalently trick its conformational state. This cross-linking allows protein-protein interaction interfaces to come together as the crosslinking substance joins together the adjoining regions of the proteins.

Three separate analyses were done on purified PI alone, Affimer 68 alone and PI-A68 complex using BS3 as a cross-linker. The cross-linked protein is then digested with trypsin, which results in a complex mixture of both modified and unmodified proteolytic peptides. These peptides are then by MS to identify any of the cross-linked adducts. All MS experiments were conducted by Dr James Ault.

2.10 Western Blotting

The following standard protocols were used for SDS-PAGE in reducing conditions. The Samples were processed on a 4-12% Bis-Tris gel (Invitrogen, Thermo Fisher Scientific, Loughborough, UK) in order to resolve smaller proteins such as Affimers or a 10% Bis-Tris gel (Invitrogen, Thermo Fisher Scientific, Loughborough, UK) for larger proteins such as PI. The Samples were reduced using the NuPAGE sample reducing agent followed by NuPAGE lithium dodecyl sulphate (LDS) sample buffer (both Invitrogen, Thermo Fisher Scientific, Loughborough, UK), before heating to 95°C for ten minutes. Samples were then loaded onto gels in a XCell SureLock electrophoresis system (Invitrogen, Thermo Fisher Scientific, Loughborough, UK). NuPAGE 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer was used to resolve small molecular weight proteins, and NuPAGE 3-(N-morpholino) propanesulfonic acid (MOPS) for mid-

size proteins (both Invitrogen, Thermo Fisher Scientific, Loughborough, UK), each diluted 1/20 in dH_2O. The gels were usually run for 45 minutes at 200 V (MES buffer) or 50 minutes at 200 V (MOPS buffer), or until the good separation of the protein standard ladder (Precision Plus protein standard, Bio-Rad Laboratories Ltd., Watford, UK).

After running, the gels were washed again for 20min in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol v/v, pH 8.3) under gentle shaking. Polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, Loughborough, UK) was made afresh by placing it in methanol for fifteen seconds, followed by keeping it for two minutes in ddH_2O, before finally placing transfer buffer until needed. A foam pad was soaked in transfer buffer and placed on the back side of a gel holder cassette in order to assemble a blot. A filter paper (also soaked in transfer buffer) was added on top, followed by the gel, the prepared membrane, a filter paper, and a final sponge (all Bio-Rad Laboratories Ltd., Watford, UK). During the process of assembly of the blotting cassette, all components were made wet with transfer buffer and bubbles removed with a roller. The assembled cassettes were then placed in the Mini Trans-Blot western tank (Bio-Rad Laboratories Ltd., Watford, UK). Western blots were run for one hour at 100 V, with an ice block placed in the tank and a magnetic stirrer.

2.10.1 PI Western blot

A total of 2 µg protein per lane was added into the wells of SDS-PAGE gel (see above for details of running an SDS-PAGE gel), (Section 2.10) followed by transfer to a PVDF membrane (Section 2.10). The membrane was then washed for almost ten minutes in Tris-buffered saline along with Tween-20 (TBST, 50 mM Tris, 150 mM NaCl, 0.1% Tween- 20 v/v), followed by placing in blocking buffer (5% w/v milk powder in TBST) for one hour at room temperature with shaking. The membrane was subsequently incubated with Affimer A68 followed by a washing step. Mouse anti-His antibody (Roche; Welwyn Garden City, UK) was then added to the membrane (as a primary antibody), diluted 1/200 in blocking buffer and incubated for one hour at room temperature with shaking, prior to four washes of fifteen minutes each with TBST. Following washing, secondary antibody, a rabbit anti-mouse-HRP (Dako, Agilent Technologies; Stockport, UK) diluted 1/1000 and StrepTactin-HRP (Bio-Rad Laboratories Ltd., Watford, UK) diluted 1/5000 were added to the membrane in blocking buffer and incubated for one hour with shaking. The membrane was washed again four times for

fifteen minutes each in TBST, before developing and visualisation of the blot. Immobilon Western Chemiluminescent Substrate (Merck Millipore, Watford, UK) was used for developing the blot, which was imaged immediately after addition of the developing agent using a G:Box Chemi XT4 imaging system (Syngene).

2.11 Rotational thromboelastometry (ROTEM)

Volunteers were selected to give the blood sample from the antecubital vein and this process was done after taking their written consent, according to the declaration of Helsinki. Ethical approval was granted by Leeds Medical School Ethical Committee, University of Leeds. The blood samples that were taken from volunteers were held for about 30 minutes at room temperature after collecting in 0.19 M sodium citrate.

A pin was injected into the cuvette while performing the ROTEM experiments on the blood sample and ROTEM reagents. There gap of 1 mm which was bridged by the blood between the pin and the bottom of the cup. The pin was moved in left and right directions and it moved freely before the blood is turned into a clot. When the blood turns into a clot, the pin's movement becomes slower and the rotation of the pin is stopped finally owing to the firmness of the clot. After this process, a ROTEM trace is generated and different clot parameters are used that are described below (Figure 13).

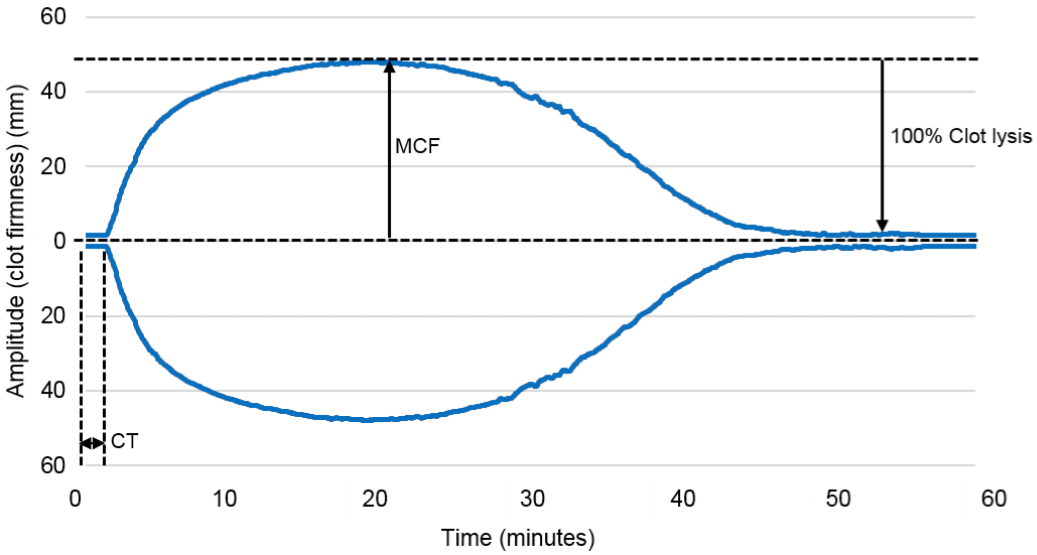

Figure 13. ROTEM trace.
This figure shows the example of a reaction curve which is generated during the ROTEM experiment. There are certain parameters of interest that can be derived from the reaction curve: clotting time (CT), maximum clot firmness (MCF) and percentage clot lysis.

2.11.1 EXTEM tests

Coagulation is activated with the help of tissue factor and this is generally done in EXTEM experiments. The collected samples were re-calcified, and this was done with the use of 20 µl star-tem reagent. The process of clotting was started with the help of 20µl ex-tem reagent and both were poured into a pre-warmed plastic cup. Then the Affimer protein was added into the cups and the total volume was 90 µl in saline solution (0.9% NaCl). These tests were conducted with and without 2.5 nM tPA, that was added to the plastic cup before the addition of blood. A total of 210 µl whole blood was added, mixed once by pipetting once, and finally the experiment was conducted. These EXTEM tests were conducted for up to 5 hours at 37°C.

2.11.2 Optimising {tPA} in EXTEM tests

In order to get an appropriate concentration of tPA to include in EXTEM assays (to assess the influence of Affimers on the whole blood lysis), a concentration curve of tPA was used (Figure 14). The experiments were conducted as mentioned above with added tPA to the reactive mixture in either 1 nM, 2 nM, 5 nM or 10 nM. The curves

which are generated are based on the ROTEM during this experiment (Figure 14), 2.5 nM tPA concentration was selected, to allow full lysis of clots during 60 minutes.

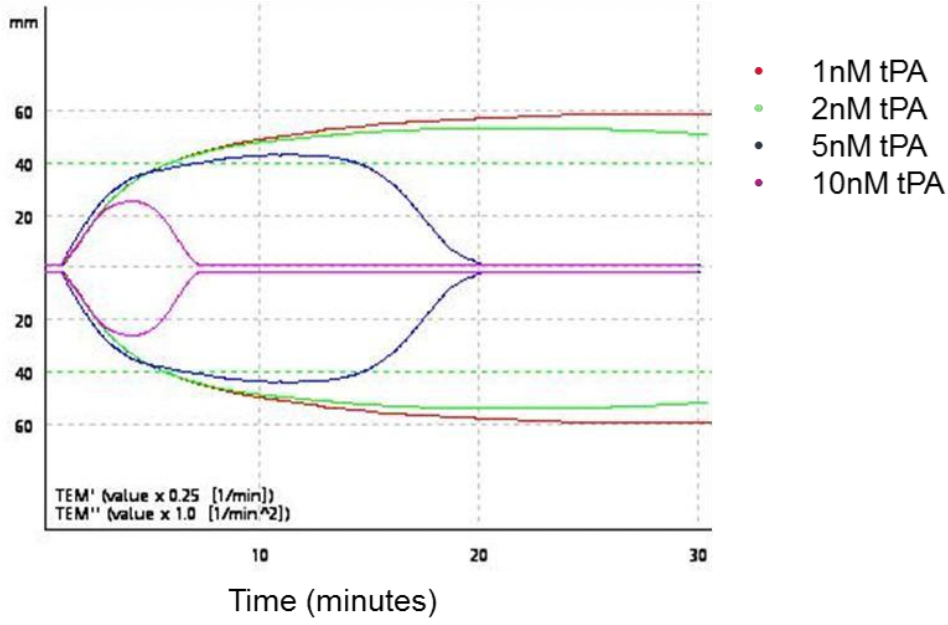

Figure 14 Tissue plasminogen activator (tPA) concentration curve.
Ascending concentrations of tPA have been utilized in ROTEM EXTEM experiments to create the conditions for lysis within 120 minutes. The traces of the ROTEM for each of the tPA concentrations that are used have been presented in different colours as indicated above.

2.11.3 ROTEM parameters

During the process of clot formation and lysis, there are multiple parameters that can be calculated from the measurements taken by ROTEM analysis. The ROTEM-calculated parameters include clotting time (CT) and maximum clot firmness (MCF). CT is the time measure from the initiation of the test until the firmness of clot amplitude of 2 mm is reached. CT parameter also reflects the time taken for generation of thrombin and the polymerisation of fibrinogen into fibrin fibres. MCF is calculated to know the maximum amplitude (in mm) that is achieved by clot formation during a test and reflects the mechanical strength of the clot. MCF is also affected by stabilisation of the clot secondary to fibrin polymerisation. With the addition of tPA, lysis time was defined as the time from MCF to time of 50% reduction in MCF.

2.12 Mutagenesis experiments

Mutagenesis experiments were performed using Site Directed Mutagenesis according to manufacturer's instructions (QuikChange® Site-Directed Mutagenesis Kit). Only Loop one AAs of Affimer A68 were mutated (total of 9 mutations were produced); loop 2 AA were kept unchanged. Mutations were introduced using the mutant primers listed in Table 4 and pET11a vector containing Affimer A68 sequence as template. PCR reactions were established using KOD Hot Start DNA Polymerase Kit (Millipore) as described in Section 2.4.1. The mutated products were digested for 1 h at 37°C with the restriction enzyme *DpnI* (New England Biolabs) to eliminate the original methylated template and to increase mutation efficiency. Supercompetent XL1-Blue cells were transformed with the digested products, plasmid DNA was purified from transformant colonies, and the presence of the mutation was confirmed by DNA sequence analysis (Section 2.4.1). BL21 star™ (DE3) cells were transformed with plasmid DNA and protein produced as described previously (Section 2.4.1).

Primer	Primer Sequence (5' to 3')
R42A.F	TTTTCCGGCATGAAGTTGTAAGCCTGTTCTTTCGCTTTAACAAC
R42A.R	GTTGTTAAAGCGAAAGAACAGGCTTACAACTTCATGCCGGAAAA
Y43A.F	GTATTTTTCCGGCATGAAGTTGGCACGCTGTTCTTTCGCTTTAACA
Y43A.R	TGTTAAAGCGAAAGAACAGCGTGCCAACTTCATGCCGGAAAAATAC
N44A.F	GTGTATTTTTCCGGCATGAAGGCGTAACGCTGTTCTTTCGCTTT
N44A.R	AAAGCGAAAGAACAGCGTTACGCCTTCATGCCGGAAAAATACAC
F45A.F	GTGTATTTTTCCGGCATGGCGTTGTAACGCTGTTCTTTCGCTTTA
F45A.R	TAAAGCGAAAGAACAGCGTTACAACGCCATGCCGGAAAAATACAC
M46A.F	ATGGTGTATTTTTCCGGCGCGAAGTTGTAACGCTGTTCTTTCG
M46A.R	CGAAAGAACAGCGTTACAACTTCGCGCCGGAAAAATACACCAT
P47A.F	CATGGTGTATTTTTCCGCCATGAAGTTGTAACGCTGTT
P47A.R	AACAGCGTTACAACTTCATGGCGGAAAAATACACCATG
E48A.F	GTAGTACATGGTGTATTTTGCCGGCATGAAGTTGTAACG
E48A.R	CGTTACAACTTCATGCCGGCAAAATACACCATGTACTAC
K49A.F	GTCAGGTAGTACATGGTGTATGCTTCCGGCATGAAGTTGTAACG
K49A.R	CGTTACAACTTCATGCCGGAAGCATACACCATGTACTACCTGAC
Y50A.F	GGTCAGGTAGTACATGGTGGCTTTTTCCGGCATGAAGTTGTAAC
Y50A.R	GTTACAACTTCATGCCGGAAAAAGCCACCATGTACTACCTGACC

Table 4 Complementary primer pairs (F and R) for generation of site-directed mutants of the Affimer of interest (loop 1 only).

2.13 Construction of Aff68DVR1 and Aff68DVR2 mutants

Two new single loop Affimers were made. PCR was used to truncate each of the two loops providing one new Affimer with Loop 1 of A68 and another Affimer with Loop 2 of A68. The new Affimers were subcloned for large scale production. Affimer 68 is constructed by having one of two variable regions 1 (Aff68DVR1) or variable region 2 (Aff68DVR2) which have been generated to decide which one of the VRs was responsible for mediating interactions of the Affimer. Aff68DVR1 and Aff68DVR2 were generated by amplifying DNA for each respective variable region from the pBSTG-Aff phagemid vector and a a wild type (WT) Affimer scaffold which has variable regions having 2 or 3 Alanine residues (Figure 15).

Splice overlap extension (SOE) reactions was set up in 0.2 ml tubes (Sarstedt) and thermal cycling carried out using a G-Storm GS2 thermal cycler. Equimolar quantities of the two DNA fragments to be spliced were added to a total DNA concentration of 40ng/µl.

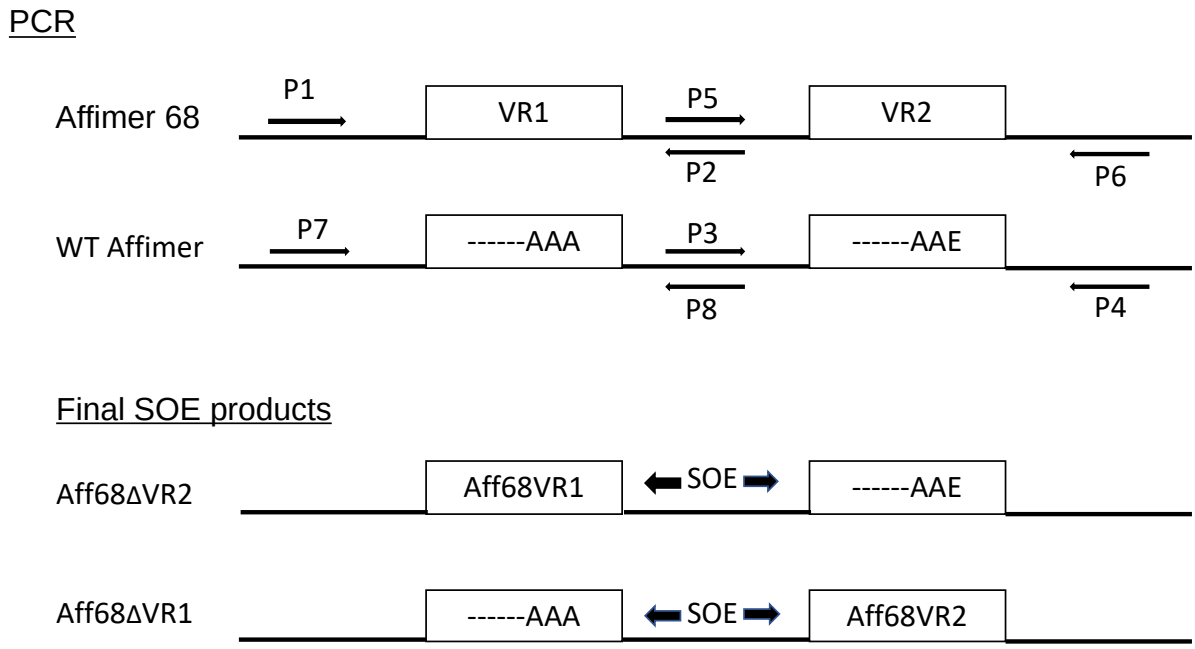

Figure 15 Generation of Aff68DVR1 and Aff68DVR2 DNA.
This scheme shows the strategy for creating Aff68DVR2 and Aff68DVR1 DNA. Template DNA was pBSTG-Aff phagemid vector that contained Affimer 68 sequence and wild-type (WT) Affimer. Primers have been listed in Table 1. VR1: variable region 1 and VR2: variable region 2.

Affimer 68 variable region DNA had been amplifiedby PCR from the pBSTG-Aff phagemid vector and that used primers P1 and P2 (for VR1), and P5 and P6 (for VR2) (primer sequences listed in Table 5). Variable region DNA from the WT Affimer framework was amplifiedby the usage of primers P3 and P4 (empty VR1), and P7 and P8 (empty VR2).

construct	Vector	Primer	Sequence
Aff68DVR2	pBSTG-Aff	P1	Forward 5’ TTCTGGCGTTTTCTGCGTCTGC 3’
		P2	Reverse 5’ CACCGTCTTTAGCTTCCAGG 3’
Aff68DVR2	WT-Aff	P3	Forward 5’ CCTGGAAGCTAAAGACGGTG 3’
		P4	Reverse 5’ GTCAGGAAACAGCTATGACC 3’
Aff68DVR1	pBSTG-Aff	P5	Forward 5’ CCTGGAAGCTAAAGACGGTG 3’
		P6	Reverse 5’ TACCCTAGTGGTGATGATGGTGATGC 3’
Aff68DVR1	WT-Aff	P7	5’ AGTAAAACGACGGCCAGTG 3’
		P8	5’ CACCGTCTTTAGCTTCCAGG 3’

Table 5 Primers for construction of Aff68DVR1 and Aff68DVR2 mutants

The particular DNA segments were then detached on agarose gels and purified as described above. It was then reunited in an SOE reaction and the end products were amplifiedusing primers P1 and P4 for Aff68DVR2, and primers P5 and P8 for Aff68DVR1 (described above). DNA was then processed with *NheI*-HF™ and *NotI*-HF™ (NEB) and ligated into pET11a vector. XL1-Blue cells were then transformed with the DNA, grown and protein was purified before being sequenced in order to confirm the correct insert.

2.14 Statistical analysis

Statistical analysis was conducted using GraphPad Prism 8 and the statistical significance was accepted at $p<0.05$. In order to make a comparison of two groups of paired data sets, the statistical significance was measured using t-test. For data sets comprising three or more groups, the statistical significance between different groups was assessed using one-way ANOVA followed by Dunnett's multiple comparison test.

Chapter 3 : Isolation of PI-binding Affimers

3.1 Introduction:

The obstructive thrombus is composed of a mesh of fibrin fibres with blood cells trapped in these networks. Current anti-thrombotic treatment strategies for primary and secondary prevention of vascular disease are mainly focused on the inhibition of platelet function, and the fibrin network is not usually targeted. However, several pieces of evidence suggest that modulating the fibrin network may have a role in decreasing thrombosis risk [242, 256]. The recent COMPASS study has shown that the addition of rivaroxaban to antiplatelet therapy reduces vascular occlusive events, but this occurred at the expense of 70% increase in bleeding events, which reduced the net clinical benefit, although this was still favourable. The increase in bleeding is secondary to excessive inhibition of the coagulation arm of thrombosis making the fibrin network unstable and thus unable to maintain a physiological blood clot. This clearly highlights the challenges faced by anti-thrombotic therapies that are ideally required to reduce thrombotic events without significantly increasing bleeding risk [257].

Fibrin clot lysis, which limits thrombus formation, is controlled by a number of factors including incorporation of anti-fibrinolytic proteins into the clot, the most powerful of which appears to be plasmin inhibitor (PI). Therefore, inhibition of PI activity will allow easier clot breakdown and given that this targets a specific component of the clot, it has the potential to limit clot instability and hence containing bleeding risk. This is not a new concept as PI has been targeted for anti-thrombotic therapies (detailed in the introduction) but the various agents described in the literature have yet to make it into the clinical field.

Affimers may have advantages over antibodies as described in the introduction section. Therefore, the first aim of my research was to isolate Affimers which are capable of binding to PI and modulating protein activity. Affimers have been described in the introduction section and they represent viable alternative to antibodies and proving to be superior in some respect, including simplicity of screening against target protein, low production costs and the potential for modification for future clinical use.

3.2 Methods

Screening of Affimers against the target protein (PI) was performed using help from individuals in the Bioscreening Technology Group, University of Leeds.

3.2.1 Affimer identification

Plasmin inhibitor (Sigma-Aldrich, Dorset, UK), was biotinylated utilising EZ-interface NHS-SS-biotin (Pierce), according to manufacturer's instructions. The biopanning with the Affimer phage library was performed as described in Chapter 2, which was conducted by one of my supervisors, Dr. Tiede. Following three rounds of panning, >1000 phage fold amplification was seen over streptavidin control wells. To refine the phage pool and select specific PI-binding Affimers. Different ER2738 colonies were chosen and Affimes were tested for PI bidning using a phage ELISA. 24 high affinity PI-binding Affimers were isolated. Out of these 24 Affimers, 7 were selected for DNA sequencing consisting of double loop unique sequencing Affimers.

3.2.2 Phage ELISA

The chosen *E.coli* colonies were grown in 100µl of 2TY growth medium (bacto tryptone and yeast extract), additionally containing 100µg/ml carbenicillin, at 37^{o}C and were shaken gently at 900rpm for 6 hours, after which a 25µl aliquot was added to 200µl of 2TY and carbenicillin as previously described. After 60 minutes, 10µl of 1011/ml M13K07 partner phage and 25µg/ml kanamycin were included and kept the medium in a shaker at 25^{o}C and 450rpm. A streptavidin covered plate (Thermo Fisher Scientific, Loughborough, UK) was blocked over night with 2x casein blocking buffer and the plate was then incubated with biotinylated plasmin inhibitor for 60 minutes, followed by one more hour with 45µl of development medium containing the phage. Subsequent to washing the wells extensively, bound phage was detected by HRP-conjugate of phage anti-phage antibody diluted 1:1000 (Seramun Diagnostica GmbH,Germany) for 60 minutes and developed with 3,3',5,5'-Tetramthylnenzidine (Seramun) and OD read at 610nm.

3.3 Affimer production

The chosen Affimers were produced utilizing the pET expression system, which delivers extensive amounts of protein in a short period of time through doctrinaire of T7 RNA polymerase and the *lac* repressor protein. The DNA sequence encoding the protein of interest was cloned into a pET11 vector that contains a T7 promoter and lac operator at the 5' end of the gene encoding the protein. Transcription of the inserted gene happens in the presence of T7 RNA polymerase thus, the vector needs to be transformed into bacterial cell that has been built to contain the gene for this enzyme, for the most part, *E.coli* strain BL (DE3). Transcription of T7 RNA polymerase inside the host cell requires activation as the host RNA polymerase is prevented from binding to the gene due to the presence of a lac promoter sequence which attaches the lac repressor protein (LacI). Lactose, or a structural copy such as isopropyl β-D-1-thiogalactopyranoside (IPTG), will link to LacI and change its adaptation, to a degree that its proclivity for DNA is significantly lessened, and in this way, the local E.coli RNA polymerase is not blocked, and translation of T7 RNA polymerase continues. Thus, it causes transcription of the gene encoding the protein of interest inside the phagemid vector (Figure 16).

Affimers undergo the amplifications of the DNA coding by PCR followed by restriction digestion with NheI and PstI to enable cloning into a pET11a phagemid. Utilizing heat shock, the phagemid is introduced into BL21 (DE3) *E.coli* cells and protein production is initiated by 0.1mM IPTG. The cells were grown for 6 hours in LB medium and after that lysed utilizing Bugbuster (Novagen). The expressed protein was purified using Ni-NTA pitch slurry (Qiagen) according to the manufacturer’s protocol.

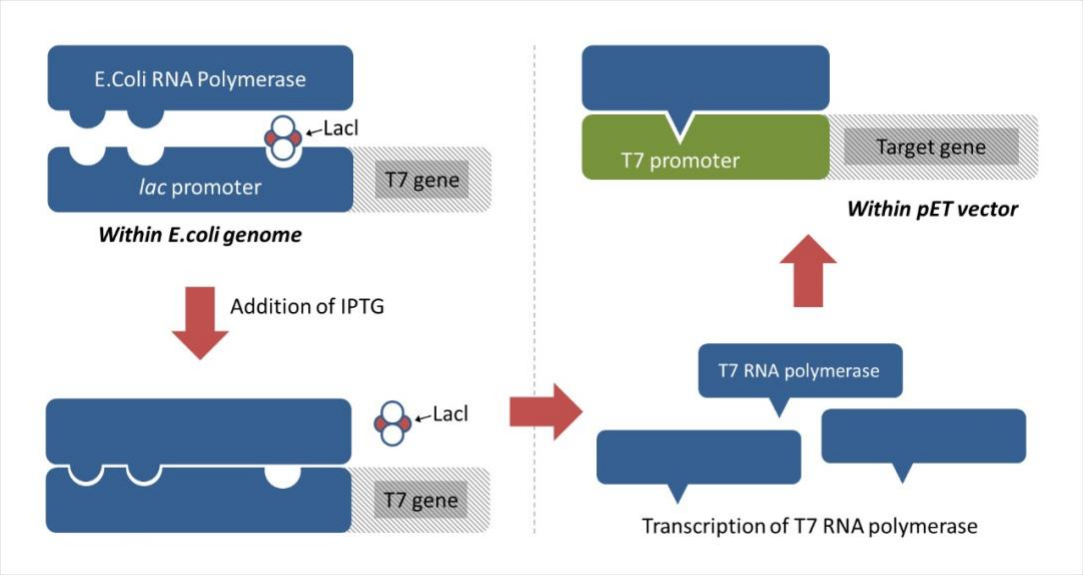

Figure 16. Illustration of the pET expression system.
In the absence of IPTG, host RNA polymerase cannot transcribe T7 RNA polymerase because of suppression by lac repressor protein (LacI) inside the lac promoter region. When IPTG is added, it alters the conformation of LacI, reducing its affinity for DNA. Transcription of T7 RNA polymerase consequently takes place followed by transcription of the target protein DNA inside the pET vector.

3.4 Turbidimetric analysis

To study the effects of selected Affimers on clot lysis/structure, a validated turbidimetric assay was employed as described in Chapter 2. Both purified proteins and plasma systems were used to analyse the effects of PI-specific Affimers. The former was used to confirm specificity whereas the latter ensured that the Affimer continued to work in the presence of plasma proteins. For plasma tests, 25µl of plasma (containing around 1µM PI) was incubated with 25µl of Affimer (0.5mg/ml or 30µM) for 30 minutes before the addition of activation mix (AM) and lysis mix (LM). In a purified system, 25µl of Affimer (0.2mg/ml or 5µM) with final concentration (0.8µM) was added to PI (1µg/ml or 1µM) with final concentration (0.16µM). To study the impacts of Affimers on PI prolongation of lysis, PI purified (2.94µM) was incubated with the Affimer (5µM) for 30 minutes. These concentrations equated to PI:Affimer molar ratio of approximately 1:1, 1:5 and 1:10 in plasma and purified systems. LM and AM were then added, and the absorbance at 390nm read at regular intervals of 12 seconds for an hour (detailed in the methods section)

3.5 Results:

3.5.1 First selection of PI binding Affimer proteins (First screen)

In the first screen, two libraries were screened. A conventional double Loop Affimer library and we also screened a single loop library to understand which of the two yields higher Affimer clones. A potential advantage of the single loop library Affimer is easier understanding of interaction sites, which may help to increase the speed of identifying novel therapeutic targets.

3.5.1.1 Isolation of PI-binding Affimers

In the first screening attempt and after completion of three rounds of phage panning, a total of 24 high affinity PI-binding Affimers were isolated from double Loop and single Loop libraries (Figure 17). Single loop Affimers are characterised by a single, rather than two, variable region. Out of these 24 Affimers, 9 had distinct sequences and were expressed in *E. coli* after being subcloned into pET11 vector from their phagmid vector.

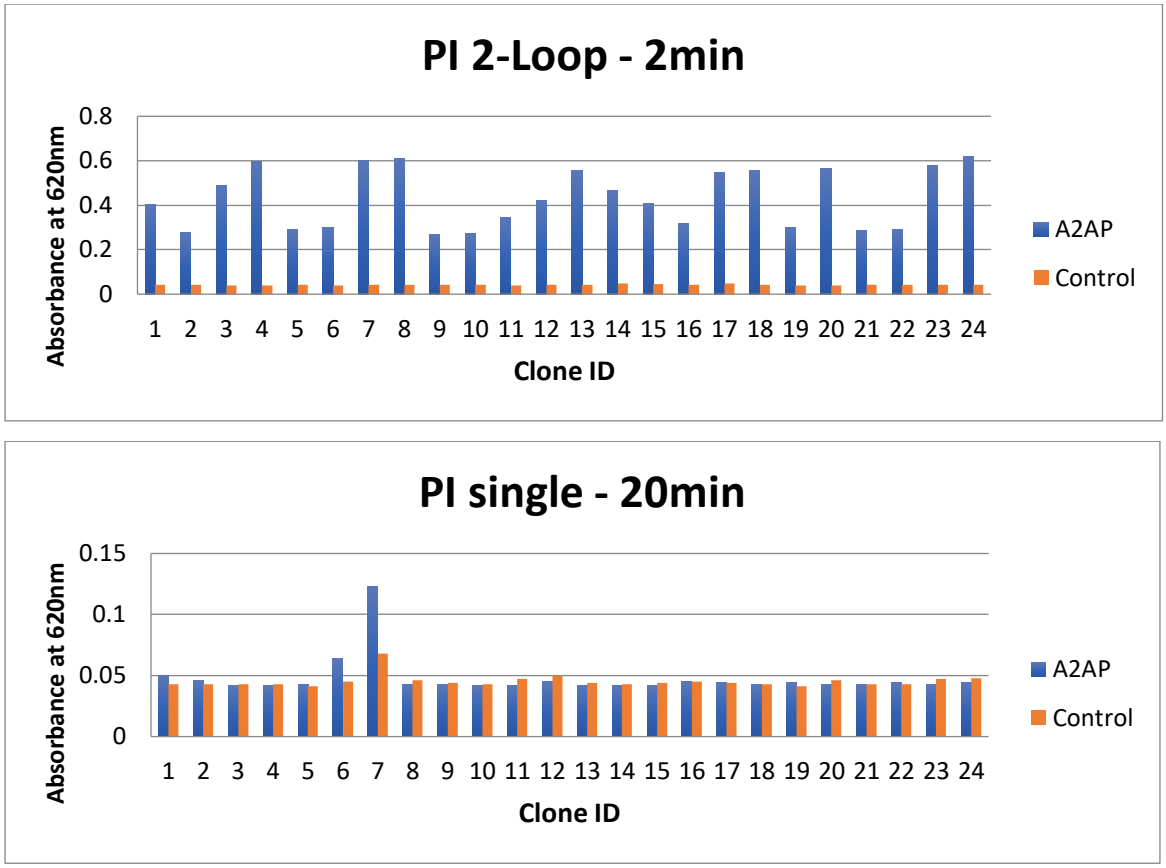

Figure 17. ELISA for PI binding Affimers.
A total of 24 phage clones in both single loop and 2-loop were added to biotinylated PI immobilised on streptavidin coated wells in blue and control wells not containing PI in red. After incubation, wells were extensively washed and developed by the addition of an HRP conjugated anti-phage antibody.

3.5.1.2 Sequence analysis of Affimers

Given the PI interacts with a number of coagulation proteins, I conducted an initial search to investigate whether any of these Affimers share sequence homology with proteins of the coagulation system. The concept behind this was related to the fact that a PI-binding Affimer may not necessarily modulate protein function but may interfere with binding to coagulation proteins, thus having an indirect effect that is worth investigating (even if no effect on PI function is demonstrated).

In order to find sequences homology with any protein involved in the coagulation system the Affimer variable regions (Table 6) were screened in NCBI BLAST that contains a large database of proteins. Table 7 summarises the main proteins that showed homology with the Affimers. Two coagulation proteins, FIX and FXIII, showed

homology with the latter being of interest as it mediates incorporation of PI into the clot.

Affimer variable regions (Table 6) were screened in NCBI BLAST to search for sequence homology against a vast protein database (Table 7).

AFFIMER	LOOP 1	LOOP 2
A1	STPWPPHIV	KWMYRENWY
A2	FAAKLVGIA	EKWWLRQDE
A3	LRWDWREQI	EPPVFTTAH
A4	HWHDVMWRH	ESAIIIQPW
A11	QVVKSWGSE	VYQNQILRL
A14	PYPGKWWDM	FRDFFGMVS
A19	YWEIWGFPA	PYWIFPIPE

AFFIMER	LOOP 1
A7	RKRRVIYVH
A6	QNRKIMTWQ

Table 6 Amino acid sequences of the variable loops

Amino acid sequences of the variable loops within the 9 Affimers which were successfully sub-cloned and purified.

Affimer	Variable region	Proteins with sequence homology, with corresponding homologous region (amino acid sequence)
A1	STPWPPHIV	None
	KWMYRENWY	Plasminogen related growth factor receptor 2 [Takifugu rubripes] WMYRE Plasminogen related growth factor receptor 2 [Dicentrarchus labrax] WMYRE

		Coagulation factor VIII [Acipenser ruthenus] FRENWY
A2	FAAKLVGIA	None
	EKWWLRQDE	Fibrinogen beta chain isoform X2 [Mastomys coucha] QKWWLMRSDE
A3	LRWDWREQI	None
	EPPVFTTAH	Coagulation factor 5/8 type domain protein [Cellulomonas terrae] ETPVFTTTAH
A4	ESAIIIQPW	Coagulation factor XIII B chain-like protein [Cricetulus griseus] SAIIIQRW Coagulation factor XII [Physeter catodon] ESAVLIPPW
	HWHDVMWRH	None
A11	VYQNQILRL	None
	QVVKSWGSE	None
A14	PYPGKWWDM	None
	FRDFFGMVS	None
A19	PYWIFPIPE	Coagulation factor IX precursor [Takifugu rubripes] PYWAFPTLPTIPE Coagulation factor IX precursor isoform X1 [Takifugu rubripes] PYWAFPTLPTIPE
	YWEIWGFPA	None
A7	RKRRVIYVH	None
A6	QNRKIMTWQ	None

Table 7 Selected results of sequence homology search using Affimer variable regions. There was no sequence homology between the variable regions of isolated double loop Affimers with PI but some of showed homology with other coagulation factors.

3.5.1.3 SDS-PAGE analysis of Affimers

The results of the SDS-PAGE gel depicted additional high molecular weight (MW) proteins in the selected Affimers (Normal MW of Affimers ~ 13kDa). However, it was observed that the MW of these additional protein bands were in multiples of the normal Affimer MW (26kDa, 39kDa, 65kDa, etc) (Figure18). Thus, it was concluded that these protein bands may corresponded to the multimers of the Affimers, although other protein impurities cannot be ruled out (investigated later). This was further investigated using immunoblotting in chapter 4 with the Affimer of interest.

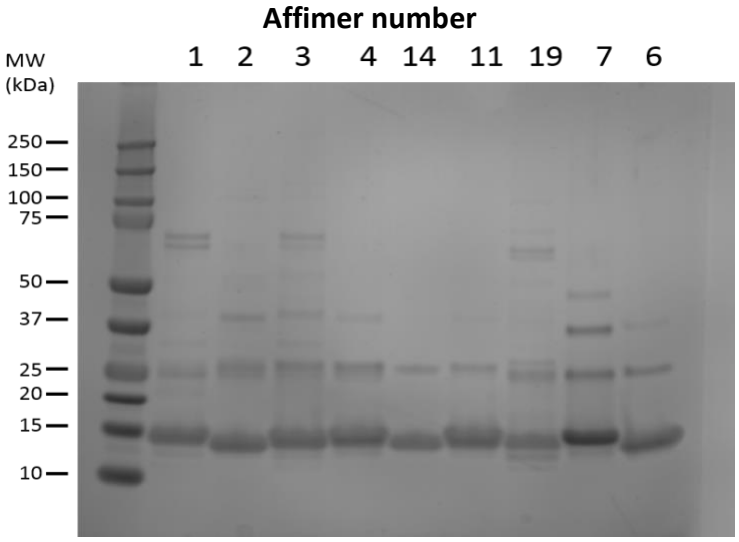

Figure 18. Molecular weight analysis of purified Affimers (1,2,3,4,14,11and 19)
on a 4-12% Bis-Tris gradient gel. The Affimers molecular weight around 13KD.

3.5.1.4 Double loop library

In the first screening attempt and after completion of three rounds of phage panning, a total of 24 high affinity PI-binding Affimers were isolated from double loop and libraries. Twenty-four phage colonies eluted from PI were tested in an ELISA to confirm binding to PI. As can be seen in Figure 18, of these 24 Affimers, 7 had distinct sequences (the remaining were simply repeat sequences). This identified 7 distinct Affimers as detailed in Table 1, which were expressed in *E. coli* after being subcloned into pET11 vector from their phagmid vector, to produce the individual proteins.

3.5.1.5 Effect of PI-binders on plasma clot structure

None of these Affimers described above had sequence homology with PI. Affimer A4 and A19 displayed sequence homology with coagulation factor XIII and coagulation factor IX respectively. It was hoped that sequence homology will be detected with PI or an area of fibrinogen that is involved in fibrinogen-PI interactions. However, the failure to find sequence homology is not necessarily surprising as protein interactions are likely to be conformational. Moreover, the sequence homology with FXIII may indicate that the Affimers picked up an area of PI-FXIII interaction. In order to understand the functional effect of Affimers on clot structure/lysis, a turbidimetric assay was conducted. Scaffold protein was used as a negative control throughout. The slight decrease in clot lysis time with Affimer A1and A2 was similar to the decrease in final turbidity induced by the scaffold protein. These differences failed to reach statistical significance in the initial concentrations tested. However, addition of A3 resulted in a significant decrease in the final turbidity. The results obtained showed that A3 reduced final turbidity when compared with scaffold protein (p=0.04). A summary of the results is provided in Figure 19.

Previous work has shown that as little as 6% change in clot lysis time may be important clinically [164]. Therefore, and in the presence of Affimer, a change in lysis time by 15% was assumed to be significant and warrants taking the Affimer forward to subsequent characterisation.

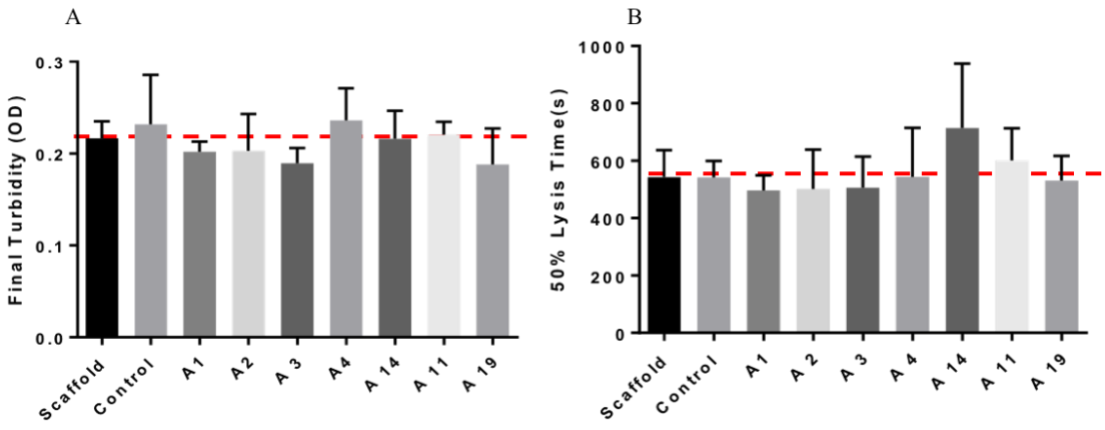

Figure 19. Effect of PI-binding Affimers on clot structure/fibrinolysis in plasma system

A. Final turbidity is reduced for A3 (p=0.04), while there is no significant reduction by other Affimers. B. Time to 50% clot lysis showed no difference in the presence and absence of Affimers. All experiments were conducted in duplicate and repeated at least 6 times.

3.6 Dose response of the Affimers (Affimer 2 and Affimer 3)

In subsequent experiments, using a fresh batch of Affimers and at higher Affimer:PI molar ratio, no significant differences were detected with the use of A3 on either clot final turbidity or lysis time. Other Affimers, chosen at random, were also studied to ensure reproducibility of the results and these showed no differences (Figure 20).

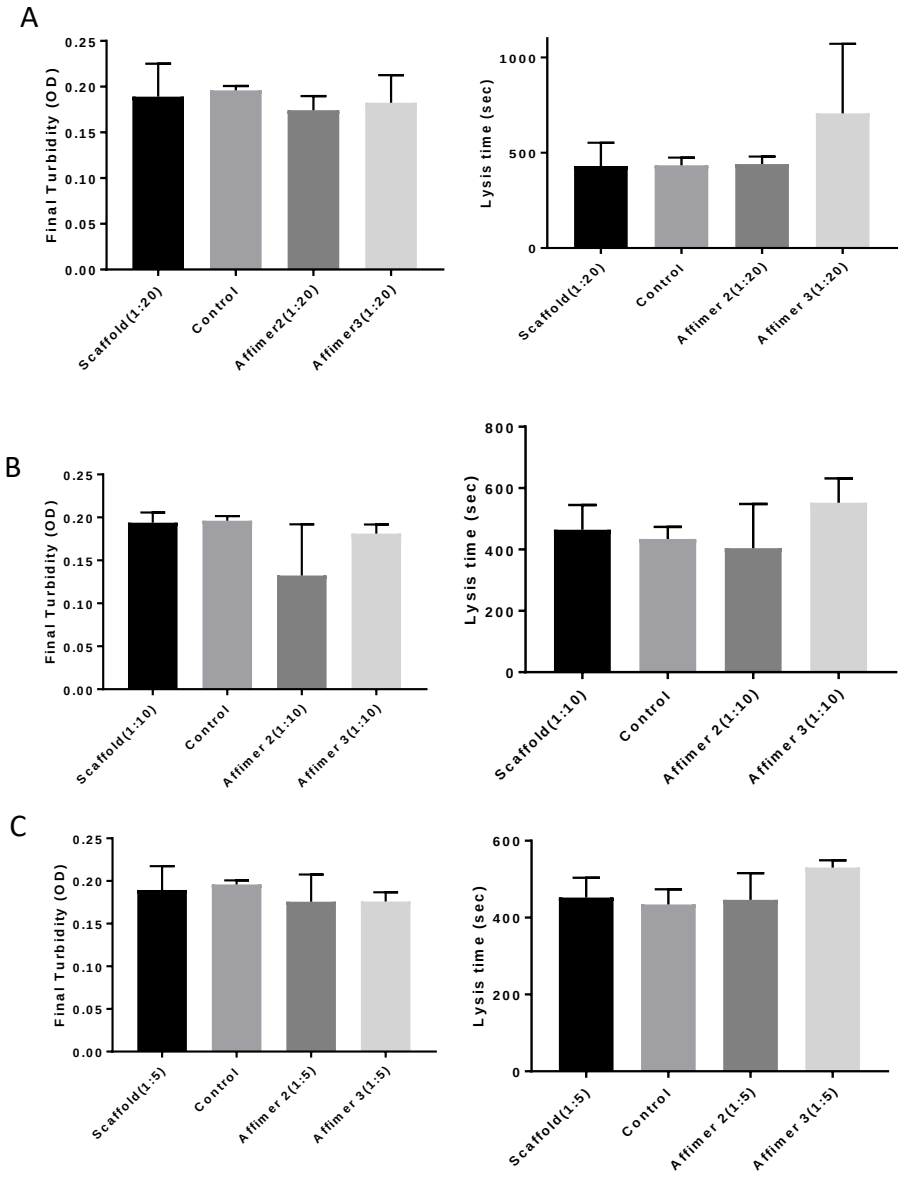

Figure 20. Turbidimetric assays different concentrations of PI-binding Affimers (at 1:5, 1:10 and 1:20 PI:Affimer molar ratio, A, B, C, respectively). No significant differences were detected. All experiments were conducted in duplicate and repeated at least 3 times. Statistical analysis was performed using one-way ANOVA, comparing each Affimer to scaffold-only control

3.6.1 Single loop library Effect of PI- binders

In addition to double loop Affimers, it was decided to study a library of single loop Affimers. Single loop Affimers are characterised by a single, rather than two, variable region. The potential advantage of this library is the easier understanding of the interaction sites given the presence of a single loop. However, a possible disadvantage is the less robust interaction, given that two loops are more likely to behave as an antibody with better “capture” of the protein. As detailed above, out of the 24 Affimers, only 2 had distinct sequences. (detailed in Table 6). These were expressed in *E. coli* after being subcloned into pET11 vector from their phagmid vector, which were subsequently produced as individual proteins.

3.6.1.1 Plasma system

The effect of the Affimer from single loop A7 and A6 had no significant effects on clot final turbidity. Although there was some decrease in clot lysis time by these Affimers, the reduction failed to reach statistical significance. (Figure 21).

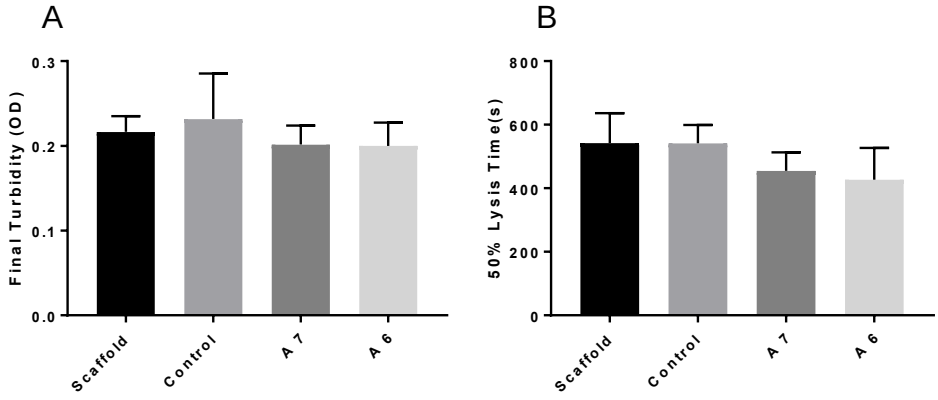

Figure 21. Effect of PI-binding Affimers on clot structure/fibrinolysis in plasma system

A Final turbidity B. Clot lysis. No significant differences were detected. All experiments were conducted in duplicate and repeated at least 3 times. Statistical analysis was performed using one-way ANOVA, comparing each Affimer to scaffold-only control.

3.6.1.2 Dose response of the Affimers (A7 and A6)

Given that only one concentration of Affimers A6&7 were used, I conducted a concentration-dependent experiment to ensure the absence of an effect (Figure 22).

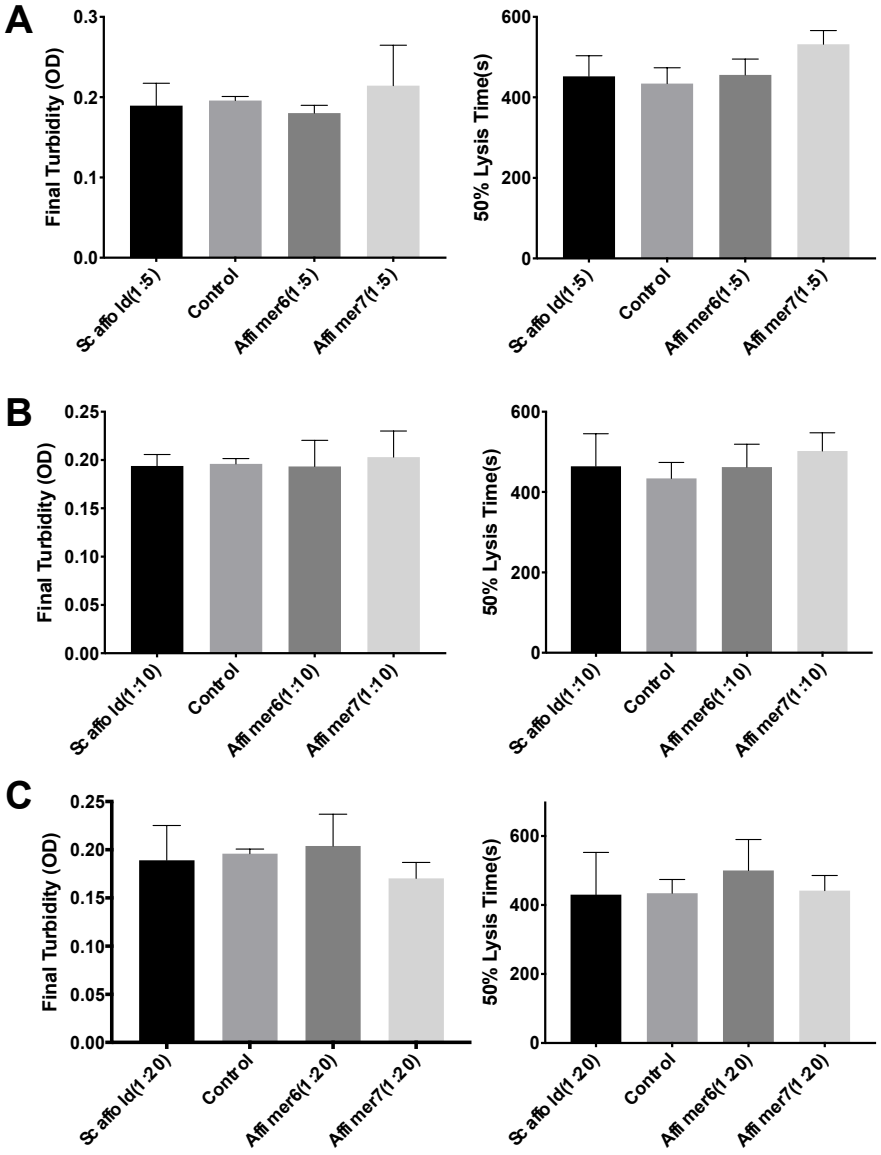

Figure 22. Turbidimetric assays different concentrations of PI-binding

Affimers (at 1:5, 1:10 and 1:20 PI:Affimer molar ratio, A, B, C, respectively). No significant differences were detected. All experiments were conducted in duplicate and repeated at least 3 times. Statistical analysis was performed using one-way ANOVA, comparing each Affimer to scaffold-only control.

3.7 Summary

Given the failure to show convincing differences in clot lysis with the 9 Affimers obtained from the first round of screening, it was decided to undertake a second screening round with a larger number of colonies picked. Also, a decision was made to test the Affimers in a purified system first before moving to plasma to ensure that any observed effect is PI-specific. Given the limited number of clones and the weak binding of single loop Affimers, these were not pursued any further.

3.8 Turbidity optimisation concentration (Purified)

In order to conduct experiments using purified protein, optimisation of turbidimetric experiments using plasma-purified fibrinogen was performed and results are shown below. The ratio of fibrinogen:PI remained largely physiological, although high concentrations of PI, up to 140µg/ml were also tested. Under these experimental conditions, the results showed that PI at a concentration of 35 µg/ml, resulted in a significant prolongation of clot lysis and, it was decided to use this concentration in purified experiments. (Figure 23)

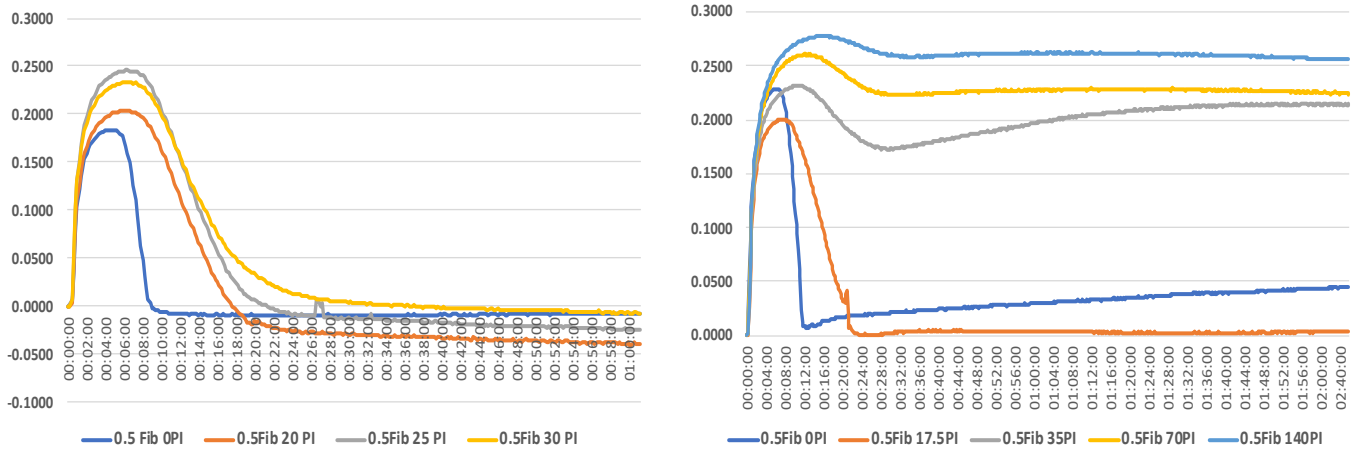

Figure 23. Effect of varying concentration of PI on clot lysis time using clots made from plasma-purified fibrinogen.
Fibrinogen was used at 0.5 mg/ml with the different concentrations of PI shown in the Figure above (all in µg/ml). Figure A shows experiments conducted with lower PI concentrations using stored protein, while Figure B demonstrates the effects of higher concentration, newly delivered PI.

3.9 Second screen (Double loop)

In the second screening, we decided to pick a much larger number of clones (a total of 144). Of the 144 clones, 16 had distinct sequences with two having a similar sequence to a clone from the first screen (which was not included in any further analysis).

AFFIMER	LOOP 1	LOOP 2
A1	VDGYWYDDE	FHYKFLWWP
A3	WQSSSGWFQ	FKRFLWFDP
A4	VLDSLMFDQ	FPWDTWLFN
A10	IHWTSHEQS	YSPAFMNSV
A25	LHWTSIEQY	HNAIFGWNA
A39	PYWTVYNQK	YAPAFKAAQ
A41	LYWDWQQQA	FYPAFTEQT
A55	IGWPWAEYQ	----------
A62	DVYTTIFEE	RPWDKFLFA
A68	RYNFMPEKY	QPQYHVEIV
A80	HLWIQHHQW	QSVGSLIPH
A109	MSASVYNWT	YSDAMSHKE
A111	PDMAWPPYW	VWFSRYDHE

Table 8 Amino acid sequences of the variable loops of the second screen.
A total of 13 Affimers were successfully sub-cloned and purified. A55 is a binder with a truncated second loop, which can occur during PCR amplification for cloning.

3.9.1 Sequence analysis of Affimers

Affimer variable regions (Table 8) were screened in NCBI BLAST similarly to the strategy adapted after the first screen (Table 9). Sequence homology was found between four out of nine amino acids in the variable loop 1 of Affimer A68 and plasminogen. No sequence homology was found between the remaining PI-binders and the protein.

Affimer	**Variable region**	**Proteins with sequence homology, with corresponding homologous region (amino acid sequence)**
A1	VDGYWYDDE	Coagulation factor 5/8 type [Amycolatopsis orientalis] GYWYD Coagulation factor V/VIII type [Amycolatopsis lurida NRRL 2430] GYWYD
	FHYKFLWWP	Fibrinogen-like protein 1 isoform X1 [Octopus vulgaris] YRFLWW Fibrinogen-like protein 1 isoform X2 [Octopus bimaculoides] YRFLWW
A3	WQSSSGWFQ	Coagulation factor V/VIII type domain protein [Paenibacillus sp. MT18] QSSGEWFQ Leukaemia inhibitory factor receptor alpha [Danio rerio] QSSSSWFH
	FKRFLWFDP	AMP-binding protein, partial [Streptomyces sp. uw30] RFLWFDP DUF2474 domain-containing protein [Rhizobiales bacterium] FKRFLWF
A4	VLDSLMFDQ	None
	FPWDTWLFN	None
A10	IHWTSHEQS	None
	YSPAFMNSV	Fibronectin/fibrinogen-binding protein [Jeotgalibacillus soli] YSPSFM Plasminogen receptor (KT) [Toxocara canis] YGPVYMNSV
A25	LHWTSIEQY	Plasminogen activator inhibitor 1-like precursor [Plutella xylostella] LNWTTIE

		Plasminogen activator inhibitor 1-like, partial [Plutella xylostella] LNWTTIEH
	HNAIFGWNA	Fibrinogen and keratin-10 binding surface anchored protein [Staphylococcus aureus] IFGWN
A39	PYWTVYNQK	Coagulation factor 5/8 type domain-containing protein [Lysobacter enzymogenes] PYWTV
	YAPAFKAAQ	None
A41	LYWDWQQQA	None
	FYPAFTEQT	None
A55	IGWPWAEYQ	None
A62	DVYTTIFEE	None
	RPWDKFLFA	None
A68	RYNFMPEKY	Coagulation factor VIII-like, partial [Scleropages formosus] YNF-PEK Plasminogen-like protein [Balearica regulorum gibbericeps] NF_PEKY Plasminogen [Pelecanus crispus] NFT_PEKY Coagulation factor V-like protejn [Pocillopora damicornis] YNFMP Coagulation factor VII isoform X3 [Grammomys surdaster] FMPDKY
	QPQYHVEIV	None
A80	HLWIQHHQW	Coagulation factor V [Ophiophagus hannah] HLWIQ
	QSVGSLIPH	None
A109	MSASVYNWT	Coagulation factor V isoform X1 [Oryzias latipes] SAS-YNWT
	YSDAMSHKE	None
A111	PDMAWPPYW	None

	VWFSRYDHE	Fibrinogen C-domain-containing protein 1-A [Drosophila willistoni] VWFAKYD

Table 9 Sequence homology between Affimers and PI or any coagulations factor.
Sequence homology was detected between at least 4 out of nine amino acids in the variable 2 loops of Affimers with PI and some coagulation factors.

3.9.2 SDS-PAGE analysis of Affimer

Similar to the Affimers in first screen, Affimers, SDS-PAGE gel depicted additional high molecular weight (MW) proteins in some selected Affimers (Normal MW of Affimers ~ 13kDa), suggesting impurities in these preparations that will require further investigation. (Figure 24)

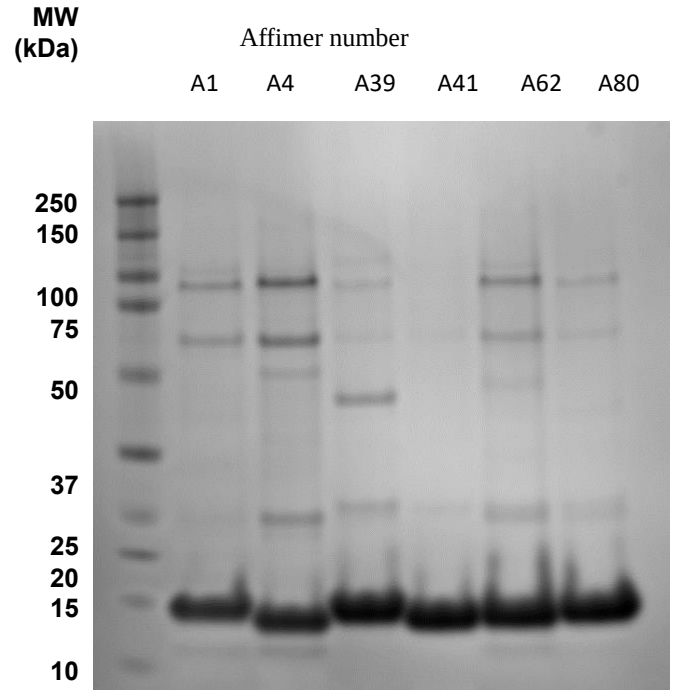

Figure 24. Molecular weight analysis of purified Affimers (1, 4, 39, 41, 62 and 80) on a 4-12% Bis-Tris gradient gel.
The Affimers molecular weight around 13KD.

3.9.3 Effect of new Affimers on clot lysis in a purified system

I used the following criteria to select Affimers of interest: i) A reduction of PI-induced prolongation of clot lysis by 15% or more was regarded as potentially important given our previous data showing that as little as 6% difference in clot lysis time can be clinically significant [164], ii) Affimer behaves similarly in plasma and purified systems and iii) Affimer does not induce significant changes to clot structure (assessed as alteration to final turbidity by more than 15%). A number of PI-binders demonstrated promising results as shown in Figure 25. Affimer A68 generated a particular interest given sequence homology with plasminogen, the significant reduction in clot lysis and the absence of an effect on clot final turbidity. Therefore, it was decided to study this Affimer in more detail with 3 additional Affimers that modulated lysis. Therefore, Affimer A68 together with A3, A55 and A111 were selected for subcloning, amplification, extraction and purification.

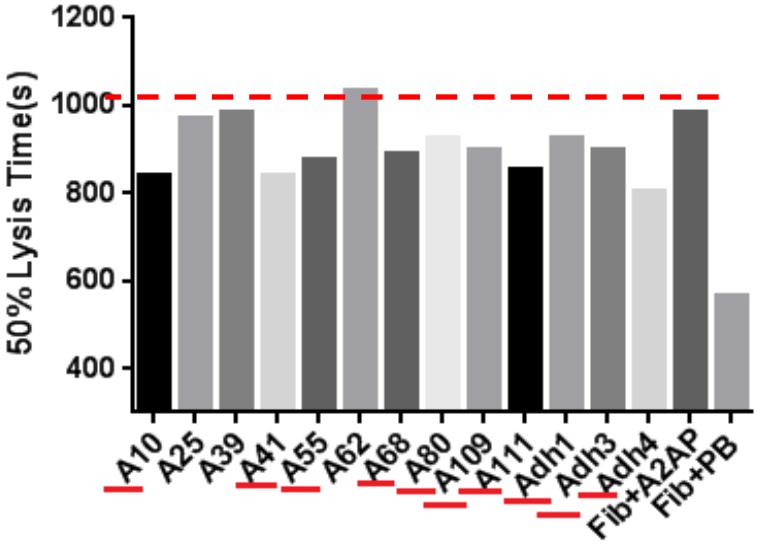

Figure 25 Effect of PI-binding Affimers on fibrinolysis in purified system
Affimers affecting lysis by more than 15% were regarded as potentially important and are underlined in red.

3.9.4 Effect of Affimer A68, A3, A55 and A111 on plasma clot lysis

I subsequently generated large quantities of 4 Affimers to test further (A68, A3, A55 and A111**)**. Using a plasma system, I employed two negative controls: scaffold only Affimer as well buffer only with no Affimer. Affimer A68 showed a reduction in plasma clot lysis time from 650±22.7 s (control) to 420±21.07 s (p=0.0017) without an effect on clot final turbidity. The numerical differences with additional Affimers (A3, A55 and A111) failed to reach statistical significance (Figure 26). However, we decided to study the effect of the rest of Affimers on clot lysis time in plasma system in case they behave differently in the presence of plasma proteins.

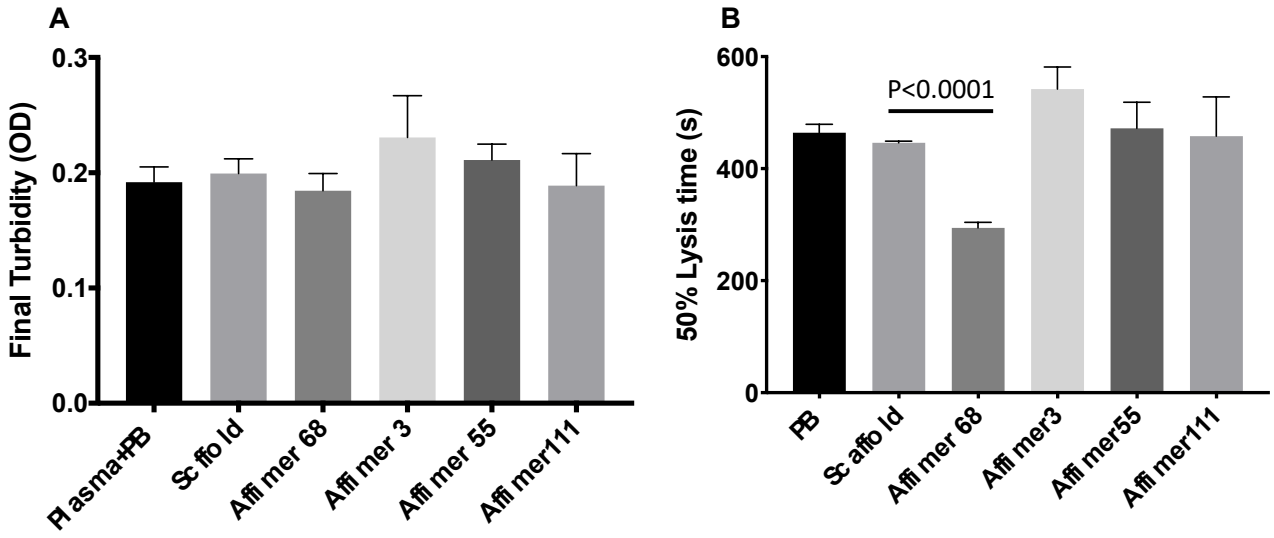

Figure 26. Effects of Affimers on clot structure and lysis time normalised to scaffold control.
A.Clot final turbidity: PI-binders A68, A3, A55 and A111 had no effect on plasma clot final turbidity. B Clot lysis time: Affimer 68 significantly decreased plasma clot lysis time by more than 35%. Experiments were done in duplicate and repeated on 6 occasions. Statistical analysis was performed using one-way ANOVA, ***P≤0.001 when compared with Scaffold control in post hoc pairwise analysis.

3.9.5 Effect of other Affimers from second screen

Affimers (1, 4, 10, 25, 39, 41, 45, 62, 80, and 109) were studied and showed no effect on clot lysis time or clot final turbidity in plasma system (Figure 27).

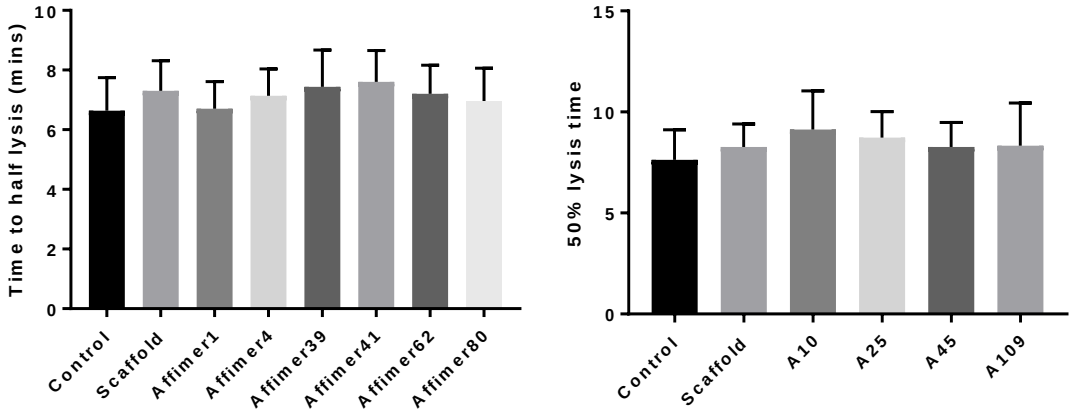

Figure 27. Effects of Affimers on clot formation/lysis time compared to scaffold control.
A Clot final turbidity: PI-binders (1, 4, 39, 41, 62 and 80) had no effect on plasma clot final turbidity. B Clot lysis time: all Affimers had no significantly decreased plasma clot lysis time. Experiments were done in duplicate and repeated on 3 occasions.

3.9.6 Effect of Affimer 68 on clot structure/lysis time

Affimer 68 affected the lysis time in the plasma as well as in the purified system. In both systems it showed a reduction in clot lysis time. The reduction in lysis time was from 650±22.7 (control) to 420±21.07 in the presence of Affimer A68 ($p=0.0017$) without a significant change in clot final turbidity, indicating that this Affimer is unlikely to be inducing major changes in fibrin network structure. Moreover, these results suggest that the effect on lysis time are not related to changes in fibrin network structure. (Figure 28).

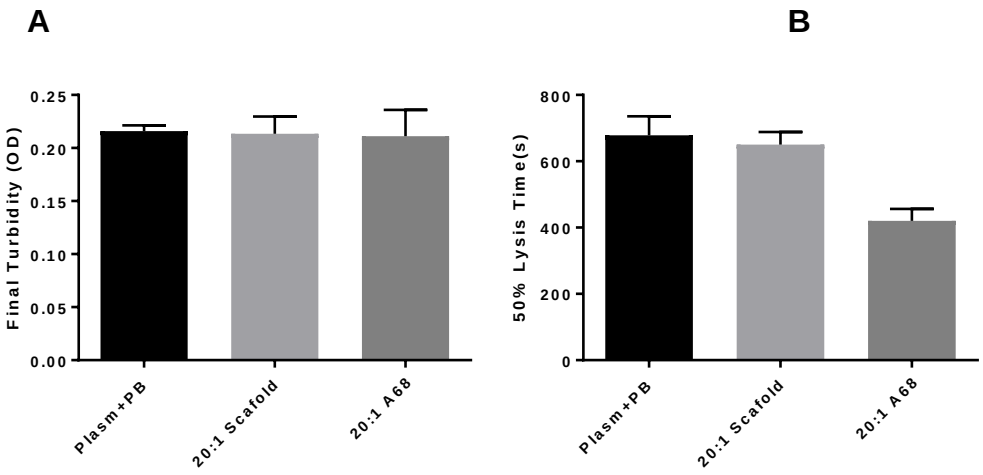

Figure 28. Effects of PI-binders on clot lysis.
A Turbidimetric assay of PI-binder Affimers 68 in plasma, showing similar final turbidity with the scaffold protein. B Affimers 68 significantly decreases 50%lysis time from 650±22.7 in the control to 420±21.07 (p=0.0017). All experiments were done in duplicate and repeated at least 3 times.

3.9.7 Effect of Affimer 68 on purified system

The effects of Affimer A68 was further tested in a purified system using PI concentrations of 35 µg/ml, showing clear inhibition of PI-induced prolongation in clot lysis. Time to 50% lysis could not be calculated (as the clot did not lyse in the presence of PI alone) and therefore lysis curves are shown in Figure 29

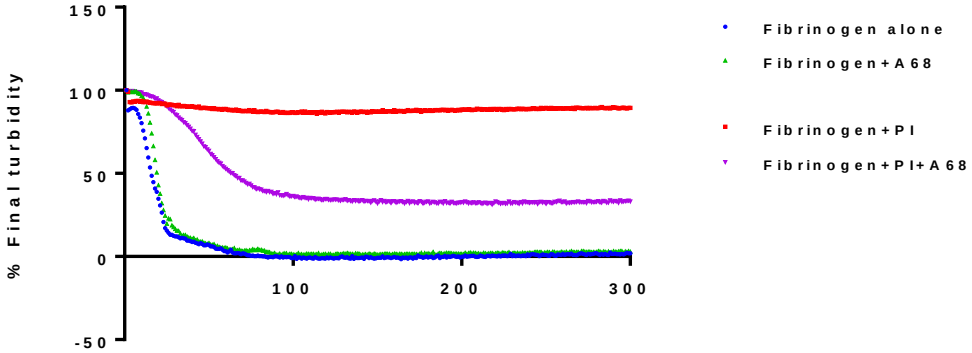

Figure 29. Effect of PI-binder A68 in purified systime.
Turbidimetric assay was conducted in the presence of higher PI concentration at 35 µg/ml. Fibrinogen was used at 1 mg/ml with PI:A68 molar ratio of 1:20.

3.10 Effect of all Affimers from first and second screening on plasma clot lysis

Using A68 as positive control to study the effect of the additional Affimers on clot lysis time on plasma system with a present of Scaffold as a negative control. Only A1.2 showed a small reduction in lysis time compare to Scaffold that lead to study the effect of A1.2 in more details (Figure 30).

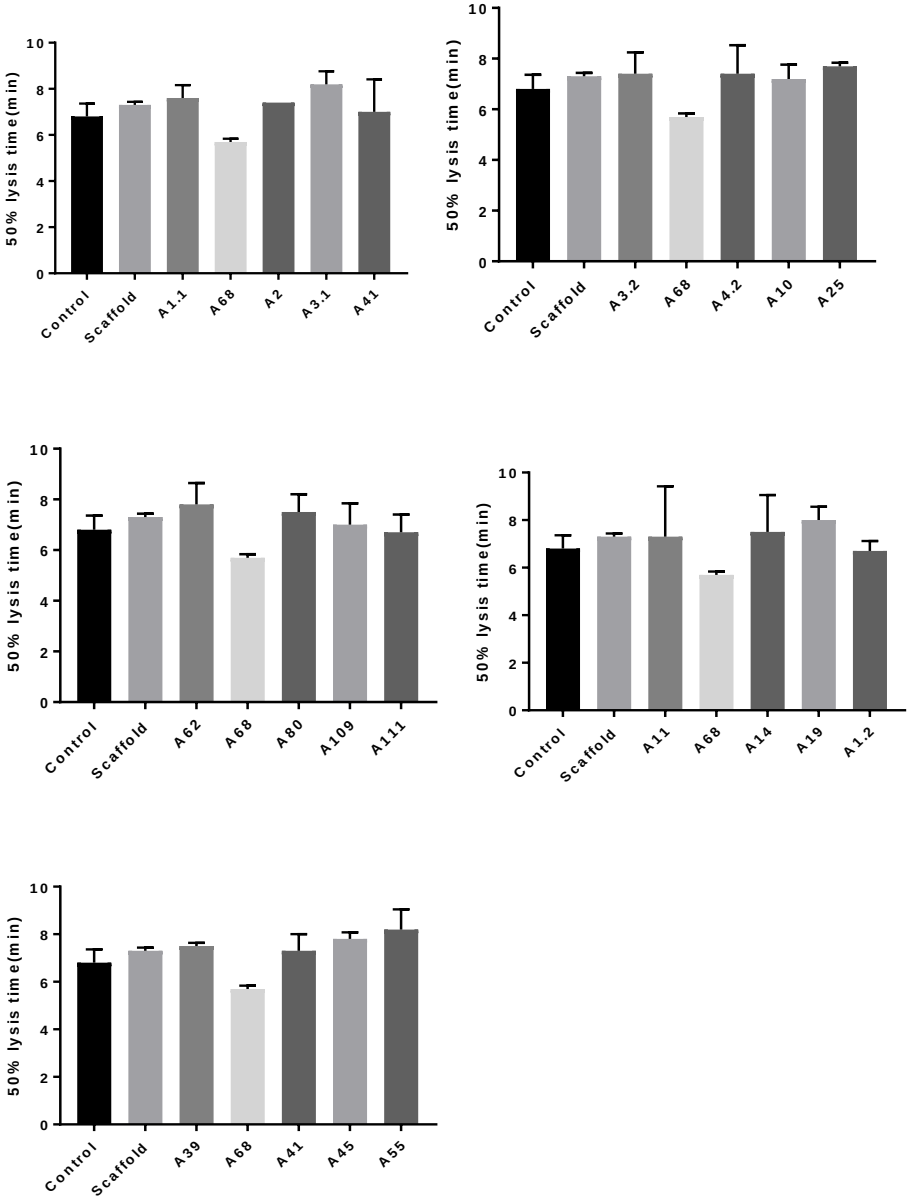

Figure 30. Effects of Affimers on clot lysis time on plasma system.

No difference was noted in lysis time compared with scaffold and PB as negative control, and A68 as positive control.

3.11 Discussion

Impaired fibrinolysis is associated with increased risk of cardiovascular events and manipulating clot lysis may offer an alternative therapeutic strategy to reduce the risk of atherothrombosis [258]. However, current modalities for long-term modulation of fibrin clot formation/lysis, such as the use thrombin and FX inhibitors [259], are too powerful and focussed on inhibiting clot formation altogether thus carry increased risk of bleeding. In contrast, my work employs a different approach by attempting to reduce the incorporation of PI into fibrin clots, consequently allowing the formation of the fibrin network while gently facilitating lysis, thus potentially having a lower risk of bleeding. Previous in vitro and in vivo work used PI-specific monoclonal antibodies to improve fibrinolysis [215] and a clinical trial was under way aiming to explore the use of such antibodies in patients with pulmonary embolism, which seems to have been withdrawn for reasons that are unclear (ClinicalTrials.gov; NCT03316729)). However, long-term use of this therapy may be problematic due to the high costs associated with the production of monoclonal antibodies and the premature termination of the study suggests off-target effect, although no details have been released to date and therefore this remains speculative.

My work has so far shown that Affimers against PI can be used as a tool to facilitate clot lysis, providing an alternative to monoclonal antibodies. Of the many PI binding Affimers isolated (total of 24 Affimers with distinct sequences), one showed inhibition of protein action (Affimer A68) thus facilitating fibrin clot lysis. Experiments using purified protein confirmed that the effects of A68 are PI-specific further supporting the validity of this approach.

In the first screening process, I failed to identify PI-binding Affimers that modulate fibrinolysis, which is probably due to the limited number of clones picked. Only one Affimer (A3), showed reduction in final turbidity when compared to the scaffold protein but had no measurable effect on clot lysis. The lack of an effect on lysis time was consistent using purified experiments or plasma samples.

In a second round of screening, a larger number of clones were picked, and this resulted in the identification of at least one PI-binding Affimer that consistently modulated fibrin clot lysis in both plasma and purified systems.

As for screening the single loop library of Affimers, these showed relatively weak binding and Affimer diversity was poor. Neither of the two Affimers showed a

significant effect on clot turbidity or lysis time. Therefore, it appears that the two loops are needed for high affinity binding and therefore the approach with single loop Affimer is not a viable strategy, at least for identification of PI-binders.

My data so far provide a proof of concept demonstrating that Affimers can be used for PI-targeted modulation of fibrinolysis. This opens the door for a new methodology to manipulate thrombosis risk with potentially exciting clinical applications. Affimer A68 reproducibly altered fibrin clot lysis without having an effect on clot turbidity suggesting no significant changes in clot structure. However, this will need to be confirmed using imaging studies employing confocal microscopy to study structure of the hydrated clot and electron microscopy to investigate clot ultrastructure in order to ensure that the architecture of the fibrin fibres is not altered. It is worth noting that one of the loops (Loop1) in Affimer A68 showed sequence homology with plasminogen, making this a particularly an interesting Affimer to investigate in detail.

In conclusion, my data so far demonstrate that Affimers represent a viable technique to modulate fibrin clot lysis by targeting a specific pathway in fibrin network resistance to lysis. This may offer future alternative treatment strategies either by directly using Affimers or through identification of novel therapeutic targets for small molecule intervention.

Chapter 4 : Plasmin inhibitor specific Affimer for modulation of fibrin clot lysis

4.1 Introduction

The previous chapter was mostly intended to assess the validity of a phage display library as a method for isolating PI-specific Affimer(s), which could modify clot formation and breakdown. The data presented in the previous chapter show that it is indeed possible to isolate PI-specific Affimers that modulate fibrin clot lysis without introducing significant changes to clot structure, as assessed by the simple turbidimetric assay.

Inhibition of PI-induced prolongation of clot lysis represents a strategy that has the potential to reduce thrombosis risk while keeping bleeding risk to a minimum. This may be a particularly effective approach in cases with increased PI incorporation into clots such as individuals with type 1 diabetes [109]. An increase in PI incorporation into clots has also been demonstrated in individuals with type 2 diabetes and, interestingly, PI quantity in the clots showed a positive correlation with glycaemic control, measured as HbA1c [4].

Previous studies have implicated PI not only in arterial disease but also in venous thrombosis and embolization, conditions where the thrombus is rich in fibrin [260]. An experimental model where acute PE was induced in mice deficient in PI showed reduced mortality rate when compared with wild type counterpart following thrombolytic therapy. This suggests that inhibition of PI, along with thrombolytic therapy, may be important in the treatment and management of thrombotic vascular occlusion [86]. However, it is important to understand that normal function of PI is imperative for maintenance of haemostasis and absence of PI can lead to severe haemorrhagic and life-threatening bleeding as seen in rare cases of congenital protein deficiency [261]. These findings also suggest that full inhibition of the antifibrinolytic effects of PI may be detrimental through increasing the risk of serious bleeding events. Therefore, while PI represents a credible therapeutic target, caution should be exercised at achieving therapeutic inhibition of action without causing unwanted side effects. Taken together, current data suggest that partial inhibition of PI activity could be more desirable clinically than full suppression of protein function in order to avoid unwanted bleeding events.

In the previous Chapter, I demonstrated that one of the PI-binding Affimers, Affimer A68, modulates PI-induced prolongation of clot lysis and reduces plasma clot lysis time. However, pervious work was performed on pooled plasma samples and using mainly one concentration of the Affimer. Also, given the use of pooled plasma samples, inter-individual variability in the effects of A68 remains unknown. Moreover, studies have been conducted on acellular systems and it remains to be seen whether the effects of the Affimer on clot lysis are maintained in the presence of blood cells. In addition, while turbidimetric assays suggest that Affimer A68 has little effect on clot structure, this needs to be confirmed using imaging techniques. Finally, the work described in the previous Chapter has shown multiple bands for Affimer A68 on SDS-PAGE. This may be due to impurity or the formation of Affimer dimers/multimers of A68 (Affimers are known to dimerise) and requires further investigation. Therefore, the aims of this Chapter were: i) investigate the effects of different concentrations of Affimer A68 on clot lysis in purified and plasma systems, ii) study inter-individual variability in response to A68 using healthy control plasma and also samples from individuals at high vascular risk, iii) investigate potential interference of blood cells in the fibrinolytic response of Affimer A68, iv) analyse the effects of Affimer A68 on clot structure using confocal microscopy and scanning electron microscopy (SEM) to make sure the Affimer does not introduce unwanted changes in clot structure and v) investigate the nature of high molecular weights products associated with A68.

4.2 Methods

4.2.1 Mass spectrometry

To investigate potential impurities in Affimer A68, mass spectrometry was used to clarify the nature of the additional HMW bands seen on SDS-PAGE. All samples were sent to a facility in Cambridge, which provides a MS service.

4.2.2 Turbidimetric analysis

Turbidimetric analysis was used to investigate the role of A68 on plasma clot lysis. These were performed as described in Chapter 2 following the addition of A68 to plasma and incubating for 30 mins before undertaking the assay. To investigate whether the effects of A68 was PI specific, a purified system was employed, fibrinogen

was added to A68 that had been pre-incubated with PI (35μM), as described in Chapter 2.

4.2.3 Rotational thromboelastometry (ROTEM) assays

To investigate the potential effects of blood cells on the fibrinolytic response to A68, ROTEM was used, which is described in detail in Chapter 2. Briefly, blood samples were collected from healthy volunteers after informed consent and incubated with Affimer A68 for 15 minutes. Blood was placed into a cup; tissue factor was added to start blood clotting followed by the addition of tPA to allow the clot to lyse.

4.2.4 Laser scanning confocal microscopy

To investigate the effect of A68 on structure of hydrated clots, confocal microscopy was employed. Clots were made using normal pooled plasma, as described in the method section.

4.2.5 Scanning electron microscopy (SEM)

To analyse the effects of A68 on fibrin network ultrastructure, plasma clots were prepared for SEM as described in the methods section in Chapter 2.

4.3 Results

4.3.1.1 Affimer integrity and purity

Affimer A68 was produced as described in Chapter 3 and analysed by SDS-PAGE to confirm integrity and purity of the protein. In addition to the expected band of approximately 13 kDa, Affimer A68 showed high molecular weight (HMW) products. The additional protein bands were in multiples of the normal Affimer MW (26kDa, 39kDa, 65kDa, etc,). Thus, this suggested that these protein bands corresponded to the multimers of the Affimer. The extra band of A68 at ~ 26 kDa was detected with the antibody indicating it is an Affimer dimer (Figure 31).

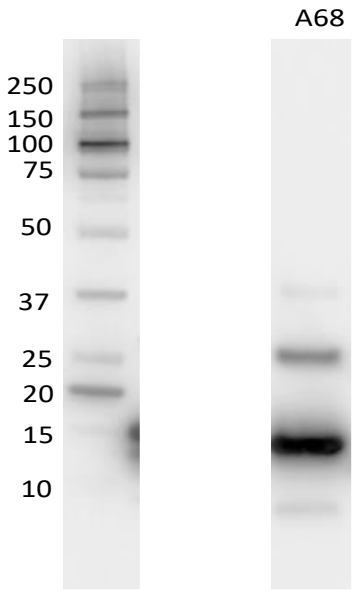

Figure 31. Western blot
using -mouse monoclonal anti-his antibody (Roche 11922416001, clone BMG-His-1) dilution 1:200, 1hour incubation. -secondary antibody: rabbit anti-mouse-HRP (dako P0260) dilution 1:1000, 1hour incubation.

4.3.1.2 Mass spectrometry (MS) to identify the extra bands

MS was performed to identify the high molecular weight extra bands. Affimer A68, and PI were run in SDS-PAGE. The areas of the gel corresponding to the extra bands were cut and sent for mass spectrometry analysis. Figure 32 shows the bands ‘’Sample A’’ (~60kDa) and ‘’Sample B’’ (~50kDa) in lane 1 (A68) and PI in lane 2. An additional immunoblot showing that Affimer A68 26 kda product is picked up with anti-His (Figure 32) and therefore that band is an Affimer dimer and does not need to be investigated further.

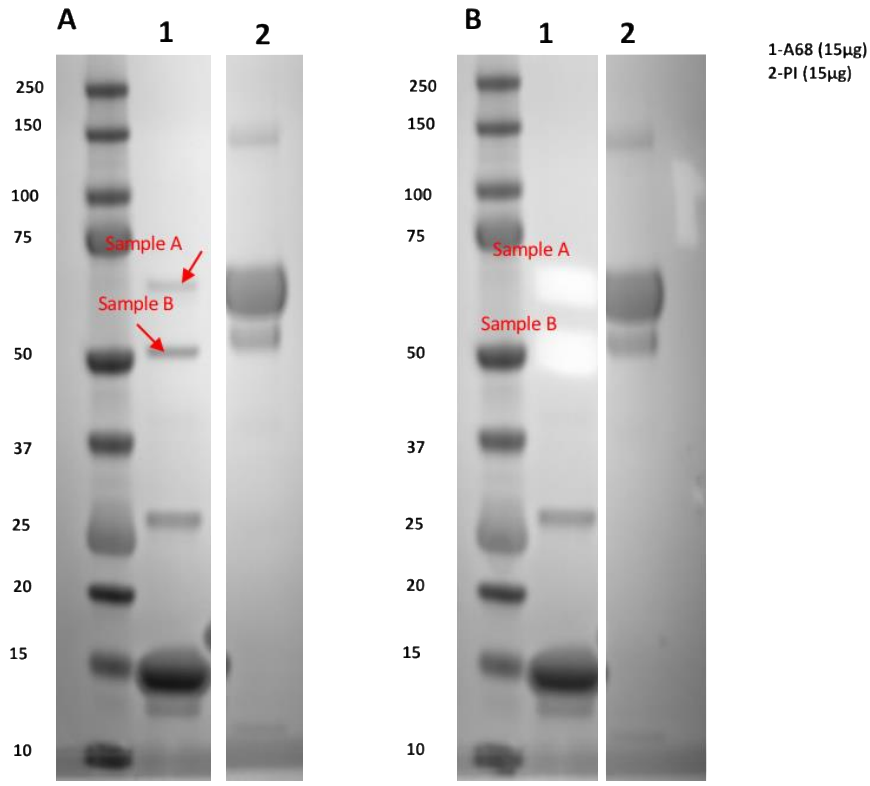

Figure 32: A SDS-PAGE of Affimer A68 and PI.
The following bands were identified (arrows, left) and then cut from the gel (right): i) "sample A'' (~60kDa), "sample B"' (~50kDa) in lane 1 (A68), '. PI was run on lane 2 as a positive control.

These gel bands were identified by Mass spectrometry in Cambridge as shown in Table 10. The proteins shown below are potential matches to each extra protein band. Some suggested proteins that are unlikely to present in the sample (as they do not correspond to the MW on the gel) were excluded. Cystatin appears to be a potential specific protein in these bands, suggesting the HMW is multimer of the Affimer. However, tubulin was also picked up suggesting contamination by *E coli* protein.

A68_ band A ~60kDa	Desmoplakin (Mass: 334021, score: 116) Tubulin alpha-1C chain (Mass: 37312, score: 72) Junction plakoglobin (Mass: 82434, score: 55)
A68_ band B ~ 50kDa	Glial fibrillary acidic protein (Mass: 57496, score: 140) Cystatin-A (Mass: 7082, score: 72) Protein Shroom 3, Desmoplakin, Myosin-7B

Table 10. Protein identification by Mass spectrometry
The three proteins with the highest score for each gel band analysed by mass spectrometry.

4.3.1.3 Binding of Affimer A68 to PI

To ensure specificity of A68 to PI and rule out non-specific binding by Affimer scaffold, comparisons were made between A68 and various controls (SQT, SQT Gly and no Affimer) in relation to PI binding using ELISA. SQT is the scaffold protein without the variable loops and SQT Gly is another control molecule which is identical to SQT but has two loops containing 9 Gly peptides. The plate was coated with PI (apart from the positive control which was coated with Affimer), blocked and then Affimer A68 was added, incubated with primary mouse monoclonal anti-his antibody (clone BMG-His-1, Roche) in a dilution of 1:200, then secondary antibody, rabbit anti-mouse-HRP (dako P0260) in a dilution of 1:1000 (Figure 33).

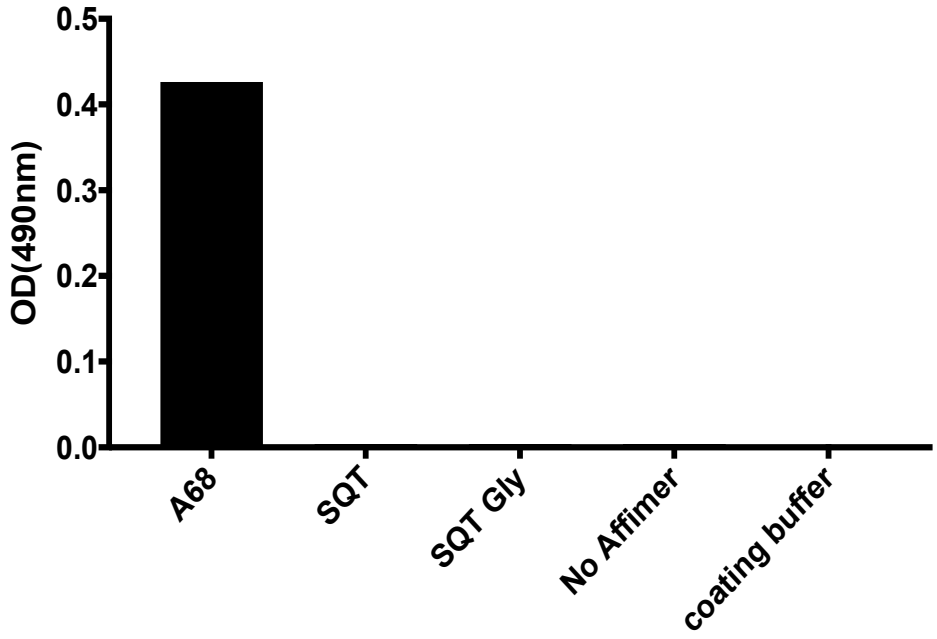

Figure 33. ELISA- Binding of Affimer to PI
demonstrated a good binding signal of A68 and fractions of A68. In contrast, the two controls, SQT and SQT Gly, showed no binding to PI.

4.3.2 Effects of different A68 concentrations on clot lysis

In the previous chapter, using purified proteins and plasma systems, Affimer A68 showed no effect on clot final turbidity but increased efficiency of clot lysis. However, a single Affimer:PI molar ratio was used and the effects of different concentrations of this Affimer on clot final turbidity (a crude marker of clot structure) and lysis time have not been established. Therefore, in the current Chapter, I investigated the effects of different A68 concentrations on clot turbidity and lysis using both purified proteins and plasma systems.

4.3.2.1 Purified proteins

Turbidimetric assays were performed in plasma and purified systems using different molar ratios of A68:PI (1:1, 2:1, 4:1, 8:1, 16:1 and 32:1). Fibrinogen was added to A68 that had been pre-incubated with PI (35μM) and results are shown in Figure 34.

Affimer A68 reduced clot lysis in a purified system in a concentration-dependent manner from (1140±84) to (960±0), (822±54), (714±6), (732±12), (600±24) and

(534±6) minutes at 0:1, 1:1, 2:1, 4:1, 8:1, 16:1 and 32:1 Affimer:PI molar ratio, respectively (Figure 34). The difference in clot lysis was statistically significant with all Affimer:PI ratios used except 1:1 (Figure 34). None of the concentrations used showed a significant effect on clot final turbidity.

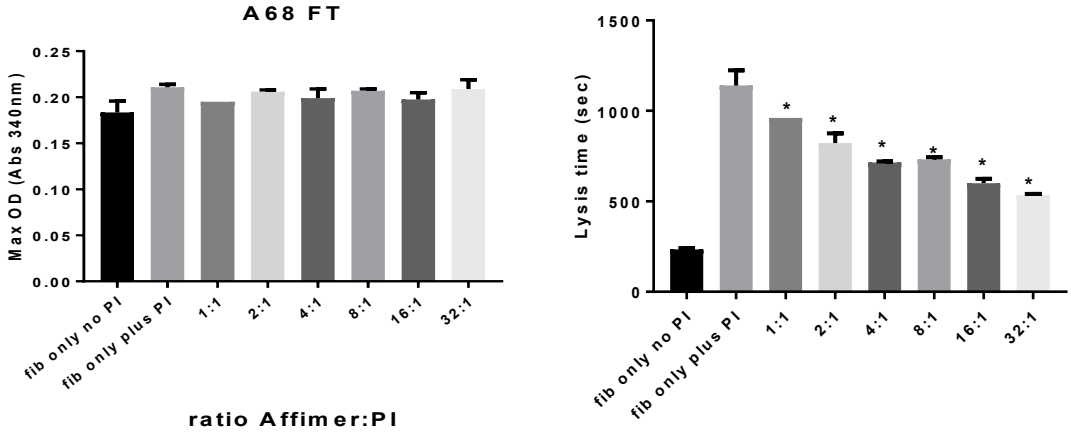

Figure 34. Effects of A68 on fibrin clot maximum absorbance and lysis time
A dose-dependent enhancement in clot lysis is demonstrated without an effect on FT. PI: plasmin inhibitor (14.3μM), fib: fibrinogen, FT: final turbidity. Numbers represent Affimer:PI molar ratios. *p<0.05

4.3.2.2 Plasma systems

The consistent reduction in lysis time by A68 was observed in clots made from pooled heathy control plasma in a dose-dependent manner (1:1, 2:1, 4:1, 8:1, 16:1 and 32:1) of A68:PI ratio. The addition of A68 in different concentrations to pooled healthy control plasma facilitated clot lysis compared to scaffold from (9.86±0.65) to (8.5±0.66), (7.42±0.76), (7.45±0.72), (6.67±0.65), (5.92±0.2) and (6.35±0.25) minutes at 0:1, 1:1, 2:1, 4:1, 8:1, 16:1 and 32:1 Affimer:PI molar ratio, respectively (Figure 35). The effect of A68 with ratio 1:1, 2:1 and 4:1 were not significant in contrast to 8:1, 16:1, and 32:1, which is not dissimilar compared with the purified system, indicating that plasma proteins have minimal interference with modulation of clot lysis by Affimer A68. Again, clot final turbidity was not affected, even when very high Affimer-PI molar ratio were used, suggesting that A68 has no significant effects on clot structure (Figure 35).

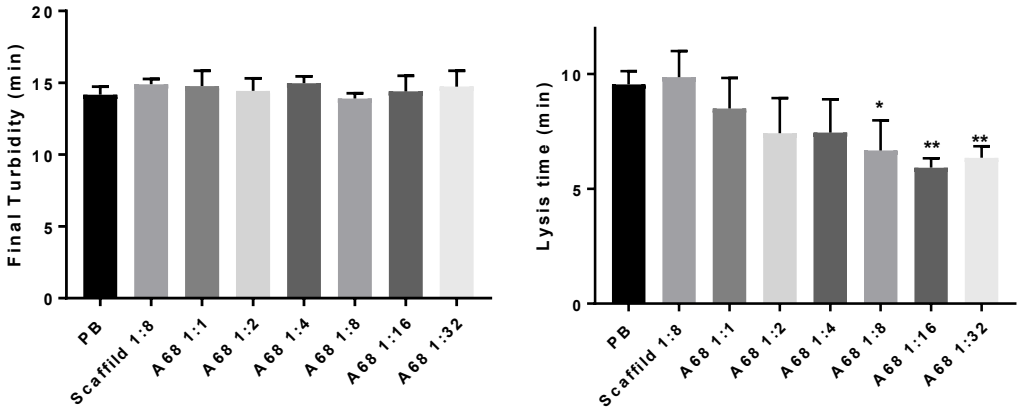

Figure 35. Effects of A68 on fibrin clot maximum absorbance and lysis time in plasma

PB: permeation buffer. Numbers represent molar ratio of Affimer:PI. Experiments were conducted in duplicate and repeated three times. Data presented as mean±SD. *p≤0.05 or **p≤0.04 Statistical analysis was performed using one-way ANOVA, comparing each Affimer to scaffold-only control.

4.3.3 Inter-individual variability in the fibrinolytic effect of Affimer A68

4.3.3.1 Type 1 diabetes T1DM

The effects of Affimer A68 on fibrinolysis were also investigated in 12 individual plasma samples from patients with type 1 diabetes. A68 reduced clot lysis time in 11 individual sample and with no effect observed in a single sample. Mean± SEM reduction in lysis time was from 9.15±0.55 min to 6.75±0.39 min (p=0.0017) in the presence of Affimer A68 (Figure 36).

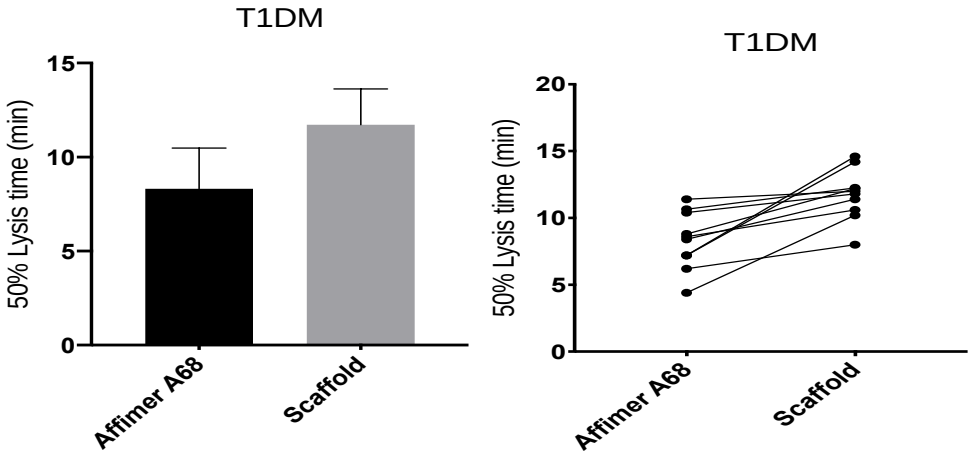

Figure 36. Effects of A68 on plasma lysis of clots formed from individual plasma samples from patients with diabetes
Affimer:PI molar ratio used was approximately 5:1 assuming concentration of PI is approximately 1 µM. Experiments were conducted in duplicate on a single occasion.

4.3.3.2 Type 2 diabetes (T2DM)

Affimer A68 was used in turbidimetric assays in 11 individual plasma samples from patients with type 2 diabetes to assess their effect on clot properties (Figure 37). Originally, 12 samples were chosen but one sample failed to show any reaction (i.e. was pre-clotted) and was therefore discarded. Affimers A68 significantly reduced clot lysis time compared with scaffold in all 11 samples from a mean of 10.15±0.64 to 7.69±0.41 minutes (p=0.004; Figure 37).

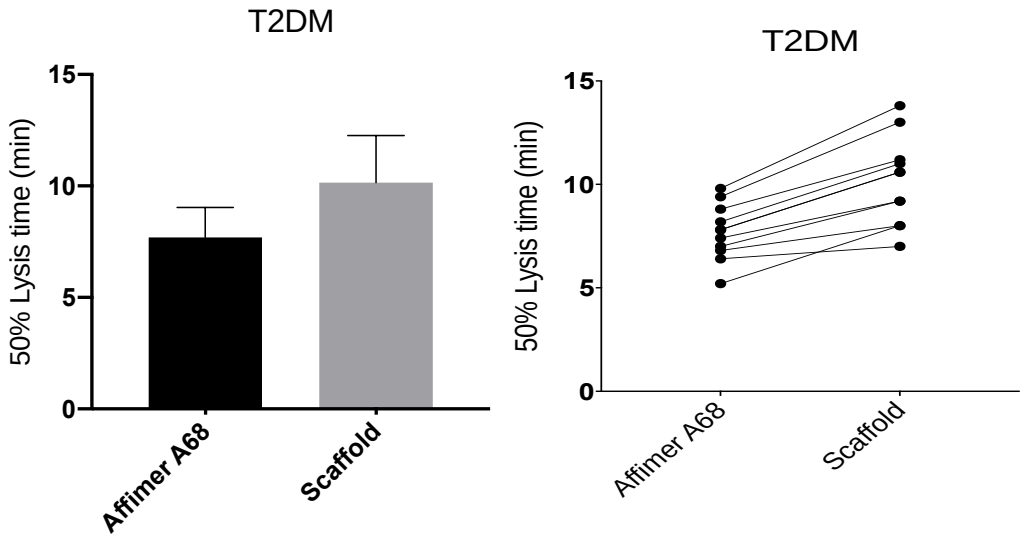

Figure 37. Effects of A68 on plasma lysis of clots formed from individual plasma samples from patients with type 2 diabetes
Affimer:PI molar ratio used was approximately 5:1 assuming concentration of PI is approximately 1 µM. Experiments were conducted in duplicate on a single occasion.

4.3.3.3 Cardiovascular disease (CV)

The effects of Affimer A68 on fibrinolysis were also investigated in 12 individual plasma samples from patients with cardiovascular disease. The reduction time in half lysis time of the mean effect of these sample from 13.20±0.76 to 9.63±0.82 minutes (p=0.004; Figure 38).

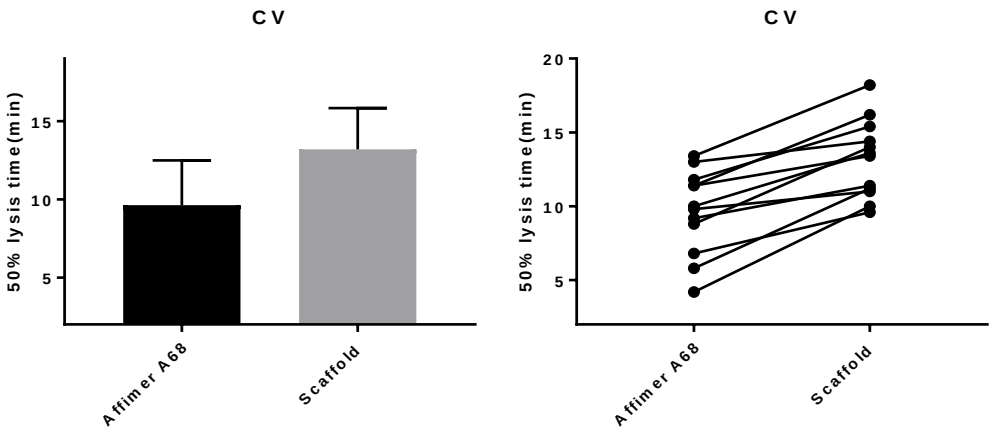

Figure 38 Effects of A68 on plasma lysis of clots formed from individual plasma samples from patients with cardiovascular disease.

Affimer:PI molar ratio used was approximately 5:1 assuming concentration of PI is approximately 1 µM. Experiments were conducted in duplicate on a single occasion.

4.3.4 Study the effects of Affimer A68 on clot structure

Affimer A68 had no effects on clot final turbidity suggesting the absence of significant changes in clot structure. However, this is a relatively crude method to study fibrin network structure and therefore imaging was performed using confocal and scanning electron microscopy. The former was employed to study the general structure of hydrated clots, whereas the latter investigated the ultrastructure of the fibrin network, including fibre thickness, of dried clots.

4.3.4.1 Confocal microscopy

Plasma clots were prepared for confocal microscopy imaging in the presence of increasing concentrations of A68. Scaffold Affimer was used as a control and this showed that Affimer A68 maintains physiological clot structure regardless of the Affimer concentration used (Figure 39).

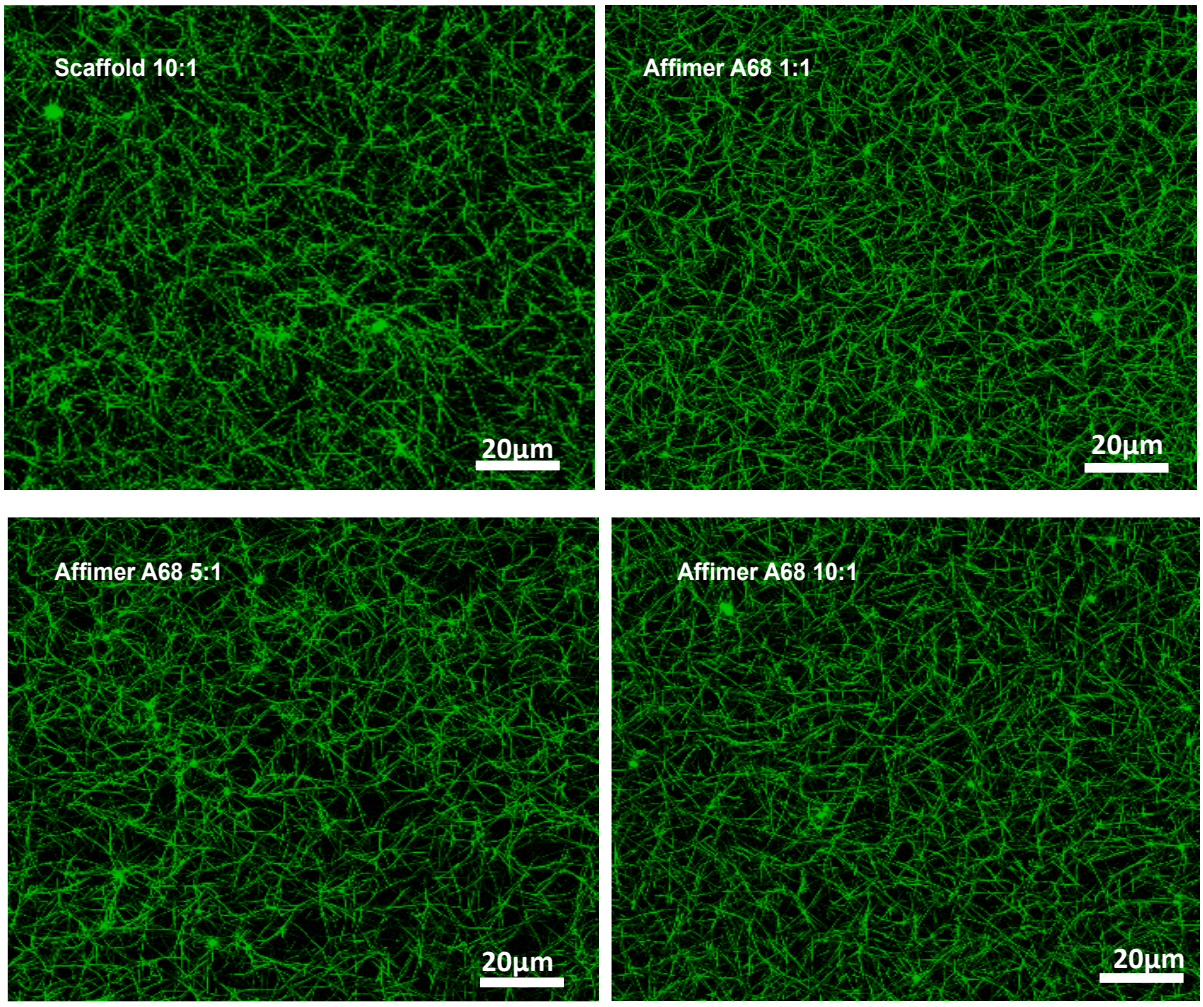

Figure 39. Effects of A68 on clot structure using confocal microscopy with Pixel intensity.
A possible increase in fibrin network density is evident in the presence of Affimer A68 but physiological fibrin network structure is maintained with all molecular ratios of Affimer:PI.1, 5:1 and 10:1 Affimer:PI molar ratio), Control was scaffold. Statistical analysis was performed using one-way ANOVA, comparing each different concentration of Affimer A68 to Scaffold. Scale bar = 20µm

4.3.4.2 Scanning Electron Microscopy (SEM)

SEM images are used to study clot ultrastructure. This showed that A68 did induce changes to the fibrin network that were not visible by confocal microscopy. More specifically, decreased fibre thickness was observed in the presence of A68 starting from 5:1 and increasing in 10:1 compared to 10:1 ration in scaffold ($p<0.001$) (Figure 40).

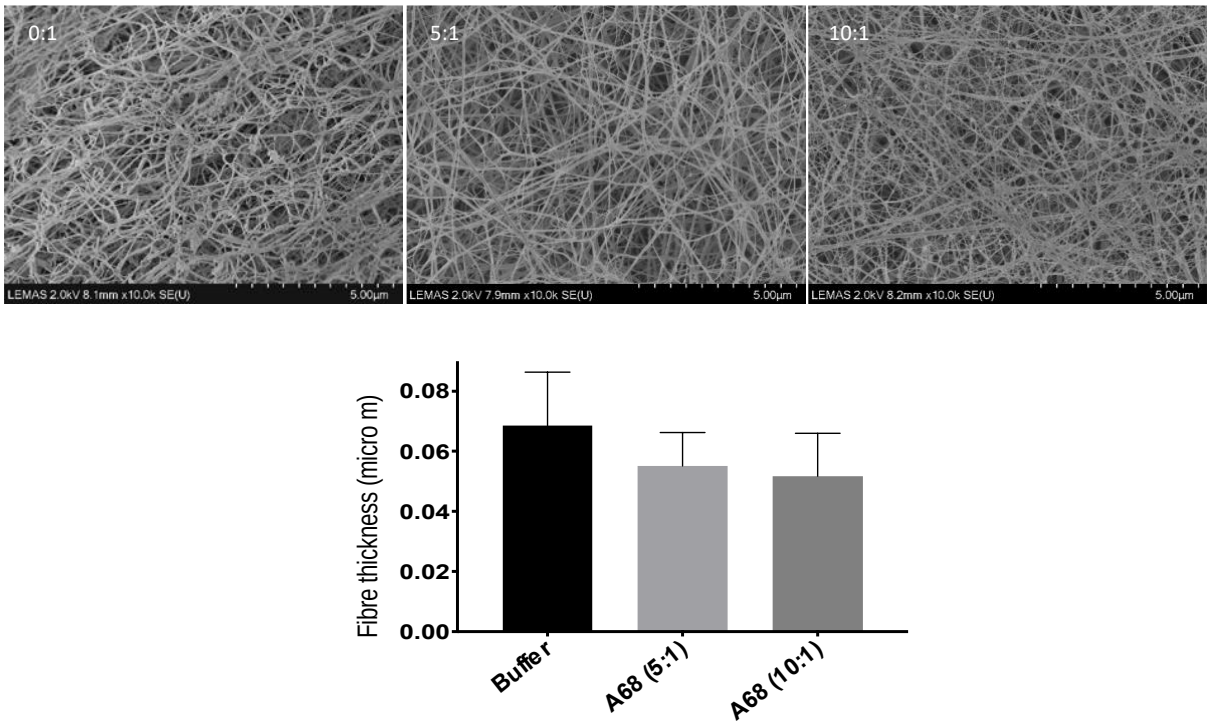

Figure 40. SEM images of plasma clots formed in the presence of different concentration of Affimer A68
Clots prepared with Affimer A68 in different concentration compared with EM image of plasma. Affimer A68 was incubated with plasma at 5:1, and 10:1 Affimer:PI ratio. Plasma sample receiving buffer only was incubated as control. A Representative SEM image of clot formed in the presence of Affimer A68. B mean fibre thickness (µm) of fibres in SEM images. To determine fibre thickness, 40 random fibres were measured in each area and results are presented as mean±SD.

4.3.5 Role of A68 in reducing PI incorporation into clots (confocal)

To clarify whether the effects of A68 are due to incorporation of the Affimer into the fibrin network, confocal microscopy was used. In this experiment, clots were made from pooled normal plasma. Fibrinogen was labelled with Alexa Fluor (red) and 5% added to the mixture while PI was labelled green. The results of this experiment are presented in Figure 41 demonstrating that Affimer A68 reduces PI incorporation into the clot compared with scaffold protein.

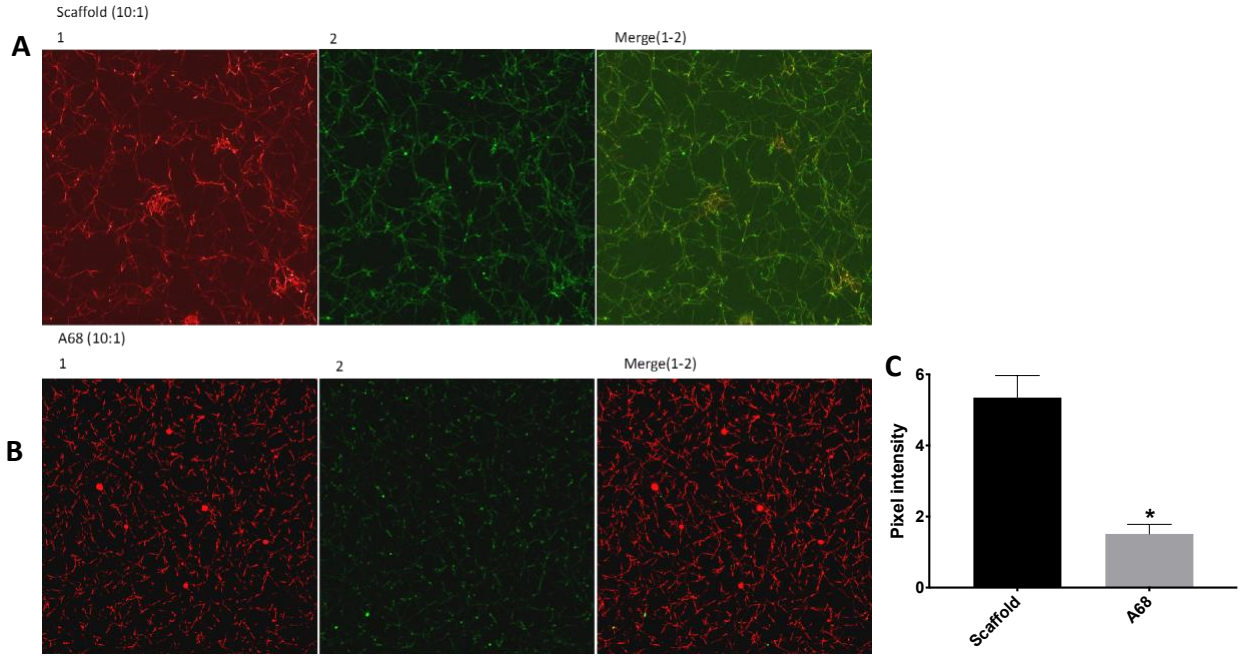

Figure 41. Confocal microscopy to analyse incorporation of PI into the clot. This demonstrates the effects of scaffold protein on PI incorporation the fibrin network (fibrinogen and PI are labelled red and green, respectively. B. This shows the effects of A68 on incorporation of labelled PI into the clot. Decreased green intensity represents decreased PI incorporation. C Mean±SEM pixel intensities of clots seen in No 2 in each A and B. *p<0.01. (-3.831 ± 0.4797). Similar results were obtained in 3 independent experiments.

4.3.5.1 Effect A68 on lysis of clots made from whole blood

In order to rule out interference in Affimer A68 activity by blood cells, I analysed lysis of clots made from whole blood samples in the presence and absence of Affimer A68. An enhancement in clot lysis was noted in the presence of A68, employing ROTEM assay. This demonstrates that blood cells do not influence the fibrinolytic effect of this Affimer. Figure 42 show Affimer A68 causing a significant enhancement in clot lysis in a concentration -dependent manner. Lysis time was reduced from (2572±200) sec in the absence of A68 to (1975±154), (1799±140), and (1800±131) sec at molar ratios of (1:1), (5:1), and (10:1), respectively ($p<0.01$ for all concentrations).

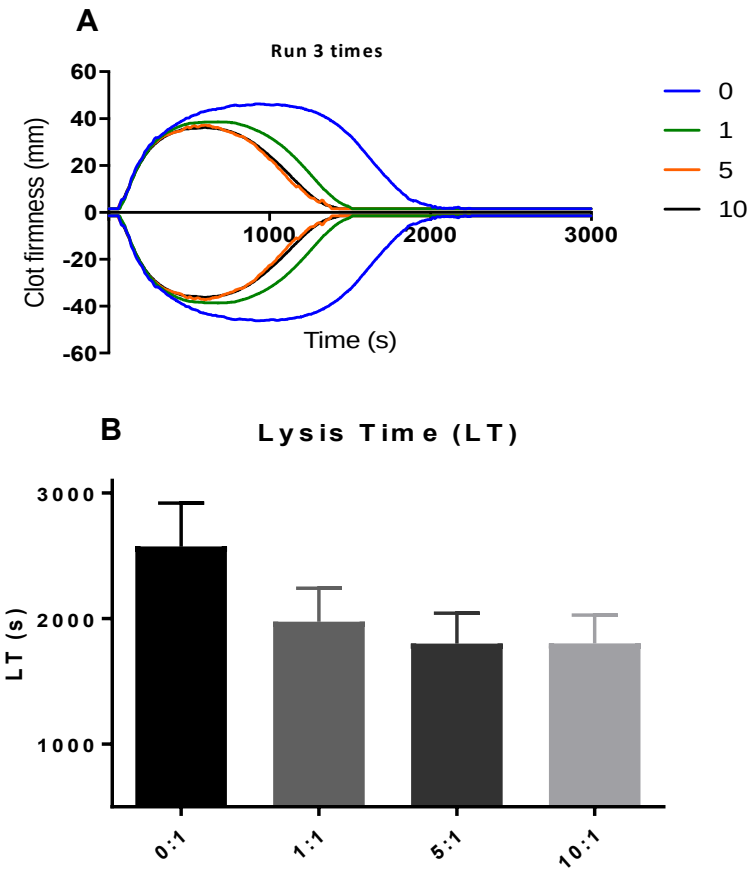

Figure 42. Effects of A68 on lysis of clots made from whole blood samples showing a concentration-dependent enhancement in clot lysis time.
A. Clot formation and lysis curves are shown in the presence of scaffold only Affimer (blue, used at 10:1 Affimer scaffold:PI molar concentration) or increasing molar concentrations of Affimer A68:PI ratio at 1:1 (green) 5:1 (orange) or 10:1 (black). B Lysis time calculated as time from full clot formation to 50% lysis. Experiments were repeated 3 times using 3 different blood donors.

4.3.5.2 Real time clot lysis using confocal microscopy

Turbidimetric experiments examine clot lysis during clot formation. To study the effects of Affimer A68 on clot lysis of fully formed clots, confocal microscopy was employed. This test serves a dual purpose: first it confirms the results of the turbidimetric assay (i.e. A68 facilitating clot lysis) and second it investigated the effects of A68 on lysis of fully formed clots, which may have future clinical implications. The addition of Affimer A68 to plasma, made the fully formed clots easier to lyse compared with scaffold protein (Figure 43)

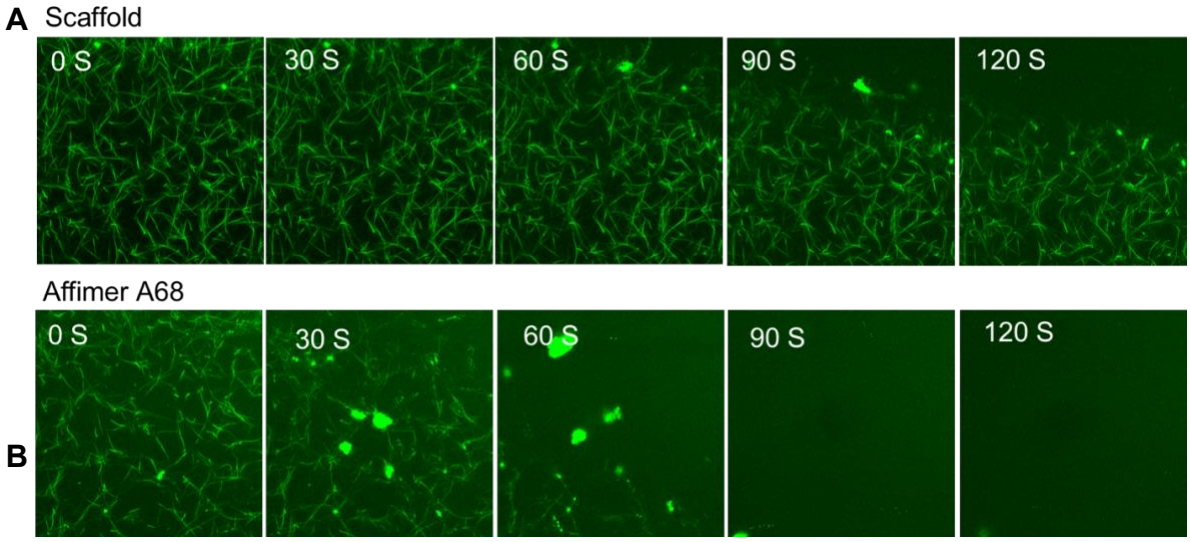

Figure 43. Real time lysis of plasma clots in the presence of Affimer A68
Clots were prepared by incubating normal pool plasma with Affimer and 488 Alexa labelled fibrinogen followed by the addition of thrombin and CaCl2. Lysis time (LT) is calculated as the time taken for the lysis mixture (containing tPA and plasminogen added to the top of the clot) to completely dissolve the fibrin networks within the viewing field. The experiment was performed on a single on 3 occasion. A. LSCM images of real time lysis of clots prepared by adding scaffold to plasma samples. B. LSCM images of real time lysis of clots prepared after addition of Affimer A68 to plasma samples. Clots made from plasma in the presence of Affimer A68 showed enhanced lysis compared to scaffold protein. This experiment was performed on a single occasion in duplicate.

4.4 Discussion

In the previous Chapter, additional bands were demonstrated when running Affimer A68 on SDS-PAGE gels, suggesting that protein multimers are forming or the preparation contains impurities, which was addressed by analysing different fractions of the Affimer isolated using size exclusion chromatography. I have also shown that Affimer A68 enhanced lysis of clots made from pooled plasma samples but inter-individual variability in the effects of A68 remains unknown. Moreover, studies have been conducted on acellular systems and it remains to be seen whether the effects of the Affimer on clot lysis are maintained in the presence of blood cells.

Identification of high molecular weight products in Affimer A68

After Affimer protein production, protein integrity and purity were assessed by analysing proteins using SDS-PAGE.

The protein bands were found in the gels that were assumed to be multiple of Affimer proteins and Western blotting with antibodies against it suggesting that this was the case, also the conformation of dimers by Affimer has been reported by others.

My immunoblotting experiments confirmed that the band at around 26 kDa was indeed Affimer-related thus representing a protein dimer. This leaves the higher MW bands that did not show on immunoblotting. They may have still been Affimer proteins not picked up on immunoblotting due to the small quantity but may equally represent other unwanted contaminants. Mass spectrometry suggested that the smaller size HMW protein is Affimer-related. However, no Affimer protein was detected in the larger size HMW, suggesting it is a contaminant. Tubulin protein is detected in E. Coli and therefore the most likely explanation that the very faint HMW band represent E Coli contaminant. However, the amount of this contaminant was very small indeed and unlikely to have affected the fibrinolysis results.

A68 concentration effect on clot lysis

I tested the effects of increasing concentrations of Affimer A68 on clot lysis and have a shown a gradual decrease in clot lysis time secondary to more efficient inhibition of PI. To further investigate the effect of A68 concentration on clot lysis, different Affimer:PI molar ratios of, 1:1, 2:1, 4:1, 8:1, 16:1 and 32:1 were used. In case of purified proteins, Affimer A68 reduced clot lysis in a purified system in a concentration-

dependent manner, results that were largely mirrored in plasma systems, although higher concentrations were needed to demonstrate an effect. This indicates that plasma proteins may interfere with the action of Affimer A68, but this interference can be easily overcome using higher concentration of the Affimer. Studies using monoclonal antibodies have demonstrated similar findings with some antibodies requiring high ratio of antibody:target protein ratio for full activity [262].

Despite using Affimers at high concentrations, clot final turbidity was not affected, suggesting that A68 has no significant effects on clot structure, although fibrin network imaging is required to confirm this. Indeed, both confocal microscopy and SEM have shown that the fibrin network maintains a physiological structure after the addition of A68, although some changes to fibrin fibre thickness were evident electron microscopy. This indicates that turbidimetric assays can fail to detect minor alterations in clot structure. Further work is required to study this in more detail, using both purified and plasma systems, to fully clarify the effects of Affimer A68 on the fibrin fibres and if a difference is found further studies are required to understand the mechanistic pathways.

Inter-individual variability in the effect of Affimer A68

As alluded to earlier, PI has been linked to increased thrombosis risk in individuals with diabetes and/or cardiovascular disease and therefore I examined plasma samples from different individuals with T1D, T2D or those with CVD.

Encouragingly, the effects of A68 were consistent across different patient populations including individuals with type 1 diabetes (n=12), those with type 2 diabetes (n=11) or patients with cardiovascular disease (n=12). These data indicate consistency of A68 action, making this a viable concept to suppress the antifibrinolytic effects of PI and facilitate clot breakdown in vivo. An advantage of this approach is the potential decreased risk of bleeding given it targets one specific pathway in coagulation. Future animal work is required to understand the effects of A68 on thrombus formation in vivo.

Real time clot lysis using confocal microscopy

To study the effects of Affimer A68 on clot lysis of fully formed clots, confocal microscopy was employed. This test serves a dual purpose: first it confirms the results

of the turbidimetric assay (i.e. A68 facilitating clot lysis) and second it investigates the effects of Affimer A68 on lysis of fully formed clots, rather clot lysis during formation (which is tested by the turbidimetric assay). The result showed that the addition of Affimer A68 to plasma, enhances clot lysis compared with scaffold protein. This indicates that A68 enhances lysis of fully formed clot, presumably due to lower incorporation of PI, supported by my data on inhibition of PI-incorporation by A68. So far, these data indicate consistency of A68 action in different individuals with variable vascular risk. This in turn makes suppression of PI activity with Affimer technology a novel method to modulate hypofibrinolysis and decrease thrombosis risk. An advantage of this approach is the potential decreased risk of bleeding given it targets one specific pathway in coagulation, particularly if used in conditions with raised levels, or enhanced activity, of PI. Future animal work is required to understand the effects of A68 on thrombus formation in vivo using appropriate models of vascular occlusion.

Chapter 5 : Molecular mechanisms (Interaction sites between Affimers and PI)

5.1 Introduction:

Early biochemical studies have shown that the interaction between plasminogen and PI happens under the strong influence of N-glycosylation of the asparagine residue in position 289 in plasminogen kringle 3 [55]. Besides, it has been shown that the interaction between PI and plasminogen may further hinder plasmin generation by actively inhibiting the adsorption of plasminogen onto fibrin [263]. On the other hand, others have found that PI-plasminogen interaction could not influence binding of plasminogen to fibrin, the breakdown of the fibrin plasminogen complex, or plasmin generation [264]. It was hypothesised in later studies that inhibition of plasmin by PI was due to plasmin-mediated generation of C-terminal lysines on fibrin, in turn reducing binding sites of plasminogen and tPA, consequently reducing plasmin generation [73]. During the process of fibrin clot formation, activated coagulation factor XIII (FXIIIa) covalently joins (crosslinks) PI into the fibrin clot where it could also directly inhibit plasmin [121]. The original crosslink is made between the glutamine residue at position 14 of PI and the lysine residue at position 303 of the fibrin α chain [204], a key site, although recent evidence indicates the existence of other interaction sites (personal communication, Dr. Ced Duval). The practical importance of crosslinking of PI to fibrin was shown in experiments using plasma from PI-deficient patients, suffering from mild to severe bleeding tendencies since early childhood (depending on the residual levels of PI) [265]. Clot lysis was enhanced in PI-deficient plasma, and when PI-deficient plasma was reconstructed with PI, the rate and extent of lysis was inversely proportional to the concentration of exogenous PI added. Further studies on whole blood and plasma have shown that the formation of fibrin-fibrin crosslink is not contributing meaningfully to the resistance of the clot to fibrinolysis, and thus the stability of thrombi to lysis depends predominantly on crosslinking of PI into the thrombus [183], and possibly other anti-fibrinolytic proteins such as complement C3 (Howes et al 2012, Hess et al 2012). Other studies have shown that PI is also able to non-covalently bind to fibrin and fibrinogen, which could possibly serve to provide the proper location of the crosslinking sites of PI and fibrin to ease the crosslinking of PI to fibrin[266]. In the previous chapters, I have shown that Affimer A68 can be used to modulate PI-induced prolongation of clot lysis. In this chapter, I attempt to identify interaction sites between Affimer A68 and PI to understand the molecular mechanisms for the effects of the Affimer. Also, it is hoped that identification

of PI interaction sites with Affimer A68 will help to design future small molecules that mimic the action of Affimer, and which be easier to administer. Given that identification of protein-protein interaction sites can be challenging, I set out to investigate the interaction sites between PI and A68 employing a number of different methodologies. First, I analysed whether the interaction is linear or conformational using Western blotting, and functional assays employing linear peptides of the variable loops, as well as pull-down assays. Second, I investigated potential interaction sites using, mass spectrometry and molecular modelling. Third I analysed the effects of Affimer A68 on lysis of plasma clots from different species (similar effects on lysis indicate interactions in a conserved region). Finally I studied the effects of each Loop of A68 on clot lysis by making single-Loop Affimers (one for each loop), in addition to undertaking mutagenesis analysis of Affimer A68.

5.2 Methods

5.2.1.1 Cross linking analysis using mass spectrometry

In order to identify potential interaction sites between Affimer A68 and PI, 3 separate analyses were done on purified PI alone, Affimer 68 alone, and PI-A68 complex The first experiment was performed using mass spectrometry was performed by Dr Mike Deery at the Cambridge Centre (University of Cambridge) without using linkers. The second and third experiments were performed locally by Dr. James Ault and linkers were used to increase the chances of detecting the interactions. Following tryptic digestion of samples, the fragments were analysed using liquid chromatography mass spectrometry, as described in Chapter 2.

5.2.1.2 Molecular modelling

Given that human PI crystal structure is yet to be described, the published crystal structure of mouse α2-antiplasmin (PDB ID 2R9Y) was used to make a homology model of human alpha-2-antiplasmin using I-TASSER and the Maestro graphical user interface was employed to check the validity of the model produced (1). The published crystal structure of the Affimer scaffold (PDB ID: 4N6T) was used as a template to create a model of Affimer A68 (2). Docking of this Affimer A68 to the homology model of human alpha-2-antiplasmin was carried out using AutoDock 4.2 (3). A total of 100 docking iterations were calculated for each predicted site, using a Lamarckian Genetic

Algorithm. The resulting poses were clustered, based on a 2 Å root mean squared deviation. The cluster with the lowest energy conformation, and also the most populated cluster pose, were further examined using PyMOL (4). The screening using AutoDock was carried out by Dr Katie Simmons, LICAMM, University of Leeds,as described in Chapter 2.

5.2.1.3 Turbidimetric analysis

Turbidimetric analysis was performed on plasma samples from different species including bovine, rat, pig, and mouse in the presence of Affimer A68 or scaffold as a negative control as described in detail in Chapter 2. The following clot parameters were analysed: lag phase, final turbidity and lysis time were measured. A mixture containing 5μM A68 or 5μM Scaffold from the same species. After 30 minutes of plasma and Affimer incubation, 50μl ofactivation mix containing 0.37U/ml thrombin, 3.3nM tPA and 7.5mM $CaCl_2$ in 0.1M NaCl, 0.05M Tris was added. Measurements were taken at 340nm every 24 seconds for 6 hours in a plate reader as described in Chapter 2.

5.2.1.4 Western blot and linear peptides of the loops of A68

Western blot was used to investigate whether the reaction between Affimer A68 or Loop 1 and PI is conformational or not (as described in Chapter 2).

5.2.1.5 Loop 1 and Loop2

Two new single loop Affimers were made. PCR was used to truncate each of the two loops providing one new Affimer with Loop 1 of A68 and another Affimer with Loop 2 of A68. The new Affimers were subcloned for large scale production as described in the methods section. The effect of each loop of A68 on clot formation and lysis was investigated using turbidimetric assays.

5.2.1.6 Site-directed mutagenesis of Loop1 of Affimer A68

My data showed that loop one of Affimer A68 is important for interaction with PI and therefore I conducted site directed mutagenesis studies to investigate the residue(s) that are crucial for the interaction. Methodology of site directed mutagenesis is described in Chapter 2.

5.3 Results

5.3.1 Type of interaction (linear or conformational)

5.3.1.1 Using A68 to detect PI

Initially, for the western blot method PI was prepared by mixing with the detergent sodium dodecyl sulphate, which reduced the protein into linear chains and coated with a negative charge. These protein molecules were then separated according to their sizes using SDS-PAGE and transferred to a blotting membrane which was followed by a blocking step using milk powder (5% w/v). The membrane was incubated with A68 to establish whether the Affimer A68 can bind to linear PI. It was then washed, incubated with anti-his antibody (as a primary antibody), washed and finally incubated with HRP secondary antibody. Affimer A68 was also loaded on the gel alongside PI as a positive control. No binding was detected between Affimer A68 and PI fragments suggesting that the interactions are conformational (Figure 44).

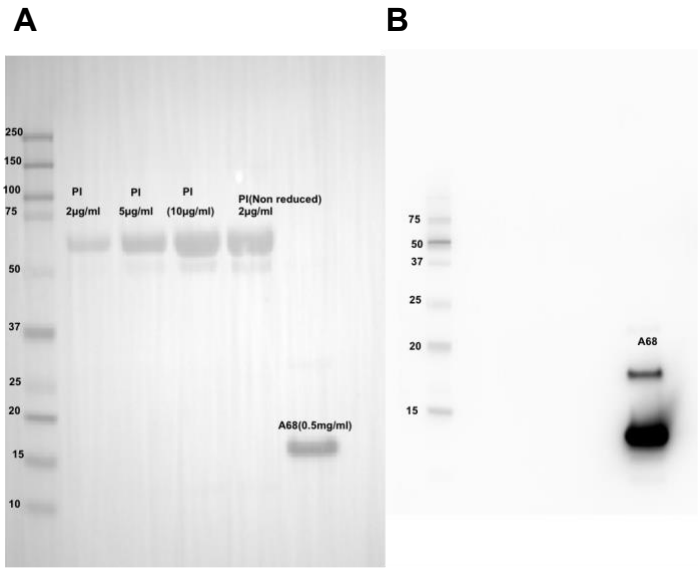

Figure 44. A SDS-PAGE of PI.
Ponceau stain was used of different protein concentrations incubated with Affimer A68. B Western blotting. Affimer A68 was detected using anti-His antibody. No interaction between PI and Affimer A68 was detected.

5.3.1.2 Pull down assays

PI pull down assays were performed to confirm that Affimer A68 binds PI in liquid phase. In this assay, magnetic beads bound-Affimer A68 was incubated with plasma and proteins bound to the Affimer were “pulled down” as described in the methods section. Proteins bound to Affimer A68 were eluted with appropriate elution buffer and the samples run on SDS-PAGE. The bands shown in the SDS-PAGE image represent proteins that bound to Affimer and/or to magnetic beads. Western blot was then performed using goat anti-human PIGA2AP-HRP (diluted 1/1000). The Western blot failed to detect PI, most likely due to the fact that the amount of PI in plasma was very small beyond the detection limit of the assay (Figure 45). The use of less diluted plasma in order to increase the chance of detecting PI was also investigated. However, this still failed to detect A68-bound PI (Figure 46).

Following the failure to pull down PI from plasma, it was felt it is necessary to confirm that Affimer A68 can “pull down” purified PI protein, to confirm the validity of the technique. When purified PI was used, Affimer A68 indeed showed binding to the protein (Figure 47)

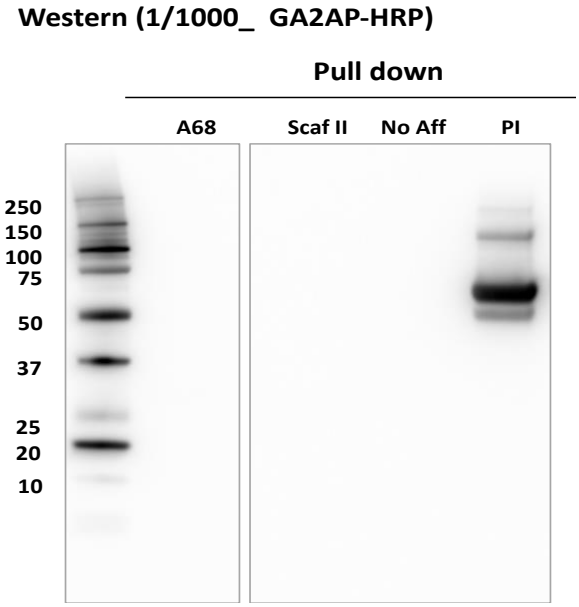

Figure 45. PI detected by A68.
Western blot to investigate PI-Affimer A68 binding (none detected using these experimental conditions).

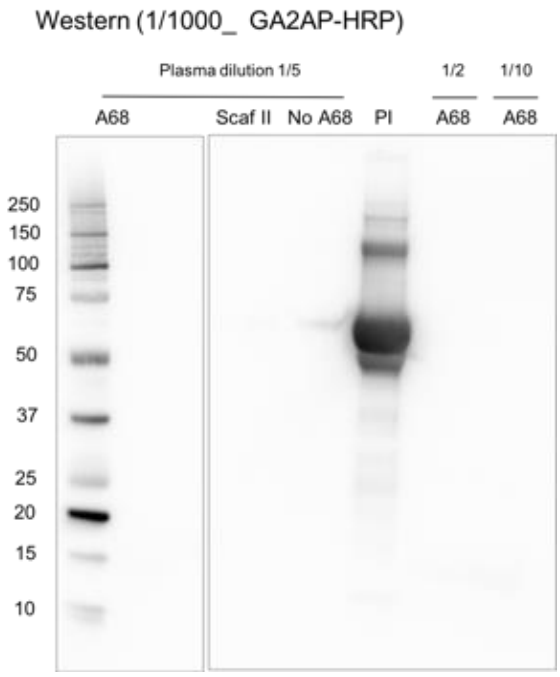

Figure 46. PI detected by A68.
Western blot to elucidate PI-Affimer A68 binding. In this second experiment, more concentrated plasma was used but again no PI-binding to Affimer A68 could be detected.

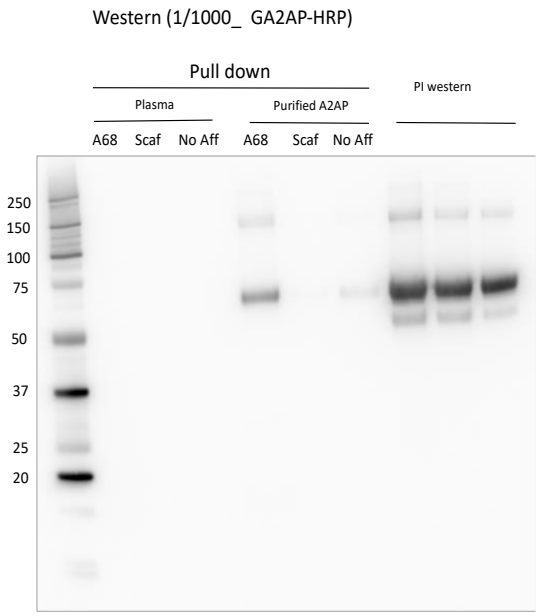

Figure 47. PI detected by A68.
Western blot to elucidate PI-Affimer A68 binding, showing successful "pull-down" on purified PI but inability to pull down the protein using plasma samples. Experiments were conducted 2 times with similar results.

5.3.1.3 Effects of linear peptides of Affimer A68 loops on clot lysis in Plasma

Linear peptides of the same sequence of the two loops of Affimer A68 were manufactured and their effect on clot formation and lysis was tested using turbidity and lysis assay employing pooled human plasma. The synthetic peptides had no significant impact on clot lysis (Figure 48). These data strongly suggest that the interaction between Affimer A68 and PI is dependent on conformation of the variable regions of the Affimer and the interaction are unlikely to be linear.

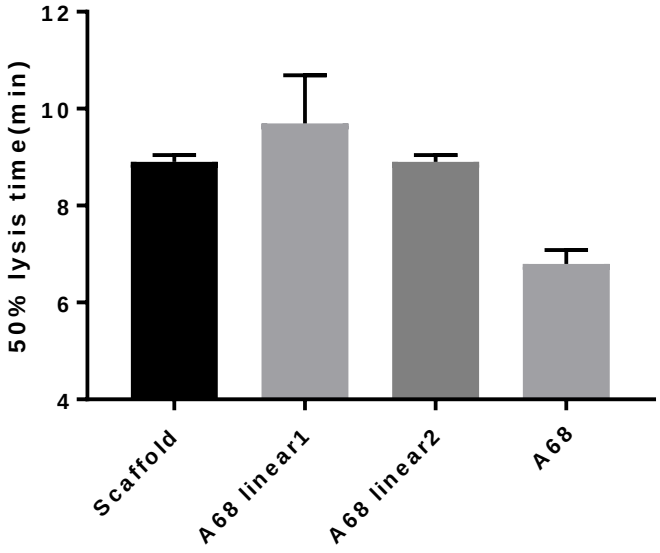

Figure 48. Effect of Linear1 and Llinear2 of A68 in plasma system.
Linear1 and linear 2 peptides of the variable regions of Affimer A68 had no significant effect in lysis time compared to Affimer A68.

Plasma system	Lysis time (min, mean±SD)
A68	6.8 ± 0.2 *
Linear1	9.7 ± 0.7
Linear2	8.9 ± 0.1
Scaffold	8.9 ± 0.1

Table 11 Effect of Linear1 and Linear2

Peptides on lysis time of clots made from plasma system. Time to 50% clot lysis is shown of three independent experiments each performed in duplicate. Data are presented as mean±SD, Statistical analysis was performed using t-test.

5.3.2 Interaction site of PI and A68

5.3.2.1 Molecular Modelling

5.3.2.1.1 Whole Affimer A68

Docking of A68 whole Affimer to the human antiplasmin model. We made the homology model of human PI based on the published crystal structure of mouse PI, and the A68 based on the published crystal structure of Affimer scaffold (PDB ID no. 4N6T). The loops showed no interaction PI in this model (Figure 49A).

The dockings had been done using the whole A68 Affimer. Both the most populated and the lowest energy pose are the same (teal) and they are in the same region as the RGD domain (green spheres) but the residues closest to PI are in the C terminus of the Affimer near the His tag. (Figure 49 B)

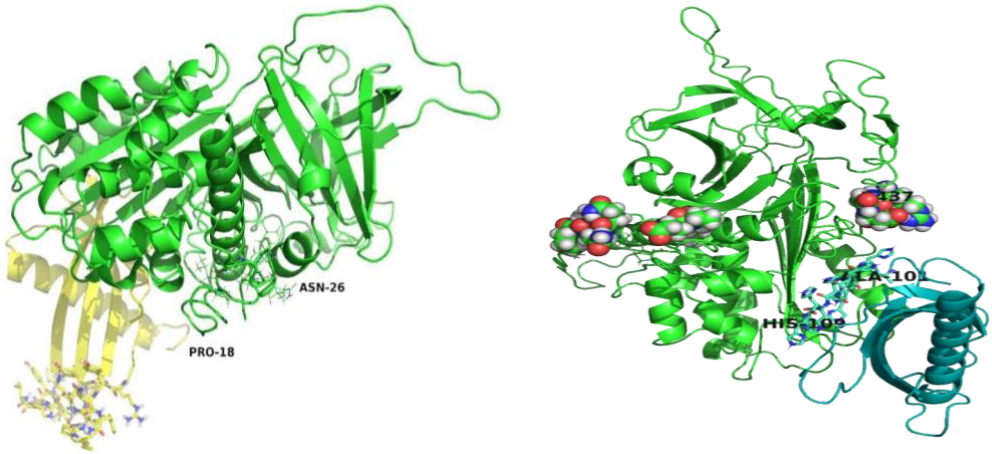

Figure 49. Modelling of Affimer A68 Interaction with plasmin inhibitor (PI).
The green represents PI and the yellow represent Affimer A68. No clear interaction was demonstrated between the two molecules.

5.3.2.1.2 Loop 1 of Affimer A68

Loop 1 of Affimer A68 showed binding to human PI (α2-antiplasmin) in between PRO-18, 29 and ASN-26, (Figure 50). With mainly the scaffold next to the Loop showing the strongest interaction.

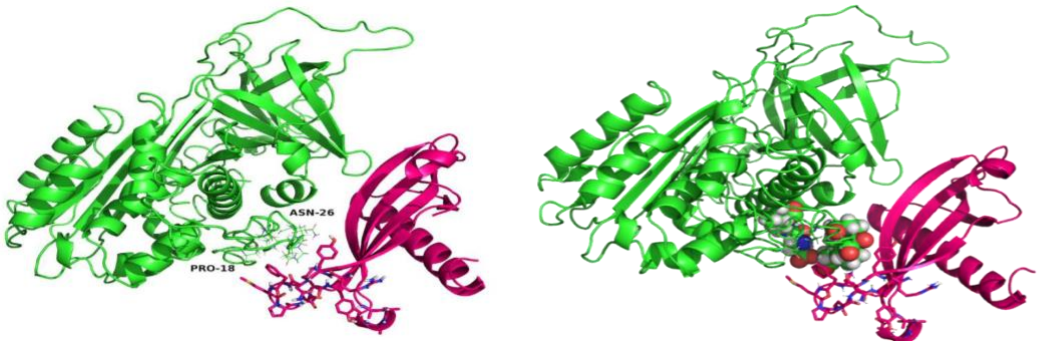

Figure 50 Modelling Loop 1 of Affimer A68 Interaction with plasmin inhibitor (PI).
The green represents PI and the magenta represents Loop 1 of Affimer A68. An interaction between Loop 1 and PI is demonstrated.

5.3.2.1.3 Loop 2 of Affimer A68

Similar to the docking of whole A68 and Loop1, Loop 2 was investigated to understand whether this interacts with PI. However, results showed that the Loop 2 is not interacting with the protein in this model. (Figure 51)

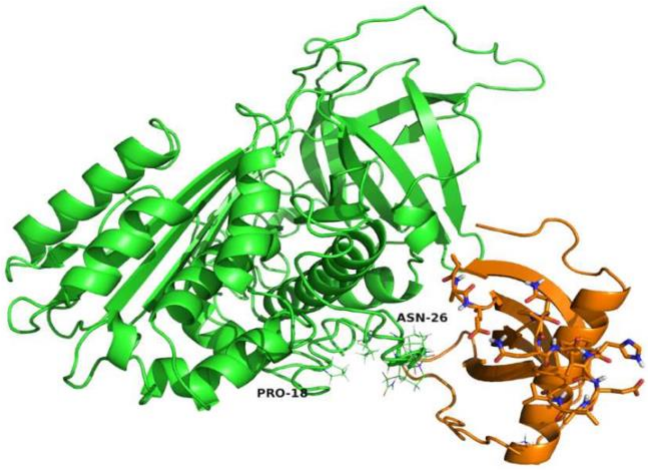

Figure 51. Modelling Loop 2 of A68 Interaction with PI.
The green represents PI and the orange represent Loop 2 of Affimer A68. No interaction between Loop 2 and PI was evident using this model.

5.3.2.2 Mass Spectrometry

In order to pinpoint interaction sites, I used mass spectrometry (MS) to investigate PI-A68 interaction. Three different experiments were undertaken to investigate interaction sites between PI and Affimer A68.

5.3.2.3 Experiment 1

This was done through a collaboration with the University of Cambridge. Trypsin was used to digest the complex of PI-A68. As proteins are digested, they lose their secondary and tertiary structure so any interaction/non-covalent association between protein molecules is lost. When digesting a protein in this way, it is rarely, if ever, to see full sequence coverage with a digest using a single protease. This is because the length and chemistry of the resulting peptides will have a bearing on whether or not

the peptide can subsequently be detected/sequenced. The protein coverage maps show areas of the proteins in which peptides were not detected. This could potentially mean that those peptides which are not detected are involved in the binding of the complex. However, it does not fully determine the sites of interaction and simply indicates where the binding may occur. Unfortunately, this approach failed to get high PI coverage map (25% coverage) and therefore results were deemed inconclusive.

5.3.2.4 Experiment 2

This was done through a collaboration with the University of Leeds (FBS). MS performed to investigate PI-A68 interaction, but the two proteins were incubated with a linker that brought together K residues in each protein. The BS3 cross linker was used, which is an 11.4 A length linker. It seems that the zero length cross linkers are either difficult to get to work and/or show cross links that are not necessarily zero length, and that they have seen distance constraints similar to BS3 in some cases. The best recommendation is a mixture of d0/d12 (BS3 where the linker either has 12 hydrogen or 12 deuterons) isotopically labelled BS3. This gives a characteristic double signal in the MS to confirm the presence of the cross linker. Subsequent fragmentation then determines the sequence of the cross-linked peptides. The treated sample was run on a gel to excise the cross linked proteins, which were then identified according to size. After tryptic digest, a strong cation exchange enrichment was performed which has higher selectivity for the generally higher charged peptides.

The data were filtered with an FDR of X (equivalent of score of 140) and output shows that any hit with a score above 140 should be considered as potential interaction site. All of these colours coded are shown in Figure 52-A. G**K**IQE to GGBU**K**KKLYE has been detected a number of times, suggesting this is a strong candidate. Furthermore, Table 2 showed the summary of the potential PI-A68 interaction sites with a scores above 140 and Table 13 demonstrates potential interaction sites with scores<140 (Figure 52 A, B).

PI sequences	A68 sequences
scores>140	
NQE	IVNFKE
NQE	FVRVVKAKE
GKIQE	GGKKKLYE
scores≤140	
NQE	IVNFKE
KLFGPD	DSLE
NQE	AKDGGKKKLYE
FLEESE	IVNFKE
QFTVPVE	HNKKE
LKLVPPMEE	DSLE

Table 12 Potential PI-A68 interaction sites.

Peptides with a hit score of more than 140 and score less than 140 are shown.

A

Score	m/z	Charge	M+H+	Calculated	Deviation ii	Peptide 1	Protein 1	From	To	Peptide2	Protein 2	From	To	Scan num	is Selected in T	Candidate	Folder Nur	Retention	miscellane	best linkag	best linkag	All linkage	Spectrum	local False discovery
217	963.5175	2	1926.028	1926.033	-2.79288	[GKIQE]	>sp|P08697|40-491	175	179	[LANINQWVKE]	>sp|P08697|40-491	162	171	2964	FALSE	16776	null	1463.32		K2	K9	217~K2~K 6834072-1	DSS/BS3#	0
208	818.4467	2	1635.886	1635.899	-8.0639	[IVNFKE]	>A68-sequence	83	88	[IVNFKE]	>A68-sequence	83	88	3024	FALSE	16815	null	1496.192		K5	K5	208~K5~K ebb63fcf-7	DSS/BS3#	0
206	818.4456	2	1635.884	1635.899	-9.35985	[IVNFKE]	>A68-sequence	83	88	[IVNFKE]	>A68-sequence	83	88	3025	FALSE	10183	null	1497.963		K5	K5	206~K5~K 05a8110-2	DSS/BS3#	0
205	774.9266	2	1548.846	1548.831	9.750647	[FKPVGD]	>A68-sequence	92	97	[IVNFKE]	>A68-sequence	83	88	2905	FALSE	6997	null	1407.498		K2	K5	205~K2~K feb6b2f1-6	DSS/BS3#	0
199	774.9232	2	1548.839	1548.831	5.360305	[FKPVGD]	>A68-sequence	92	97	[IVNFKE]	>A68-sequence	83	88	2854	FALSE	10125	null	1407.207		K2	K5	199~K2~K 464eff6c-7	DSS/BS3#	0
188	774.9219	2	1548.837	1548.831	3.733286	[FKPVGD]	>A68-sequence	92	97	[IVNFKE]	>A68-sequence	83	88	2704	FALSE	20998	null	1409.342		K2	K5	188~K2~K 17127035-	DSS/BS3#	0
184	774.9215	2	1548.836	1548.831	3.100555	[FKPVGD]	>A68-sequence	92	97	[IVNFKE]	>A68-sequence	83	88	2856	FALSE	13389	null	1410.529		K2	K5	184~K2~K 7444c3ea-	DSS/BS3#	0
184	638.8261	2	1276.645	1276.642	2.32294	(NQE)	>sp|P08697|40-491	0	3	[IVNFKE]	>A68-sequence	83	88	2624	FALSE	10098	null	1284.028		{0	K5	184~{0~K‡e7a3b287-	DSS/BS3#	0
183	719.3688	3	2156.092	2156.087	2.253406	[GKIQE]	>sp|P08697|40-491	175	179	[DDLANINQWVKE]	>sp|P08697|40-491	160	171	3132	FALSE	16881	null	1554.971		K2	K11	183~K2~K d5c9827e-	DSS/BS3#	0
174	801.9464	2	1602.886	1602.885	0.33781	(NQE)	>sp|P08697|40-491	0	3	[FVRVVKAKE]	>A68-sequence	32	40	2514	FALSE	16583	null	1214.961		{0	K6	174~{0~K‡52e958e1-	DSS/BS3#	0
165	654.7203	3	1962.146	1962.142	2.083382	[IVNFKE]	>A68-sequence	83	88	[FVRVVKAKE]	>A68-sequence	32	40	2409	FALSE	20806	null	1254.392		K5	K8	123~K5~K 7be589ef-e	DSS/BS3#	0
164	642.6838	3	1926.037	1926.033	1.944127	[GKIQE]	>sp|P08697|40-491	175	179	[LANINQWVKE]	>sp|P08697|40-491	162	171	2962	FALSE	16775	null	1462.239		K2	K9	164~K2~K 47c4300c-	DSS/BS3#	0
156	801.9453	2	1602.883	1602.885	-1.0971	(NQE)	>sp|P08697|40-491	0	3	[FVRVVKAKE]	>A68-sequence	32	40	2544	FALSE	6890	null	1217.32		{0	K6	156~{0~K‡3d273342-	DSS/BS3#	0
149	654.7203	3	1962.146	1962.142	2.083382	[IVNFKE]	>A68-sequence	83	88	[FVRVVKAKE]	>A68-sequence	32	40	2569	FALSE	13225	null	1256.839		K5	K8	101~K5~K afd7d1ce-5	DSS/BS3#	0
144	545.3069	3	1633.906	1633.916	-5.9983	[GKIQE]	>sp|P08697|40-491	175	179	[GGKKKLYE]	>A68-sequence	62	69	2492	TRUE	16580	null	1202.065		K2	K3	144~K2~K d461cee0-	DSS/BS3#	0
144	801.9456	2	1602.884	1602.885	-0.66039	(NQE)	>sp|P08697|40-491	0	3	[FVRVVKAKE]	>A68-sequence	32	40	2331	FALSE	20780	null	1212.789		{0	K6	144~{0~K‡f1771ff6-4	DSS/BS3#	0
143	801.9431	2	1602.879	1602.885	-3.84217	(NQE)	>sp|P08697|40-491	0	3	[FVRVVKAKE]	>A68-sequence	32	40	2328	FALSE	20779	null	1211.331		{0	K6	143~{0~K‡81921375-	DSS/BS3#	0
141	565.7231	5	2824.587	2824.587	-0.22517	[FVRVVKAKE]	>A68-sequence	32	40	[AKVWVKQPQYHVE]	>A68-sequence	70	82	2286	FALSE	20739	null	1189.117		K8	K2	128~K6~K abb15947-	DSS/BS3#	0
140	642.6859	3	1926.043	1926.033	5.21507	[GKIQE]	>sp|P08697|40-491	175	179	[LANINQWVKE]	>sp|P08697|40-491	162	171	2960	FALSE	10165	null	1463.904		K2	K9	140~K2~K a3710da7-	DSS/BS3#	0
139	969.9549	2	1938.903	1938.9	1.250403	[IVDFKE]	>A68-sequence	83	88	[MASGNENSLE]	>sp|P08697|40-491	0	10	3423	FALSE	17267	null	1710.505		K5	S8	127~K5~K‡37170b70-	DSS/BS3#	0
137	817.4546	2	1633.902	1633.916	-8.58331	[GKIQE]	>sp|P08697|40-491	175	179	[GGKKKLYE]	>A68-sequence	62	69	2614	TRUE	16628	null	1269.75		K2	K3	137~K2~K afff045c-5	DSS/BS3#	0
134	719.3666	3	2156.085	2156.087	-0.80769	[GKIQE]	>sp|P08697|40-491	175	179	[DDLANINQWVKE]	>sp|P08697|40-491	160	171	3189	FALSE	7129	null	1553.568		K2	K11	134~K2~K d105cf49-C	DSS/BS3#	0
134	551.2758	2	1101.544	1101.542	1.887859	(NQE)	>sp|P08697|40-491	0	3	[GKIQE]	>sp|P08697|40-491	175	179	2086	FALSE	10020	null	1003.12		{0	K2	134~{0~K‡407671bc-	DSS/BS3#	0
131	545.3084	3	1633.91	1633.916	-3.33596	[GKIQE]	>sp|P08697|40-491	175	179	[GGKKKLYE]	>A68-sequence	62	69	2520	TRUE	6887	null	1205.513		K2	K4	131~K2~K 14aba628-	DSS/BS3#	0.013333
130	719.3693	3	2156.093	2156.087	2.893456	[GKIQE]	>sp|P08697|40-491	175	179	[DDLANINQWVKE]	>sp|P08697|40-491	160	171	3134	FALSE	10202	null	1554.818		K2	K11	130~K2~K 4fabc18f-4	DSS/BS3#	0.024691
130	638.8279	2	1276.649	1276.642	5.205481	(NQE)	>sp|P08697|40-491	0	3	[IVNFKE]	>A68-sequence	83	88	2606	FALSE	13248	null	1276.867		{0	K5	130~{0~K‡c9b362de-	DSS/BS3#	0.024691
129	706.8956	4	2824.561	2824.587	-9.4385	[FVRVVKAKE]	>A68-sequence	32	40	[AKVWVKQPQYHVE]	>A68-sequence	70	82	2308	FALSE	20755	null	1201.409		K6	K2	129~K6~K dffb27d-4	DSS/BS3#	0.022989
129	638.8286	2	1276.65	1276.642	6.239432	(NQE)	>sp|P08697|40-491	0	3	[IVNFKE]	>A68-sequence	83	88	2660	FALSE	6919	null	1279.315		{0	K5	129~{0~K‡6bea4f37-f	DSS/BS3#	0.022989
127	817.4576	2	1633.908	1633.916	-4.9111	[GKIQE]	>sp|P08697|40-491	175	179	[GGKKKLYE]	>A68-sequence	62	69	2590	TRUE	13228	null	1267.327		K2	K3	127~K2~K bce1e941-	DSS/BS3#	0.022222
126	732.8616	2	1464.716	1464.71	3.790639	[GKIEE]	>sp|P08697|40-491	175	179	[FLEQSE]	>sp|P08697|40-491	139	144	3386	FALSE	13627	null	1685.866		K2	S5	126~K2~S 9247e4aa-	DSS/BS3#	0.020833
126	817.4564	2	1633.906	1633.916	-6.37998	[GKIQE]	>sp|P08697|40-491	175	179	[GGKKKLYE]	>A68-sequence	62	69	2589	TRUE	10091	null	1265.114		K2	K3	126~K2~K 2bd42f3e-b	DSS/BS3#	0.020833
125	514.6176	3	1541.838	1541.832	3.910203	[HNKKE]	>A68-sequence	22	26	[IVNFKE]	>A68-sequence	83	88	2211	FALSE	13035	null	1067.696		K4	K5	125~K3~K b9ccbf6d-c	DSS/BS3#	0.019048
125	638.826	2	1276.645	1276.642	2.16628	(NQE)	>sp|P08697|40-491	0	3	[IVNFKE]	>A68-sequence	83	88	2443	FALSE	20840	null	1271.423		{0	K5	125~{0~K‡c7e9d6e6-	DSS/BS3#	0.019048
125	638.8248	2	1276.642	1276.642	0.239347	(NQE)	>sp|P08697|40-491	0	3	[IVNFKE]	>A68-sequence	83	88	2597	FALSE	3635	null	1287.972		{0	K5	125~{0~K‡59b41f06-a	DSS/BS3#	0.019048
124	638.8248	2	1276.642	1276.631	9.038523	[DSLE]	>A68-sequence	7	10	[KLFGPD]	>sp|P08697|40-491	429	434	2597	FALSE	3636	null	1287.972		S2	K1	124~S2~K 59b41f06-a	DSS/BS3#	0.018519
124	588.6449	3	1763.92	1763.917	1.554991	(NQE)	>sp|P08697|40-491	0	3	[AKDGGKKKLYE]	>A68-sequence	59	69	1924	FALSE	16364	null	900.833		{0	K7	101~{0~K‡5049c7b7-	DSS/BS3#	0.018519
119	796.4092	2	1591.811	1591.81	0.614533	[DSLEIE]	>A68-sequence	7	12	[IVNFKE]	>A68-sequence	83	88	3282	FALSE	16931	null	1635.89		S2	K5	119~S2~K 9fc6e018-4	DSS/BS3#	0.018018
118	820.9081	2	1640.809	1640.794	8.997178	[IVDFKE]	>A68-sequence	83	88	[FLEESE]	>sp|P08697|40-491	139	144	3459	FALSE	17278	null	1729.739		K5	S5	118~K5~S 83d4ae36-f	DSS/BS3#	0.017544
115	806.4208	2	1611.834	1611.838	-2.04362	[HNKKE]	>A68-sequence	22	26	[QFTVPVE]	>sp|P08697|40-491	221	227	2394	FALSE	13184	null	1162.964		K4	T3	-1~K3~T3~6378e032-0	DSS/BS3#	0.017094
114	491.2923	4	1962.147	1962.142	2.666222	[IVNFKE]	>A68-sequence	83	88	[FVRVVKAKE]	>A68-sequence	32	40	2406	FALSE	20805	null	1252.797		K5	K6	114~K5~K 769af5e3-5	DSS/BS3#	0.02439
114	980.1782	3	2938.52	2938.516	1.458632	[NSLEIE]	>A68-sequence	7	12	[LARFAVDEHNKKENALLE]	>A68-sequence	14	31	3153	FALSE	16898	null	1567.34		S2	K12	112~S2~K 5c934980-	DSS/BS3#	0.02439
112	836.4239	2	1671.841	1671.84	0.474136	[DSLE]	>A68-sequence	7	10	[LKLVPPmEE]	>sp|P08697|40-491	435	443	3258	FALSE	16924	null	1623.83		S2	K2	112~S2~K 17c5030d-c	DSS/BS3#	0.02381
111	732.8616	2	1464.716	1464.71	3.790639	[GKIQE]	>sp|P08697|40-491	175	179	[FLEESE]	>sp|P08697|40-491	139	144	3386	FALSE	13626	null	1685.866		K2	S5	111~K2~S 9247e4aa-	DSS/BS3#	0.031008
111	788.9034	2	1576.8	1576.808	-5.24533	[HNKKE]	>A68-sequence	22	26	[EHNKKE]	>A68-sequence	21	26	2732	FALSE	10114	null	1340.9		K4	K5	-1~K3~K4 bd4390a7-	DSS/BS3#	0.031008
110	811.4098	3	2432.215	2432.233	-7.4814	[FLEESE]	>sp|P08697|40-491	139	144	[QVSPLTLLKLGDEE]	>sp|P08697|40-491	4	17	2710	FALSE	10110	null	1329.918		S5	K9	110~S5~K 79f6ec05-6	DSS/BS3#	0.030303
109	545.3069	3	1633.906	1633.916	-5.9983	[GKIQE]	>sp|P08697|40-491	175	179	[GGKKKLYE]	>A68-sequence	62	69	2490	TRUE	16579	null	1201.112		K2	K4	108~K2~K 8514551e-1	DSS/BS3#	0.028986
109	801.9449	2	1602.883	1602.885	-1.53382	(NQE)	>sp|P08697|40-491	0	3	[FVRVVKAKE]	>A68-sequence	32	40	2436	FALSE	3543	null	1219.598		{0	K6	109~{0~K‡7b63d140-	DSS/BS3#	0.028986

B

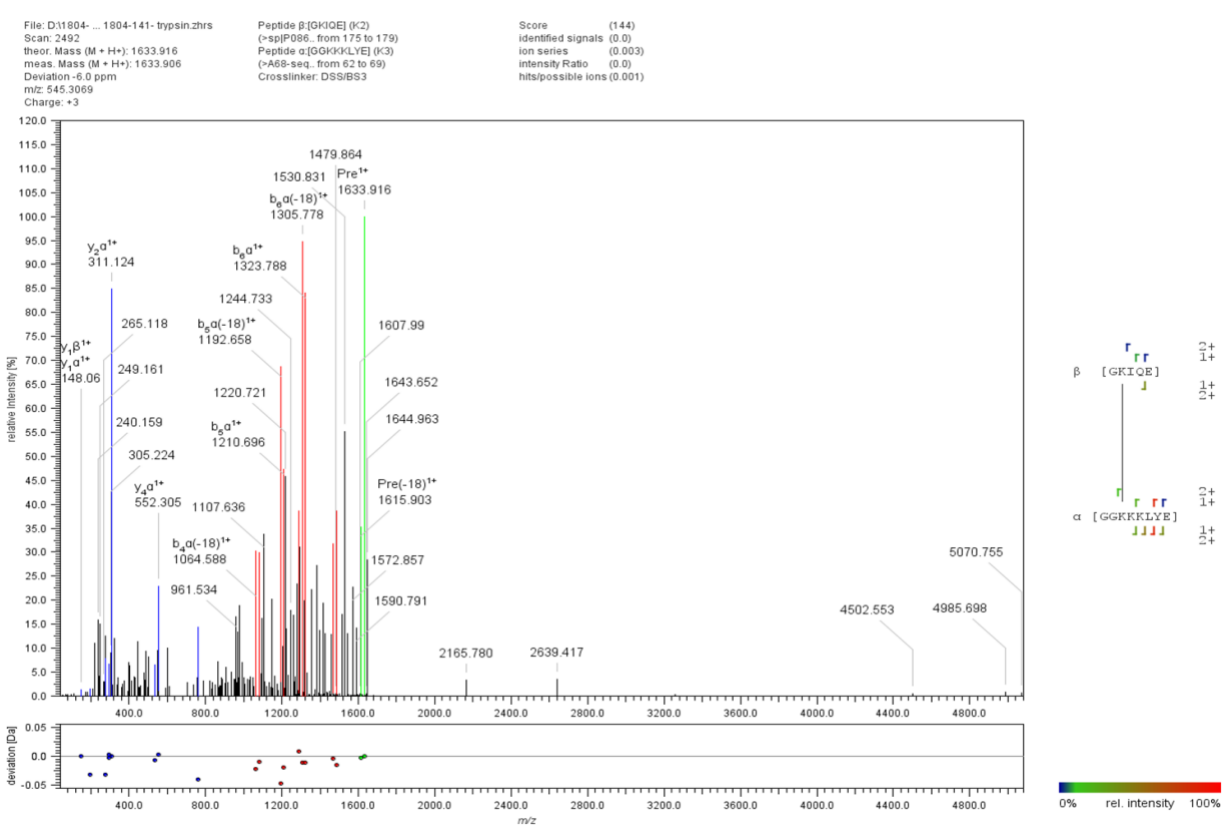

Figure 52. mass spectrometry

A. the PI-A68 digest using protease Glu-C. B In the spectrum red is for b ions and blue is for y ions. In the peptide fragmentation schematic the colour represents the intensity of the ion in the spectrum.

PI

MALLWGLLVLSWSCLQGPCSVFSPVSAMEPLGRQLTSGPNQEQVSPLTLLKLGNQEPGG
QTALKSPPGVCSRDPTPEQTHRLARAMMAFTADLFSLVAQTSTCPNLILSPLSVALALSHLA
LGAQNHTLQRLQQVLHAGSGPCLPHLLSRLCQDLGPGAFRLAARMYLQKGFPIKEDFLEQ
SEQLFGAKPVSLTGKQEDDLANINQWVKEATEGKIQEFLSGLPEDTVLLLLNAIHFQGFWRN
KFDPSLTQRDSFHLDEQFTVPVEMMQARTYPLRWFLLEQPEIQVAHFPFKNNMSFVVLVPT
HFEWNVSQVLANLSWDTLHPPLVWERPTKVRLPKLYLKHQMDLVATLSQLGLQELFQAPD
LRGISEQSLVVSGVQHQSTLELSEVGVEAAAATSIAMSRMSLSSFSVNRPFLFFIFEDTTGL
PLFVGSVRNPNPSAPRELKEQQDSPGNKDFLQSLKGFPRGDKLFGPDLKLVPPMEEDYPQ
FGSPK

Affimer A68

MASGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQRYNFMPEKYTMYYLTLEAKD
GGKKKLYEAKVWVKQPQYHVEIVNFKELQEFKPVGDAAAAHHHHHHHH

Figure 53. Sequence of PI and Affimer A68.
Highlighted the Potential PI-A68 interaction sites. Peptides with a hit score of more than 140 and score less than 140 are shown.

5.3.2.5 Experiment 3

This was done through a collaboration with the University of Leeds (FBS). After the two different experiments described above, a new approach was devised. The cross linking was repeated as above followed by trypsin digestion but additionally SCX fractionation was employed (Cross linked peptides are more highly charged so can be separate by SCX from the non-XL peptides). Only 3 of the 398 crosslinks were not dead-end reactions (the reagent has only reacted with a peptide at one end; the other end has been hydrolysed an is not attached to a peptide), and these showed very low scoring matches questioning their importance. A total of 4 of the 398 crosslinks achieved a score above 100 and were considered to have good quality spectra from which to determine the crosslink. Of note, all of these crosslinks were dead-end reactions with H_2O. Very few intra- and inter- peptide crosslinks have been detected. We know the crosslinking reaction has occurred because a large number of dead-end crosslinks reacting with H_2O have been detected. There are a number of other proteins identified particularly by the digest with the protease trypsin, suggesting contamination by other proteins.

5.3.3 Interaction sites of PI

5.3.3.1 Effect of A68 on plasma clot lysis in different species

The effect of Affimer A68 on clot lysis was investigated using plasma samples from different species, which would help to narrow down potential interaction sites and will also help to design appropriate future animal in vivo experiments in order to develop the Affimers for clinical use. PI sequence homology in the different species studied is shown in Figure 10. Of note, C-terminus of the protein shows differences between species with 67% identity displayed comparing human and bovine PI, dropping to 61% comparing human and mouse species. Overall, it appears the highest degree of homology is found between human and bovine PI. When turbidimetric assays were employed, Affimer A68 had no effect on lag time or clot final turbidity of clots made from different species (Figure 54). However, it significantly shortened bovine plasma clot lysis without affecting lysis of clots made from other species.

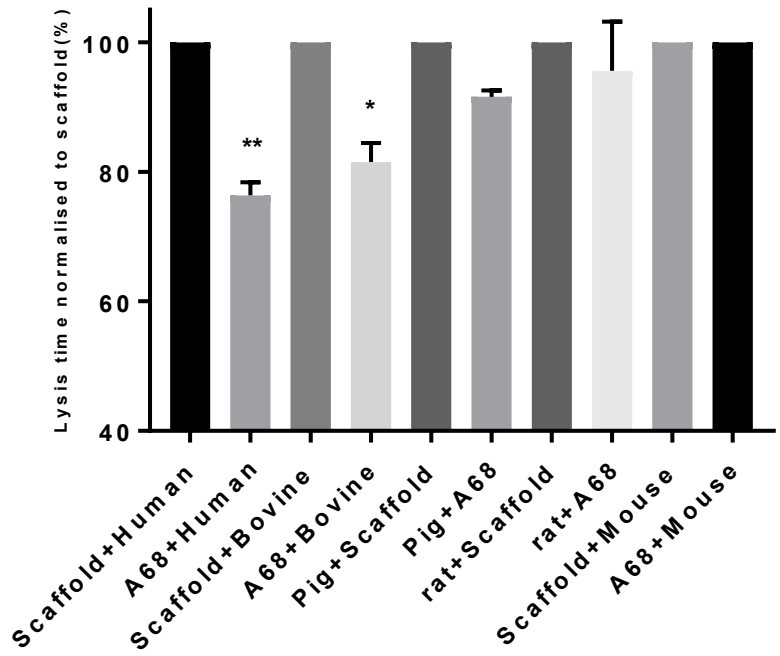

Figure 54. Effect of A68 on fibrinolysis of plasma clots made from different species
Results are shown adjusted to lysis time of plasma samples in the presence of scaffold. Affimer A68 reduced plasma clot lysis of bovine samples (p=0.0125) without having a significant effect on clots made from mouse, rat or pig plasma.

		Lysis time(min,mean±SD)
Bovine plasma	A68	5.4 ± 0.1 ** (21% reduction)
	Scaffold	6.9 ± 0.1
Mouse plasma	A68	2.3 ± 0.1 (0% reduction)
	Scaffold	2.3 ± 0.1
Pig plasma	A68	135.1 ± 3.9 (8% reduction)
	Scaffold	147.5 ± 3.1
Rat plasma	A68	101.1 ± 2.9 (5% reduction)
	Scaffold	105.9 ± 2.9
	A68	7 ± 0.35 (35% reduction)
Human plasma	Scaffold	10.8 ± 0.3

Table 13. The effects of A68 in bovine and mouse plasma.

A total of three independent experiments were performed in duplicate, data presented as mean±SD. Statistical analysis was performed using t-test.

5.3.3.2 Sequence alignment

In the previous experiment, Affimer A68 failed to modulate lysis of mouse clot but appears to have an effect on bovine clot. So, the interaction appears to occur in an area conserved in human and bovine PI. Furthermore, Amino acid sequences of PI (Human, Mouse, and Bovine) are shown in a sequence alignment (Figure 55). The potential interaction sites are (MEPL), (NQEP), (RCL), and the only one site in C-terminus is (PRGD).

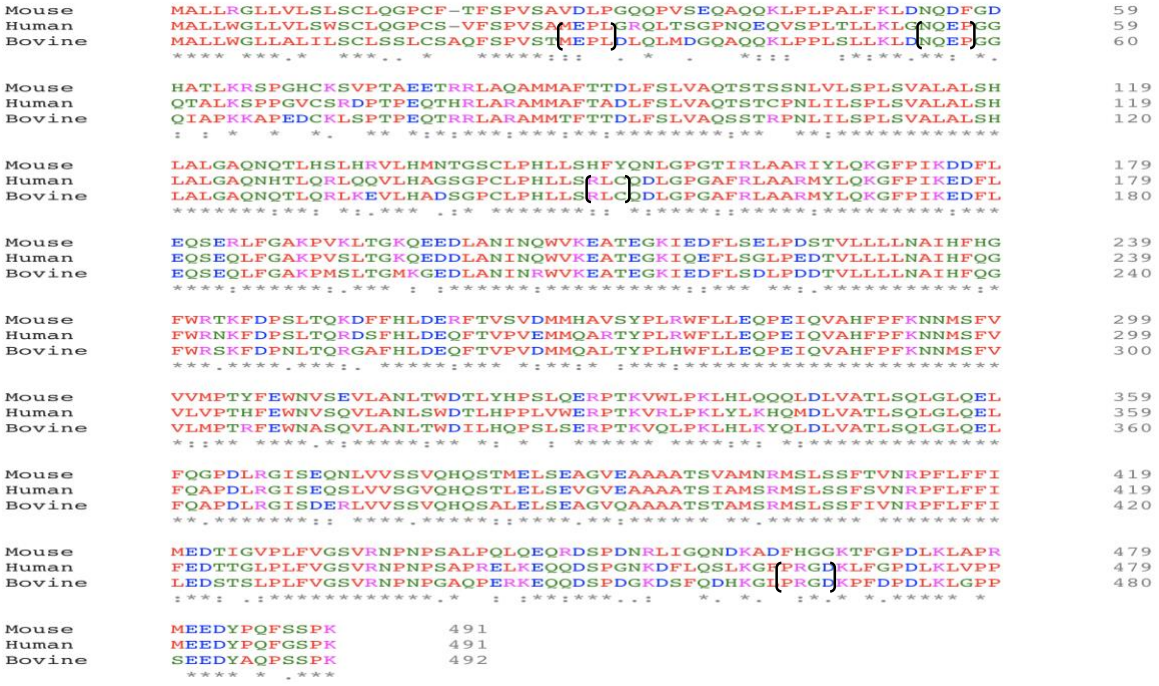

Figure 55. sequence alignment of PI (Human, Mouse, and Bovine).

5.3.3.3 Effect of PI and PI without C-terminus in purified system

In order to investigate wither C-terminus of PI involved to interact with Affimer A68 or not, turbidimetric assays were used. Purified PI without C-terminus (ART PI) and purified PI full length (normal PI) were tested to study the effect of Affimer A68 on both. The effect of Affimer A68 was tested using purified system with 20:1 PI:Affimer A68 ratio. Affimer A68 modulated clot lysis time in both but the effects on full length of PI was more pronounced than clots made in the presence of C-terminus truncated PI (Figure 56).

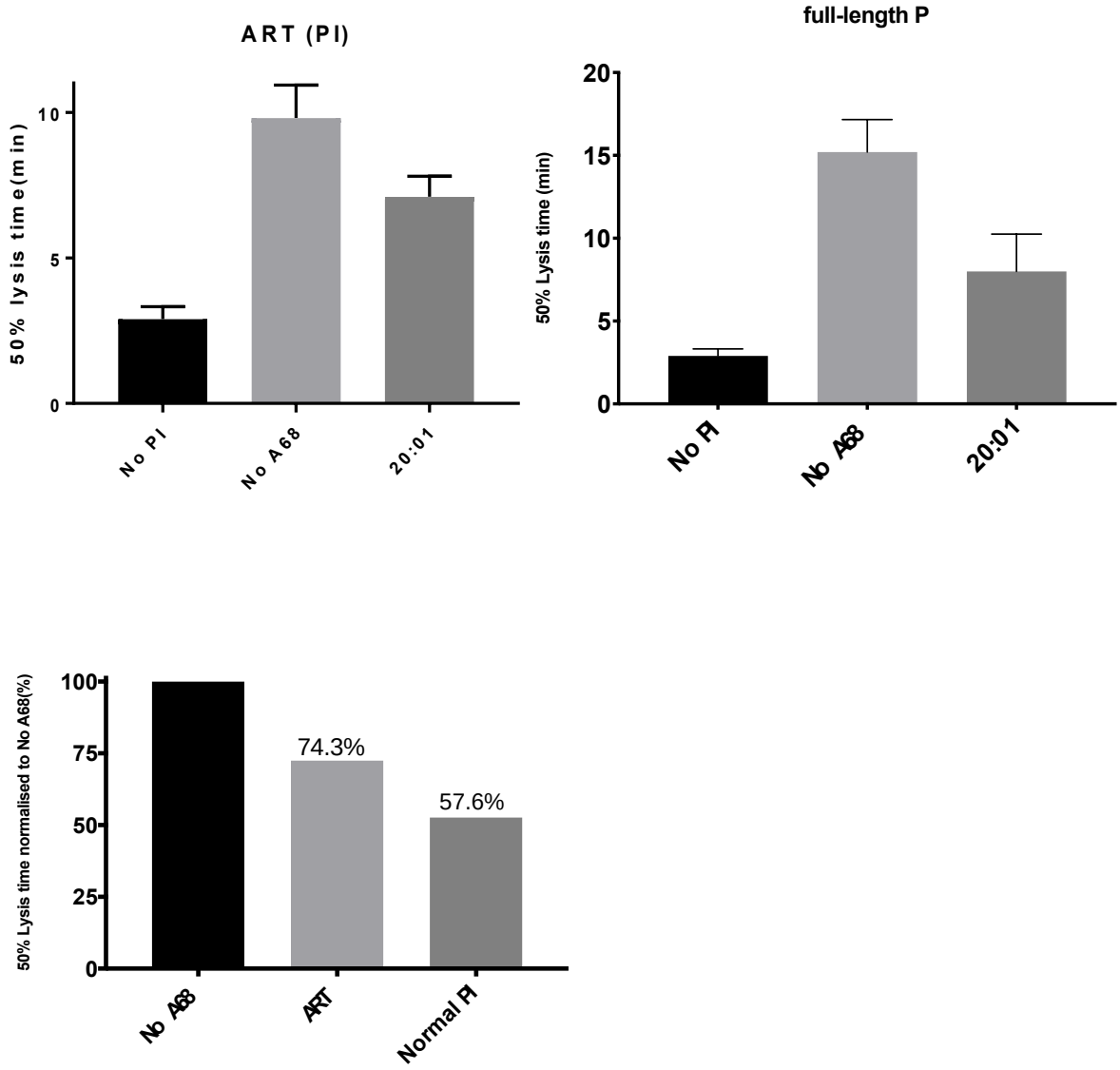

Figure 56 Effect of PI and PI without C-terminus in Purified system. A Clot lysis time in the presence of C-terminus truncated (ART PI). B. Clot lysis time of full-length PI. In both figure NO PI: clots made from purified fibrinogen in the absence of PI. Affimer A68 was used at 20:1 Affimer:PI molar concentration and all experiments contained FXIII at 22 µg/ml.

5.3.4 Interaction sites of A68

5.3.4.1 Effects of Loop1 and Loop2 of A68 on plasma clot lysis

In order to investigate whether both loops of A68 are required for binding or just one of them, two new constructs were designed. I produced two constructs (pET11 plasmid) A68 (Loop1/AAE) and A68 (AAAA/Loop2). The original cystatin molecule has four amino acids in the first variable region and three amino acids in the second variable region. To silence one of the variable regions, we decided to replace the first

variable region with four alanine (same AA as the original) and the second with two alanine plus E (AAE). Alanine was chosen as it is a non-bulky and a chemically inert amino acid. For the second variable region PW was replaced by AA as these two amino acids are part of the inhibitory function of the original cystatin. We kept the glutamic acid E, therefore AAE. The constructs were analysed by SDS-PAGE (Figure 59). The effects of Loop1 and Loop2 of A68 were investigated using turbidimetric assay in plasma samples. Loop1 showed significant reduction in clot lysis time ($p=0.04$), while Loop2 had no effect ($p=0.4$). Affimer A68 with both loops and scaffold were also tested as positive and negative control, respectively. Taken together, A68-Loop1 appears to be the one responsible for inhibition of PI activity (Figure 58). In order to further confirm that Loop 2 is not involved in enhancing the fibrinolysis, different concentration of the Affimer were used at 10:1, 20:1, 40:1, and 80:1 Loop2:PI molar ratio. In 4 individual experiments, each performed in duplicate, Loop 2 failed to enhance fibrinolysis and, if anything, it showed prolongation in lysis although this was not significant. Of note, the effects of loop 1 on lysis enhancement were consistently inferior to double loop A68. This suggests that although loop 2 has no effect on lysis, it may have a role in stabilising A68-PI interaction, thus making the fibrinolytic effect of loop 1 more pronounced.

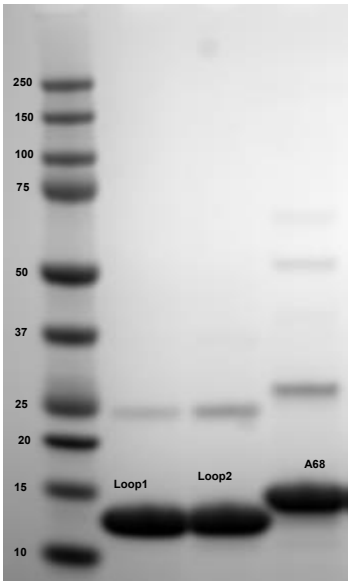

Figure 57. SDS-PAGE Molecular weight analysis of purified Loop1 and Loop 2 of A68 Affimers on a 4-12% Bis-Tris gradient gel.

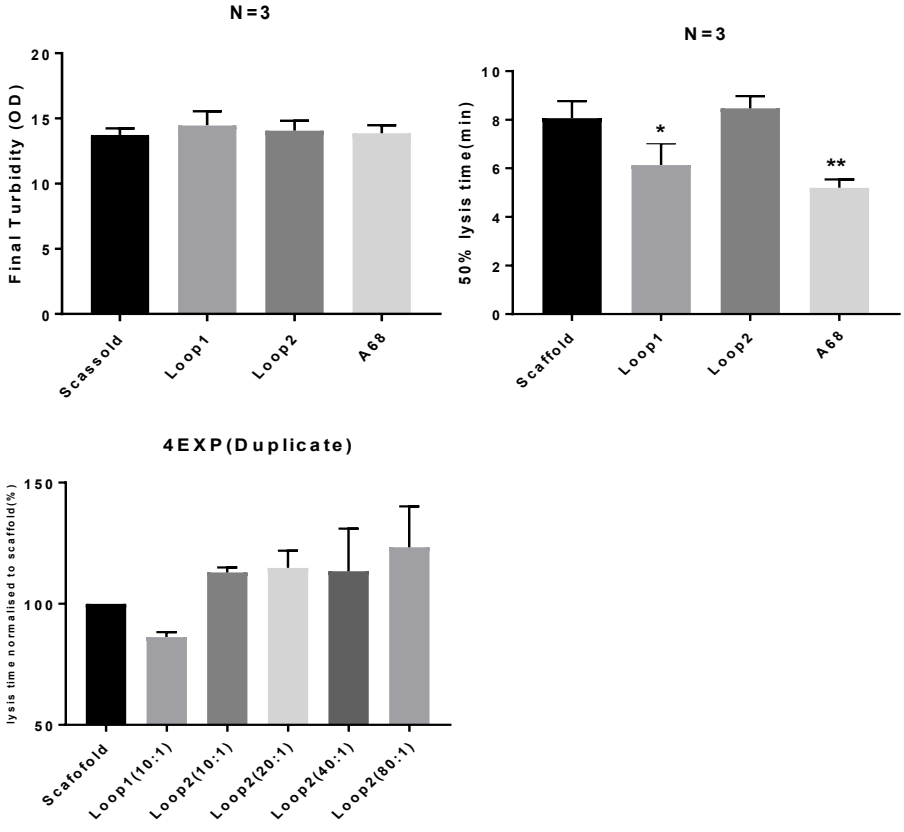

Figure 58. Effect of Loop1 and Loop2 of A68 in plasma system on fibrin network characteristics.
Affimer A68, Loop1 and Loop2 did not show an effect on clot final turbidity clot structure. B. A68 and Loop1, but not Loop2, had a significant effect on clot lysis time. C. Loop2 had no effect on lysis time even when used at very high concentrations.

Plasma system	Lysis time (min, mean±SD)
A68	5.2 ± 0.2 **
Loop1	6.133 ± 0.5207 *
Loop2	8.467 ± 0.2906
Scaffold	8.067 ± 0.4055

Table 14 The effects of Loop1 and Loop2 of A68 on plasma clot lysis. A total of three independent experiments were performed in duplicate, data presented as mean±SD. Statistical analysis was performed using t-test.

5.3.4.2 Site-directed mutagenesis of Loop1

Affimer A68-Loop1 has thus far been shown to enhance clot lysis, and therefore mutagenesis studies were conducted to clarify the functional residue(s) responsible for inhibiting PI function. Turbidimetric assays were then performed to understand the effect of each mutation on clot lysis time. The mutant that had no effect in clot lysis time suggest that this residue (the missing residues) is involved in the interaction with PI. On other hand, the mutant that continued to alter clot lysis time indicates that this residue is not involved in the interaction. The results showed that 5 key residues on Loop1 had no significant effect in clot lysis which are (N, F, P, K and Y50). These data suggest that 5 residues in Loop1 of A68 are important for the interaction and enhancement of fibrin clot lysis (Figure 59).

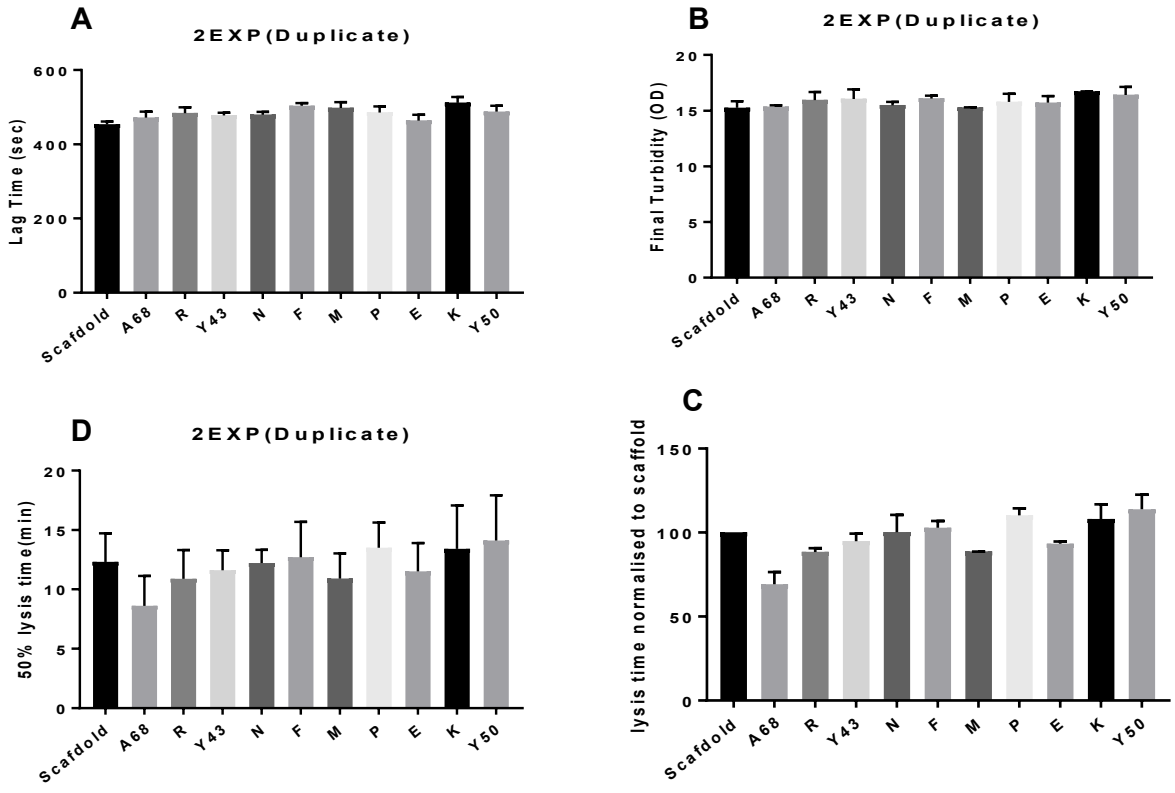

Figure 59. Effect of each mutation on clot lysis time of A68-Loop1 in clot structure/lysis

A Lag time B Final Turbidity (OD) C. Lysis time. D. Lysis time normalised to scaffold. The 5 residues that appear to be important for the function of A68 are underlined in red.

5.3.4.3 Molecular Modelling of mutated Loop1

Experimental data showed that mutation of many of the amino acids in loop1 of Affimer A68 affected its interaction with PI. The amino acids contained within loop1 were subjected to energy minimisation, with the rest of the scaffold frozen. An energy minimised version of the original A68 Affimer was used to compare to each of the alanine mutants.

Mutants were made in the Affimer scaffold containing both loops and also just loop1, to see if mutations in loop1 affected residues in loop2. Each mutation is discussed in Table 15.

Amino acid	**loop1**	**both loops**
R42A	looks identical to A68 except for loss of Arg side chain at start of loop	looks identical to A68 except for loss of Arg side chain at start of loop. R42 is not near loop2 so doesn't affect conformation.
Y43A	loss of Tyr side chain and slight movement of Asn44. Twist of Phe45 and Tyr50	loss of Tyr side chain and slight movement of Asn44
N44A	looks identical to A68 except for loss of Asn side chain at start of loop. N44 Makes H-bond to K49 so loss of this may affect loop conformation	slight twist of Tyr43. N44 Makes H-bond to K49 so loss of this may affect loop conformation
F45A	lots of movement of R42-N44. Loss of Phe45 side chain.	lots of movement of R42-P47. Loss of Phe45 side chain
M46A	looks identical to A68 except for loss of Met side chain at start of loop.	looks identical to A68 except for loss of Met side chain at start of loop.

P47A	movement of Met46 and slight movement of Phe45	big movement of Met46 and slight movement of Phe45
E48A	lots of movement of N44-P47. Loss of Glu48 side chain.	lots of movement of N44-P47. Loss of Glu48 side chain but not close enough to Q78 in loop2 to affect this.
K49A	very similar to A68, slight twist in Phe45	very similar to A68, slight twist in Tyr43
Y50A	big movement in R42	big movement in K49 F45 and Y43

Table 15 Effect of changes in AA of loop1 on Affimer conformation analysed as a single or double loop Affimer.

5.4 Discussion

Identifying molecular mechanisms for PI-inhibitory effect of Affimer A68 was the main focus here and in particular identifying Affimer PI interaction sites, since results will undoubtably help in the design of future small molecules that can mimic the action of the Affimer while being easier to manufacture and administer. This procedure was not straightforward and different methodologies were used to pinpoint interaction sites. Results in general were effective in constructing a general understanding of the potential interactions between PI and Affimer A68. Four major corners were investigated to build a holistic picture of such interaction: type of interaction, cross linking analysis, interaction sites of PI and interaction sites of A68. Results showed that the interaction between PI and Affimer A68 that results in functional changes to the proteins is mainly located within Loop1 but a role of Loop2 at stabilising the binding cannot be ruled out.

Data from Western blot analysis as well as the linear peptide experiments, strongly suggest that the interaction is conformational. In retrospect, I should have used both linear peptides of Loop1 and Loop2 together to conclusively rule out that these peptides have no effect on lysis. However, given that Loop1 seems to be the “functional” loop, it is unlikely that combining the two linear peptides would have changed the results.

Despite screening Affimers against PI in order to isolate PI binders, it was important to determine the specificity of Affimer A68. While ELISA results described in Chapter 3 show specificity of A68, it was necessary to confirm that Affimer A68 interacts with PI in solution and the pull-down assay was used for this purpose. While these assays managed to “pull down purified PI, it failed to detect the protein in plasma. This is likely due to the small amount of PI in plasma, beyond the detection limit of the assay. Alternatively, it may be due to plasma protein interfering with the interaction. However, the latter scenario is unlikely given that Affimer A68 consistently reduces plasma clot lysis.

Mass Spectrometry was used to analyse cross linking between PI and Affimer A68, A procedure was designed and conducted by incubating Affimer A68 with PI to uncover interaction sites between the two molecules. Initial experiments were conducted without a linker, but protein coverage was very poor to provide conclusive results. Therefore, the methodology was refined using linkers in order to maintain the

conformation of mixture. This technique has shown potential interaction sites: NQE on PI binding to IVNFKE on A68, NQE from PI binding to FVRVVKAKE on Affimer A68 and finally, GKIQE on PI binding to GGKKKLYE on Affimer A68. However, these experiments suggested some interaction sites, but these were conserved in human and mouse PI, casting doubt on their importance (given that A68 does not affect mouse PI, as detailed below). Overall these experiments failed to give conclusive results and therefore more work is this area is required.

Affimer A68 failed to reduce clot lysis time in mouse plasma demonstrating that A68 interacts with PI in an area that is not conserved between human PI and mouse PI. In addition to lysine residues, the C-terminal region of human PI also contains arginine glycine-aspartic acid (RGD). This RGD sequence is conserved in human and bovine PI but this does not extend to other mammalian species such as mice, rates or rabbits [73]. My results confirmed Affimer A68 ability to reduce lysis time of bovine but not mouse or rat clots with some effect observed with pig clots, albeit non-significant. These data, together with my findings that Affimer A68 effects may be more prominent in whole blood, suggest that RGD area may be one interaction site.

The effect of Affimer A68 was tested in different molecular forms of PI non-plasminogen-binding forms (ART-PI) and full-length PI on purified system. The results showed that Affimer A68 block PI-induced prolongation of clot lysis with full length PI more than truncated version of the protein. This suggests that: i) one interaction site may well be at the C-terminus of the protein, which is further supported by the fact that this Affimer has an effect on bovine but not mouse plasma clot lysis (C-terminus in mice is different), and ii) there is possibly more than one interaction site between Affimer A68 and PI given that the Affimer modulated inhibition of lysis by truncated PI (albeit to a lesser degree). Finally, I note that the effects of Affimer A68 was particularly pronounced in whole blood environment (section 4.3.6), which may be related to blockade of the RGD sequence on the C-terminus of PI [81].

Therefore, I have a tentative interaction site on PI, which leaves the question of interaction site on A68. Having established Loop1 responsibility for inhibiting PI function, mutagenesis studies were carried out to understand the key AA responsible for this effect. N, F, P, K and Y50 appeared to be important for Affimer function. This indicates that more than one AA is important for Affimer A68 function. Further mutagenesis studies may be an option to enhance the fibrinolytic capacity of Affimer A68 and this remains an area for future research.

Molecular modelling further supported a role for Loop1 in PI inhibition by showing potential interaction with the protein.

Chapter 6 : General Discussion

Impaired fibrinolysis is associated with increased risk of cardiovascular events and manipulating clot lysis may offer an alternative therapeutic strategy to reduce the risk of vascular occlusive disease [258]. However, current modalities for long-term modulation of fibrin clot formation/lysis, such as the use thrombin and FX inhibitors carry increased risk of bleeding [259].

The structure of the fibrin network and its breakdown have been the focus of much research, as clot susceptibility to lysis has implications for both bleeding and thrombotic disorders. Vascular occlusive disease, including myocardial infarction, stroke and venous thrombosis, remain the main cause of mortality in the Western population [267]. Treatment is aimed at reperfusion to regain blood flow through the affected vessel followed by prophylactic therapy to prevent re-occlusion. Potential therapies aimed at facilitating clot lysis by targeting the fibrin network have been explored [109] and this strategy may have particular benefit in subpopulations at increased risk of thrombosis due to hypofibrinolysis.

My work utilises an alternative methodology by endeavouring to reduce the incorporation of PI into fibrin clots, therefore facilitating lysis. Moreover, this PI-specific mode of action carries the theoretical advantage of lower risk of bleeding given the targeted approach. Earlier in vitro and in vivo studies, utilising PI-specific monoclonal antibodies to improve fibrinolysis, have shown promise [268] and a clinical preliminary trial has been in progress investigating the role of such antibodies in patients with pulmonary embolism (study has not reported to date). However, use of antibodies may be problematic due the potential large cost and the risk of developing anti-idiotypic antibodies given the large size of antibodies, and employing smaller proteins may reduce the risk of such complications. Moreover, the potential short in vivo half-life of Affimers can be an advantage in acute vascular occlusion. Some may argue that this short half-life is a potential weakness but Affimers are easily amenable to modification to lengthen half-life and therefore can, in theory at least, be used in a variety of vascular conditions to include acute and chronic disease.

Isolation of Affimer A68

My work has so far shown that Affimers against PI can be used as a tool to facilitate clot lysis, providing a viable alternative to monoclonal antibodies.

A total of one hundred and sixty-seven high affinity PI-binding Affimers were isolated, of which twenty-two had distinct sequences, and these were subsequently expressed in *E.coli*. One Affimer, termed A68, consistently inhibited the prolongation of PI-induced clot lysis in a purified system (confirming specificity) and plasma system (ruling out significant interaction with other proteins).

In the first screening process, I was unsuccessful at identifying PI-binding Affimers that modulate fibrinolysis, which is probably due to the limited number of clones picked. Only one Affimer (A3), showed reduction in final turbidity when compared to the scaffold protein but had no measurable effect on clot lysis. The lack of an effect on lysis time was consistent using purified experiments or plasma samples.

In a second round of screening, a much larger number of clones were selected which resulted in the identification of at least one PI-binding Affimer that consistently modulated fibrin clot lysis (Affimer A68). In particular, this Affimer showed consistent effects in plasma samples, including individual samples from different medical conditions, and also showed an effect using purified proteins (confirming specificity) and whole blood samples from different individuals (confirming the absence of significant interaction from bloods cells or plasma proteins). Interestingly, the effect of Affimer A68 on fibrin clot lysis when studied in fully mature clots (i.e. confocal microscopy) is consistent. Lysis conducted on mature clots allows more time for incorporation of PI into the clot thus the “blocking effects” of Affimer A68 become more obvious.

This consistency of action indicates the possibility of using this Affimer in clinical settings, although safety data are required before going down that route. A disadvantage of Affimer A68 is the fact that the scaffold is a plant origin, making direct clinical use potentially problematic. However, early work indicates that immunogenicity of Affimers having plant-based scaffold and human-based scaffold are not dissimilar (unpublished work by) and therefore clinical use of A68 remains a possibility. Moreover, the structure of this Affimer may be used to develop human scaffold based Affimers or simply employed to identify new therapeutic targets, where small molecule technology can be used.

Importantly, my results gave a proof of concept confirming that Affimers can be used for PI-targeted modulation of fibrinolysis. This opens the door for a new methodology to manipulate thrombosis risk with potentially exciting clinical applications.

Affimer A68 and clot structure

Affimer A68 reproducibly altered fibrin clot lysis without having an effect on clot turbidity, indicating no significant changes in clot structure. This was indeed confirmed with confocal and SEM experiments which showed that Affimer A68 ensures the presence of physiological clot structure, although subtle differences in fibre thickness were detected, which were not picked up by the turbidimetric analysis. This emphasises the importance of using multiple techniques when analysing clot structure as reliance on a single methodology may draw inaccurate conclusions. The mechanisms for altered fibrin thickness by Affimer A68 is not entirely clear but may be related to altered incorporation of PI into the clot or the direct effect of the Affimer on fibre thickness. This can be investigated by comparing purified clots in the presence and absence of PI or Affimer A68.

Affimer A68 interaction sites

Affimer A68 sites

After doing a blast screen, it became apparent that one of the Loops in Affimer A68 shared sequence homology with plasminogen. This provided the possibility that this Affimer enhances lysis by competing with plasminogen binding to fibrin(ogen). However, this scenario is unlikely as my experiments failed to provide evidence that A68 facilitates clot lysis in the absence of PI. Therefore, the effects are likely to be related to inhibitory effect on PI, related to reduced incorporation into the clot. This concept is supported by my confocal microscopy data showing reduced incorporation of PI into fibrin clots in the presence of Affimer A68.

To clarify Affimer A68-PI interaction sites, I first investigated linear peptides of the variable Loops on the assumption that if these have an effect on lysis, it would be much easier to investigate interaction sites. However, none showed an effect confirming that the interactions are conformational, which was not surprising.

I then moved to produce a single Loop Affimer to understand which of the two Loops is responsible for the PI-inhibitory action. Two additional Affimers were produced, each containing just one of the Loops of Affimer A68 (either Loop 1 or Loop 2) to understand

which of the two Loops is essential for Affimer function. My data show that Loop one inhibits PI activity, whereas Loop two has no effect. However, the effects of Loop one was weaker than Affimer A68, suggesting that Loop two is important to stabilise Affimer A68-PI interactions, even if it shows no effect on protein function.

After establishing that Loop 1 of Affimer A68 is important for protein function, I undertook in depth analysis of functional residue(s) responsible for the fibrinolytic effect using site-directed mutagenesis. I found five key residues on Loop1 that seemed to be crucial for the function of Affimer A68 (N, F, P, K and Y50). Therefore, there are multiple AA that are essential for the activity of Affimer A68.

Finally, a homology model of human PI based on the published crystal structure of mouse PI was investigated for interaction with Affimer A68 (based on the published crystal structure of Affimer scaffold). Using a molecular modelling of whole A68 (with both Loops 1&2), the Affimer with just Loop1 and the third Affimer with Loop2 only demonstrated that Loop1 interacts with PI while Loop2 fails to show such an interaction. Interestingly, all these ducking share the same area in PI which is between PRO-18 and ASN-26.

PI interaction sites

To pinpoint the interaction sites of Affimer A68 on PI, I used a number of methods. The quickest was studying clot lysis in plasma samples from different species. This showed that Affimer A68 has largely similar effects in bovine and human PI, and to a lesser extent pig, but failed to demonstrate an effect in mouse or rat plasma. There are conserved areas in the N- and C-terminus of bovine and human PI which are different in mouse and rat PI. In particular, the presence of a RGD area in the C-terminus (as detailed Chapter 5, page 54) of bovine and human, but not mouse and rat, together with the suggestion that Affimer A68 had more of an effect in whole blood than plasma systems (by inhibiting interaction with platelets), suggests that this area in human PI is important for interaction with Affimer A68. Moreover, molecular modelling data suggest that this is indeed a possible interaction site adding weight to this concept. Future work will be required to investigate this possibility, including the use of RGD sequences to “absorb-out” the effects of Affimers.

Mass spectrometry has been equally inconclusive regardless as to whether we used simple conditions or supplemented the method with the use of linkers. However, one caveat in this work is the failure to establish inter-molecular links within the protein

suggesting issues with the methodology. Unfortunately, due to the limited time, I have been unable to investigate the possibility of using different linkers and this remains an area for future work.

In conclusion, my data show that the Affimer technology is a viable methodology to inhibit specific proteins in the coagulation system in order to modulate the fibrinolytic process. Affimer A68 has consistently shown enhancement in clot lysis while maintaining physiological clot structure. This opens a new avenue in the treatment of thrombotic conditions by targeted modulation of anti-fibrinolytic proteins. Suitable Affimers may be used directly in vascular occlusive disease, after appropriate safety studies, or can be employed as reagents to identify novel therapeutic targets that are amenable for small molecule intervention.

Future work

Although Affimers and other engineered protein scaffolds are relatively new when compared to antibodies, they have already proven to be useful as molecular biology reagents in a variety of environments, including imaging, purification of proteins, and altering protein function [212]. Moreover, protein-based engineering scaffolds have been designed for human clinical trials, and the concept of scaffold-based treatments has already been proven with the approval of Ecallantide (Kalbitor) an agent used for the treatment of hereditary angioedema [224].

My work has shown that Affimers represent a tool to modulate clot lysis. The way forward is clear:

- Further characterisation of the mechanistic pathways for Affimer A68 reduction of clot lysis is required. This is likely to involve complex crystallisation studies, which will help to clarify various interaction sites.
- Generation of PI mutants in areas conserved in human and bovine PI but not mouse or rat. If mutated PI fails to bind Affimer A68 then this will help to pinpoint interaction sites, some of which may be suitable for small molecule intervention. One caveat of this approach is that mutating PI may cause conformational changes in the molecule altering interaction sites outside these areas.
- Most importantly, future work should focus on studying the in vivo effects of Affimer A68 using suitable animal models of thrombosis. This should also

include studies on pharmacokinetics, immunogenicity and toxicology to ensure the safety of the Affimer. Also, studies may be needed to identify ways to prolong the half-life of the Affimer such as PEGylation. One difficulty is the absence of an effect in mouse plasma and therefore if mice are used, it is best to use PI-deficient mice supplemented with human PI. An alternative approach is to supplement wild type mice with human PI as work from my colleagues within the team has shown that this approach results in further prolongation in clot lysis and may be easier that breeding PI-deficient mice, at least for initial experiments.

- Given the results of the mutagenesis studies, suggesting that 5 key AA residues in loop 1 of Affimer A68 are important for inhibiting PI function and the fact that loop 2 does not possess an inhibitory activity, future work may involve: i) structuring a new library of Affimers that contain the 5 key AA in Loop 1 with random AA in the rest 4 AA in the same Loop, which may increase the inhibitory activity of the Affimer, ii) explore changing AA in Loop 2, including the possibility of mirroring Loop 1 residues, thus creating a new PI-specific Affimer with increased efficacy. Overall, this will allow the use of lower Affimer concentrations thus making it even more cost effective for use in the clinical field.
- The fact that the scaffold is of plant origin remains a concern in case Affimer A68 is to be used clinically. Therefore, consideration should be given to developing a human scaffold-based Affimer that has similar variable regions compared with A68 that can be used clinically.

In order of importance, I feel the next step should be animal in work to ensure that the effects of Affimer A68 are evident in vivo. This can then be followed by steps to improve the efficacy of A68 and “humanise” the scaffold protein to make it suitable for potential human studies. Crystallography can be done simultaneously as this technique is both time consuming and challenging with success never guaranteed. Taken together, this highlights the difficulties and time commitment needed to develop new therapeutic agents that classically takes 10-15 years, even in the best equipped centres.

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