The role of complement C3 in Diabetes

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The work in Chapter 3 of the thesis has appeared in publication as follows:

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I was responsible for turbidity and lysis experiments and contributed to and edited the manuscript.

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Abstract

The prevalence of diabetes worldwide continues to rise and with it the burden of cardiovascular disease (CVD). This is related to clustering of cardiovascular risk factors in diabetes, coupled with a deranged haemostatic system, generating a pro-thrombotic environment. Matters are compounded further by increased incorporation of proteins such as plasmin inhibitor (PI) and complement C3 into the clot contributing to a hypofibrinolytic state. C3 is a key component of the complement pathways and elevated plasma levels are seen in diabetes and correlate with clot lysis time in type 1 patients, which may relate to increased incorporation of C3 into the clot compared to controls. *Ex-vivo* analysis demonstrates that C3 causes a prolongation in clot lysis, and this is more pronounced in diabetes. Targeted interference with fibrinolysis, such as inhibition of fibrinogen interactions with C3, may lead to the development of diabetes specific therapeutic agents. The aims of my work were therefore to i) further study the role of C3 in hypofibrinolysis in relation to diabetes ii) elucidate potential binding sites between fibrinogen and C3 and iii) modulate fibrin clot lysis by targeted interference with fibrinogen-PI and fibrinogen-C3 interactions.

My work has helped demonstrate that complement C3 plays an important role in hypofibrinolysis in type 2 as well as type 1 diabetes, an effect that shows wide inter-individual variability and may relate to differing rates of glycation of C3 in diabetes. I have also identified potential interaction sites of this protein with the β chain of fibrinogen. Moreover, I have helped develop a new method, using Adhiron technology, for the identification of novel therapeutic targets that can be used to enhance the fibrinolytic process and reduce thrombosis risk.

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List of abreviations

α_2-AP	Alpha-2-antiplasmin
AGE	Advanced glycation end product
AM	Activation mix
BMI	Body mass index
BSA	Bovine serum albumin
CAD	Coronary artery disease
cDNA	Complementary deoxyribonucleic acid
CFT	Clot formation time
COX-1	Cyclooxygenase-1
CRP	C-reactive protein
CVD	Cardiovascular disease
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EB	Elution buffer
EC	Endothelial cell
ECM	Extra cellular matrix
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
GP	Glycoprotein
FV	Factor 5
FVII	Factor 7
FVIII	Factor 8
FIX	Factor 9
FX	Factor 10
FXI	Factor 11

FXII	Factor 12
FXIII	Factor 13
FXIIIa	Activated factor 13
FpA	Fibrinopeptide A
FpB	Fibrinopeptide B
HDL	High density lipoprotein
IGF-1	Insulin-like growth factor-1
IHD	Ischaemic heart disease
IL-6	Interleukin 6
LDL	Low density lipoprotein
LM	Lysis mix
LSCM	Laser scanning confocal microscopy
LC-MS	Liquid chromatograph mass spectrometry
LT	Lysis time
MA	Maximum absorbance
MAC	Membrane attack complex
MASP	MBL associated serine protease
MBL	Mannose binding lectin
MetS	Metabolic syndrome
MI	Myocardial infarction
MG	Macroglobulin
MMP	Matrix metalloproteinase
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
OD	Optical density
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2

PB	Permeation Buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PDGF	Platelet derived growth factor
PG	Prostaglandin
PI	Plasmin Inhibitor
ROS	Reactive oxygen species
PTM	Post translational modification
SB	Starting buffer
SD	Standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SPR	Surface plasmon resonance
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TAE	Tris-acetate-EDTA
TAFI	Thrombin activatable fibrinolysis inhibitor
TBS	Tris buffered saline
TCC	Terminal complement complex
TED	Thioester containing domain
TF	Tissue Factor
TFPI	Tissue factor pathway inhibitor
TMB	Tetramethylbenzidine
TNFα	Tissue necrosis factor alpha
tPA	Tissue plasminogen activator

Tris	Tris(hydroxymthyl)aminomethane
TSH	Thyroid stimulating hormone
U/ml	Unit per millilitre
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
vWF	Von Willebrand factor

Publications and presentations

Publications

K Hess, S H Alzahrani, JF Price, MW Strachan, N Oxley, R King, T Gamlen, V Schroeder, PD Baxter and RA Ajjan. **Hypofibrinolysis in type 2 diabetes: the role of the inflammatory pathway and complement C3**. Diabetologia. 2014 Aug; 57 (8): 1737-41.

Adhirons: A novel methodology for modulation of fibrin clot lysis

R King, C Tiede, K Smith, A Tang, K Simmons, S Ponnambalam, CWG Fishwick, MJ McPherson, DC Tomlinson, RA Ajjan

Under review

Oral presentations

Inhibiting complement C3 and fibrinogen interaction: A potential novel therapeutic target to reduce cardiovascular disease in diabetes

R King, C Tiede, V John, A Trehan, K Simmons, CWG Fishwick, MJ McPherson, DC Tomlinson, RA Ajjan

Health Education Yorkshire and Humber Academic Presentation Day, Goole, June 2014

Inhibiting complement C3 and fibrinogen interaction: A potential novel therapeutic target to reduce cardiovascular disease in diabetes

R King, C Tiede, V John, A Trehan, K Simmons, CWG Fishwick, MJ McPherson, DC Tomlinson, RA Ajjan

North European Young Diabetologist meeting, Devon, May 2014.

Inhibiting complement C3 and fibrinogen interaction: A potential novel therapeutic target to reduce cardiovascular disease in diabetes

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Leeds Institute of Genetics, Health and Therapeutics Postgraduate Symposium, March 2014, Leeds, UK

Poster presentations

Inhibiting complement C3 and fibrinogen interaction: A potential novel therapeutic target to reduce cardiovascular disease in diabetes

RJ King, C Tiede, K Simmons, V John, A Trehan, C Fishwick, D Tomlinson, RA Ajjan.

Diabetes UK Annual Professional Conference, March 2014, Liverpool, UK

Fibrinogen targeted Adhirons can successfully modulate fibrin clot lysis in the presence of α2-AP

RJ King, C Tiede, D Tomlinson, RA Ajjan

XXIV Congress of The International Society on Thrombosis and Haemostasis (ISTH), July 2013, Amsterdam, NL

Chapter 1

Introduction

Cardiovascular disease (CVD) is a major cause of death in Western populations. In the UK in 2013, CVD was eclipsed only by cancer as the leading cause of death, accounting for 28% of all deaths registered (ons.gov.uk). Diabetes mellitus (DM) is known to increase the risk of developing CVD, which remains the main cause of mortality in these individuals[1]. Not only is the risk of developing a first cardiovascular event increased in diabetes, these individuals also have a poorer prognosis following treatment compared to those without diabetes, regardless of the treatment given in the acute stage [2-5]. With the number of patients with diabetes reaching 3 million in the UK alone in 2012 (www.diabetes.org.uk) an increased understanding of the disease processes involved in DM and improved therapeutic strategies will be required to reverse this unnerving trend, This strong association between DM and CVD is multifactorial and is related to clustering of classical cardiovascular risk factors, including hyperglycaemia, obesity, hypertension, dyslipidaemia and increased thrombotic potential. The pro-thrombotic environment that is associated with DM arises secondary to a combination of increased platelet activation and enhanced fibrin clot formation along with compromised clot breakdown, or hypofibrinolysis. Chronic inflammation contributes to these disease processes and is also involved in the development of atherosclerotic plaques. Recent evidence suggests a close link between the inflammatory and coagulation pathways[6], and therefore inflammation plays key roles early and in late stages of the atherothrombotic process.

1.1 Coagulation and fibrinolysis

The formation of blood clots and their subsequent destruction, or lysis, is a vital physiological function aimed at preventing acute blood loss following trauma.

This is termed haemostasis, and comprises finely balanced interactions between the vessel wall (in particular the endothelium), platelets, along with procoagulant and anticoagulant proteins. The resultant plug of platelets, red blood cells, leucocytes and fibrin fibres seals the vessel and prevents leakage of blood from the vascular tree, and subsequently allows the formation of new tissue and wound healing. In contrast to this, thrombosis should be viewed as a pathological state, where the formation of a blood clot in a diseased blood vessel results in partial or complete blood flow obstruction, consequently resulting in end organ damage.

1.1.1 Blood vessels

The blood vessel provides the setting and personnel essential to the ordered sequence of events involved in coagulation and haemostasis, and is also the scene of deleterious thrombosis related to atherosclerotic plaque rupture. Knowledge of blood vessel biology and anatomy is therefore crucial to understanding these mechanisms. Arteries and veins throughout the body are lined by a single layer of endothelial cells (EC). Supporting the EC is a loose network of tissue, the extracellular matrix and together they form the intima. Below this layer is the media (consisting mainly of smooth muscle cells) and then the adventitia. The latter two layers are less developed in veins compared to large and intermediate arteries and are not visible in smaller arterioles and capillaries[7].

1.1.1.1 Endothelial cells

EC play a vital role in haemostasis through the production of numerous proteins and surface receptors that help regulate vascular tone, clot formation and cell

adhesion. Constriction and dilation of blood vessels is controlled by the release of several proteins, the most important of which are endothelin, and angiotensin II, which mediate vasoconstriction, whereas nitric oxide (NO) and prostacyclin are responsible for vasodilation. They synthesise and secrete coagulation proteins von Willebrand factor (vWf) and tissue factor (TF) essential for the initiation of clot formation and platelet activation. Several anticoagulant proteins and those involved in clot lysis are also produced including tissue factor pathway inhibitor (TFPI), thrombomodulin and tissue plasminogen activator (tPA). Receptors present on the surface of EC also help activate other fibrinolytic and anti-fibrinolytic proteins such as protein C and thrombin activatable fibrinolytic inhibitor (TAFI). These proteins will be discussed in more detail in section 1.3. The EC are therefore equipped to initiate the coagulation pathway plugging the breached vessel wall and help confine the clot to the site of injury through regulatory pathways. Adhesive receptors are also present and these are responsible for adhesion of inflammatory cells to EC. The function of EC and their morphology can vary depending on their anatomical position, for example cells within the brain form very tight junctions, contributing to the blood brain barrier, whereas in the liver, gaps between cells are much wider to facilitate soluble and cellular flow between blood and the extravascular space[7].

1.1.1.2 Extracellular matrix

The extra cellular matrix (ECM) is a complex structure that contains numerous proteins, which are involved in maintaining vessel wall structure, platelet adhesion and sustaining of matrix proteins. The most abundant protein is collagen (of which there are numerous types) which together with elastin provides structural support to the blood vessels. Numerous adhesion proteins,

such vWf, fibronectin, vitronectin and laminin (together with collagen) promote platelet adhesion and contribute towards fibrinolysis. Proteoglycans such as heparin sulphate also exhibit anticoagulant properties and may also contribute to the structural integrity of the vessel by combining with collagen. A group of enzymes, named matrix metalloproteinases (MMP) are responsible for the degradation and repair of most of the ECM proteins[7].

1.1.1.3 Smooth muscle cells

Smooth muscle cells contained within the media layer play a role in vessel contractility in response to molecules such as NO and endothelin. They produce a number of the proteins seen within the ECM such as collagens, elastin, glycoproteins, proteoglycans and TF. They also produce several growth factors including vascular endothelial growth factor (VEGF) and insulin-like growth factors (IGF) that are involved in smooth muscle cell generation[7].

1.1.1.4 Adventitia

This final layer consists of a loose network of cells made up of fibroblasts, adipocytes and mast cells. These cells are responsible for synthesising collagens, glycoproteins, elastin, TF and lipids[7].

1.1.2 The coagulation cascade

The final step of the coagulation cascade is the formation of a cross-linked fibrin clot, which follows a sequence of enzymatic reactions and activation of inactive precursors. Until recently, these reactions were divided into two separate pathways, intrinsic and extrinsic, with differing coagulation proteins, or factors involved in each pathway, which ultimately converge to a common pathway culminating in the conversion of soluble fibrinogen into insoluble fibrin networks.

The intrinsic pathway results in sequential activation of factors (F) XII, XI, IX, VII and V, whereby the extrinsic pathway is dependent upon FVII being activated by tissue factor (TF). The common pathway involves activation of FX which converts prothrombin (II) to thrombin (IIa) and finally the formation of fibrin[8]. Although these systems represented *in vitro* coagulation, it failed to explain the clinical manifestations of clotting disorders such as a defect in FVIII seen in haemophilia. Increased understanding of the interplay between different coagulation factors and also their interaction with platelets, particularly thrombin generation, has led to a more contemporary model of coagulation[9]. This model is divided into three phases, initiation, amplification and propagation, underpinned by plasma exposure to TF[10] (Figure 1-1).

Following injury to the vascular system, EC bearing activated TF are exposed to the circulating plasma which contains all the necessary zymogens and cofactors required for clot formation. Initiation occurs following the formation of a FVIIa/TF complex on the cell surface with subsequent activation of factors IX and X. A second complex is formed, Xa/Va which is able to produce a small amount of thrombin. Amplification of this process occurs following the activation of platelets by thrombin, releasing activated vWF and clotting factors V, VIII and XI. The propagation phase take place on the surface of activated platelets which are now able to bind factors IXa, VIIIa, Va and Xa. In similar fashion to the initial TF bearing cell, Xa is formed following the formation of a FIXa/VIIIa complex which then forms a complex with Va thus generating a surge in thrombin production if sufficient platelets are recruited to the site of injury[9]. The generation of thrombin causes the production of an insoluble fibrin clot through

its action on fibrinogen and also through activation of FXIII which will be discussed below.

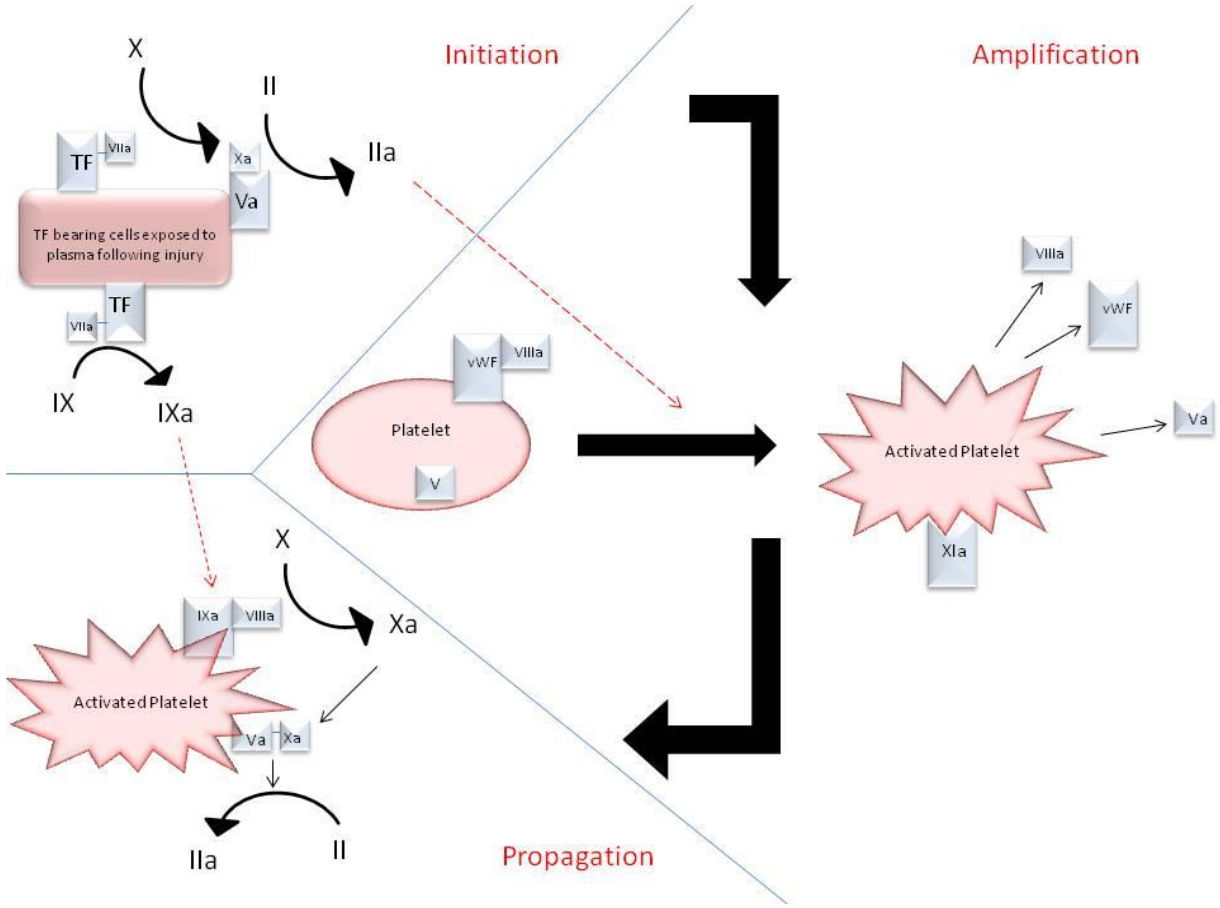

Figure 1-1 Illustration of coagulation cascade following tissue injury.

Cells bearing TF bind FVII and activates factors X, IX and thrombin (IIa). Insufficient thrombin is produced to permit fibrin formation. Amplification sees activation of platelets by thrombin, releasing vWF, FVIIIa and FVa. On the surfaces of activated platelets complexes of FIXa-VIIIa are formed, activating FX and forming FVa-Xa complex which is able to produce increased quantities of thrombin (assuming sufficient platelets are recruited) and thus fibrin clot formation.

1.1.3 Platelets

As described above, platelets play a crucial role in the formation of the fibrin clot. Despite their diminutive appearance they are complex cells without which haemostasis would not be possible. Their roles can be divided into three separate functions, namely 1) platelet adhesion (at the site of vessel injury), 2) activation and 3) aggregation, resulting in an insoluble plug[10]. The three phases will be described in more detail below.

1.1.3.1 Adhesion

At the site of vessel injury, platelets rolling along the endothelium adhere to the subendothelial matrix through the interaction of numerous surface receptors on platelets and proteins within the subendothelial matrix, such as vWF, collagen, and fibronectin. These receptors include integrins (such as glycoproteins (GP) IIb/IIIa and GP Ia/IIa) leucine rich glycoproteins and immunoglobulins[7].

1.1.3.2 Activation

Activation of platelets occurs following adhesion to matrix bound proteins and interaction with soluble agonists such as thrombin which binds to the GP IIb/IIIa receptor[10]. This triggers a cascade of complex reactions that exposes surface phospholipids, alters the structure of the platelets and culminates in the release of granules. Numerous coagulation factors are now able to bind platelets via the GP receptors or phospholipids[9]. A variety of compounds are released from the granules including ADP, ATP, calcium, platelet derived growth factors (PDGF) and transforming growth factor beta (TGFβ), fibrinogen, FV, platelet factor 4, vWf and thrombospondin. These molecules cause further platelet adhesion and activation, promote tissue repair and initiate platelet aggregation. One of the key

reactions within platelets following activation is the conversion of arachidonic acid to prostaglandin endoperoxides G_2 and H_2 (PGG_2 and PGH_2) by the action of cyclo-oxygenase 1 (COX-1). PGG_2 and PGH_2 in turn are converted to thromboxane A_2 (by thromboxane synthesis) which is thought to be involved in granule secretion by aiding the fusion of intra-cellular granules with the cell membrane[7;11]. Thromboxane A_2 is also involved in platelet aggregation and causes vasoconstriction of smooth muscles. The antiplatelet agent aspirin (acetylsalicylic acid) acts by acetylating COX-1 thus inhibiting its action, reducing thromboxane A_2 synthesis and platelet aggregation, generating fibrin clots that are less resistant to lysis.[12;13]

1.1.3.3 Aggregation

The final role of platelets in the formation of the haemostatic fibrin plug is to aggregate and form a dense mass. This occurs again via the action of one of its GP receptors. GP IIb/IIIa of one platelet binds to a fibrin molecule within the clot which in turn binds to the GP IIb/IIIa receptor of another platelet. The end result of these interactions is a dense accumulation of platelets which forms a physical plug around the site of injury[9]. Therefore, in addition to forming the skeleton of the blood clot, fibrin acts as a bridge between platelets facilitating growth of the platelet plug.

1.2 The fibrin network

As mentioned earlier, the fibrin network forms the backbone of the blood clot and is essential for maintaining integrity of the thrombus. Until recently, fibrinolysis was the main treatment modality for acute myocardial infarction[4], emphasising the importance of the fibrin clot in disease pathogenesis. The main

proteins involved in clot formation and resistance to fibrinolysis are discussed in this section.

1.2.1 Fibrinogen

Fibrinogen is a glycoprotein that is synthesised in the liver (a process which takes roughly 5 minutes) and circulates in the plasma at concentrations of 2-4 mg/ml (~6-12μM) but can reach double these concentrations in pathological states. It is a dimeric protein made up of two symmetrical sets of identical polypeptide chains, Aα,Bβ and γ. The α chain contains 610 residues, the β chain 461 and γ chain 411 residues. The presence of 4 carbohydrate clusters (one on each β and γ chain) combine to form a protein of 340,000Da. All 6 chains are held together by a total of 29 disulfide bonds [14;15]. Three separate regions make up the molecular structure of fibrinogen, a central, E region and two peripheral, D regions. The amino termini of all six chains are contained in the E region, while the carboxy termini of the Bβ and γ chains are found in the D region. The α chain carboxy termini end in a separate αC domain[16]. Extending from the E region, the chains intertwine and from a helical coiled coil structure which lead to the D regions. The alpha chains extend back from the D regions and are thought to bind to β chain residues 1-66 within the E region[17] (Figure 1-2).

1.2.1.1 Conversion to fibrin and clot formation

Following activation of the coagulation cascade fibrinogen is converted to a fibrin monomer and subsequent insoluble fibrin clot through the actions of thrombin. Thrombin binds to the central E region and removes peptides from the N-termini of the α and β chains. Cleavage of the α chain occurs first,

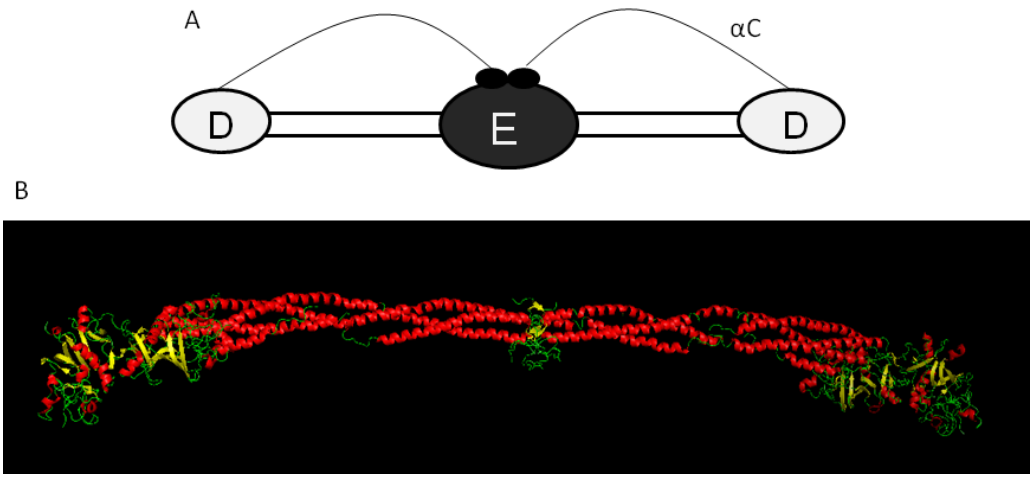

Figure 1-2 Structure of the fibrinogen molecule

A) Schematic representation of the fibrinogen molecule with the central E domain and 2 peripheral D domains from which the αC regions originate. B) ribbon representation of the crystallised fibrinogen molecule (Protein Data Bank [PDB] code: 3GHG)[18]**.** *The αC regions are not obvious due to their mobile properties.*

releasing a 16 amino acid peptide, fibrinopeptide A (FpA), thus generating a new N terminal sequence which is termed the “A” site. A non-covalent bond occurs between this “A” site within the E region and an “a” pocket within the γ chain of the D region of a neighbouring fibrin molecule. This initial interaction produces a half-staggered chain of double stranded protofibrils and elongates the fibrin chain. A second peptide, fibrinopeptide B (FpB) is now released from the N terminal of the β chain again by the action of thrombin. The removal of these terminal 14 amino acids again produces a new N terminal sequence; this time termed the “B” site. In a similar manner to the A:a interaction of the α and γ chains, the “B” site binds to a “b” pocket found within the β chain of the D region of another molecule [19-21] (

Figure 1-3). It is thought that this B: b interaction of 2 fibrin molecules promotes lateral aggregation of protofibrils and thereby generates thick fibrin fibres. Interestingly, however, lateral aggregation may occur in the absence of FpB release, casting doubt on the exact role of FpB in clot formation[22]. Around the time FpB is released the αC domains disengage with the central E region and subsequently interact with other protofibrils, and in so doing lateral aggregation occurs and a fibrin scaffold is formed[14].

1.2.1.2 Stabilisation of the fibrin clot

1.2.1.2.1 Factor XIII

Much needed stability is added to the clot through the action of factor XIII, a member of the transglutaminase family. It is a tetrameric proenzyme (320,000Da) that contains 2 A chains and 2 B chains, and circulates in plasma bound to fibrinogen. The B chains are produced in the liver and are inert acting primarily as a carrier for the A chains. The latter are produced largely in the bone marrow and combine with the B chains upon contact in plasma[14]. In addition to its action in cross linking fibrin fibres, activated FXIII can also bind to platelets and is thought to supplement other ECM proteins in the adhesion of platelets to the damaged EC wall[23]. This coagulation factor is also unique in that it appears to have extra- and intracellular functions. FXIII exists as a dimer of the active A chains within platelets, monocytes, macrophages, dendritic cells, chondrocytes, osteoblasts and osteocytes[24;25].

1.2.1.2.2 Activation of FXIII

The active, cysteine, site of FXIII is concealed within the A chain of inactive FXIII and thus a series of activation steps are required to form the

transglutaminase. Firstly thrombin cleaves a 37 residue peptide from the N terminal of the A chain, weakening the bond between the A and B chains, a process that occurs at the same time as fibrinopeptides are released from fibrinogen[26]. The B chain is dissociated from the A chain following the binding of 2 calcium ions to the A subunits, and in doing so fully exposes the active cysteine site, producing active FXIIIA. The B subunits also play an important role in correctly orientating the A subunits within the fibrin matrix[14].

1.2.1.2.3 Cross linking

Once activated, FXIIIa is able to form covalent bonds between the γ and α chains of neighbouring fibrin molecules. The γ chains are cross linked first in a very timely fashion. Dimeric γ chain structures are produced through the formation of a bond between lysine 406 of one gamma chain and glutamine 398 (or 399) of another. The cross linking of α chains proceeds at a relatively slower pace and results mainly in the formation of large α chain polymers[27]. Numerous glutamine and lysine residues have been identified as possible cross linking sites within the α chain and this is speculated to be an evolutionary adaptation to the heterogeneous nature of the αC domain[14]. The physical presence of these crosslinks increases the fibrin fibre thickness, conferring stability and elasticity to the clot, as well as making the clot more resistant to lysis by interfering with the action of plasmin within the D and E regions as it attempts to degrade fibrin. Further resistance to fibrinolysis is provided by the cross linking of the anti-fibrinolytic proteins plasmin inhibitor (PI), plasminogen activator inhibitor-2 (PAI-2) and thrombin activatable fibrinolysis inhibitor (TAFI) to the α chain of fibrin[26], and these are further discussed below.

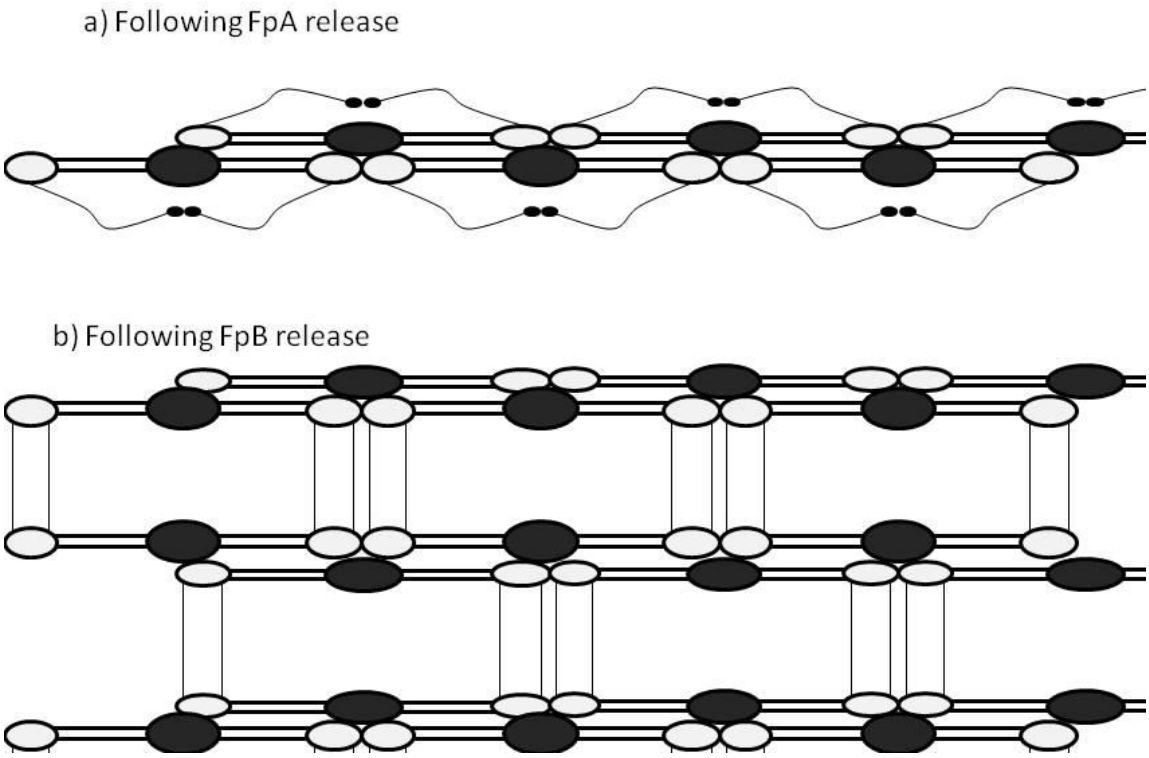

Figure 1-3 Protofibril formation following action of thrombin.

Cleavage of fibrinopeptide A (FpA) generates a new N terminal sequence, termed the "A" site. A non-covalent bond occurs between this "A" site within the E region and an "a" pocket within the γ chain of the D region of a neighbouring fibrin molecule. This produces a half-staggered chain of double stranded protofibrils and causes elongation of the fibrin chain. Following the release of fibrinopeptide B (FpB) from the β chains a new N terminal sequence is formed, this time termed the "B" site. In a similar manner to the A:a interaction of the α and γ chains, the "B" site binds to a "b" pocket found within the β chain of the D region of another molecule.

1.3 Control of clot formation

1.3.1 Natural anticoagulants

1.3.1.1 Protein C pathway

Protein C is a vitamin K dependant zymogen, similar to factors VII, IX, X and prothrombin and is synthesised in the liver with a molecular weight of 59,000 Da. However, upon activation on the surface of EC, protein C acts as an anticoagulant rather than a procoagulant. In order for it to be activated, thrombin has to form a complex with thrombomodulin on the surface of EC[28]. This alters the structure of thrombin abolishing its ability to activate platelets or convert fibrinogen to fibrin, but can activate protein C which subsequently proteolytically cleaves factors Va and VIIIa. The major function of this pathway is to control coagulation and prevent extension of the clot beyond the site of injury within the vessel wall[29].

1.3.1.2 TF pathway inhibitor (TFPI)

As its name suggests TFPI inhibits TF and thus the initiation of coagulation. It is also able to inhibit FXa and TF-VIIa-Xa complex to lesser degrees[9]. It is synthesised by EC and can circulate bound to plasma lipoproteins, on vessel wall proteoglycans or in platelets. Two isoforms exist, TFPIα and TFPIβ, with only the former present in platelets which may be ideally placed to inhibit thrombin generation that follows platelet activation. The β isoform is expressed on endothelial cells where its inhibitory actions are likely to be important when endothelial TF is present, for example during inflammation[30].

1.3.1.3 Heparin-antithrombin pathway

This pathway involves another of the serine protease inhibitors, antithrombin III. It is also produced in the liver, and is found predominantly on the vascular endothelium. Along with its ability to inhibit thrombin, it is also able to inhibit clotting factors, Xa, IXa, Xia, XIIa and VIIa when in complex with TF and with varying efficacy[28;29]. By combining with heparin, the activity of antithrombin is increased by a factor of around 1000, and thrombin inhibition is also only achievable if thrombin itself is in complex with heparin, which is in contrast to the other clotting factors. Antithrombin deficiency represents the most severe clinical manifestation of all natural anticoagulant deficiencies[31].

1.3.1.4 Other pathways

Other molecules secreted by EC which have an anticoagulant effect are prostacyclin and nitric oxide (NO) which are potent vasodilators and are instrumental in the control of vascular tone. They can also exert and anticoagulant effect by inhibiting platelet aggregation[7;32].

1.3.2 Fibrinolysis

The fibrinolytic system is triggered following the generation of fibrin and ensures that coagulation does not continue unchecked[14].

1.3.2.1 Plasminogen

Plasminogen is a single chain zymogen (92,000 Da) that is synthesised in the liver. It is converted to its active form, plasmin, by the action of tissue plasminogen activator (tPA), which cleaves the Arg561-Val562 peptide bond, removing a 77 residue peptide. Plasmin consists of a heavy (residue 79-562) and light chain (residue 562-791) which are linked by a disulphide bond[33]. An

active serine site is contained within the heavy chain. Plasminogen binds fibrinogen but the affinity to fibrin is increased 1000 fold, to prevent unwanted and excessive clot formation. Plasmin begins dissolution of the fibrin clot by hydrolysis of arginyl and lysyl bonds within the coiled coil region of the D and E domains as well as the αC region. The first consequence of plasmin digestion cleaves a 4kDa fragment from the Aα chain, producing fragment X (which may still be clotted by thrombin). Further cleavage within the D and E regions produces fragments Y and D, the former is further degraded to produce a second fragement D and fragment E. Thus each fibrin molecule yields 2 molecules of fragment D and one of fragment E (*Figure 1-4*) [34]. Plasmin is also able to degrade other proteins involved in coagulation such as FVIII, FV and vWf.

1.3.2.2 tPa

This serine protease is predominantly produced within EC as a single chain glycoprotein and circulates in plasma at 5-10ug/ml. It is activated into a 2 chain protein by the cleavage of bond Arg275-Ile276 by plasmin and FXa and the formation of a disulphide bridge between the 2 chains[33]. It usually circulates in association with its inhibitor plasminogen activator inhibitor-1 (PAI-1), and the initiation of coagulation causes this complex to dissociate. Upon formation of a fibrin clot, free tPA and plasminogen bind to fibrin and this complex increases the action of tPA by a factor of 1000[35].

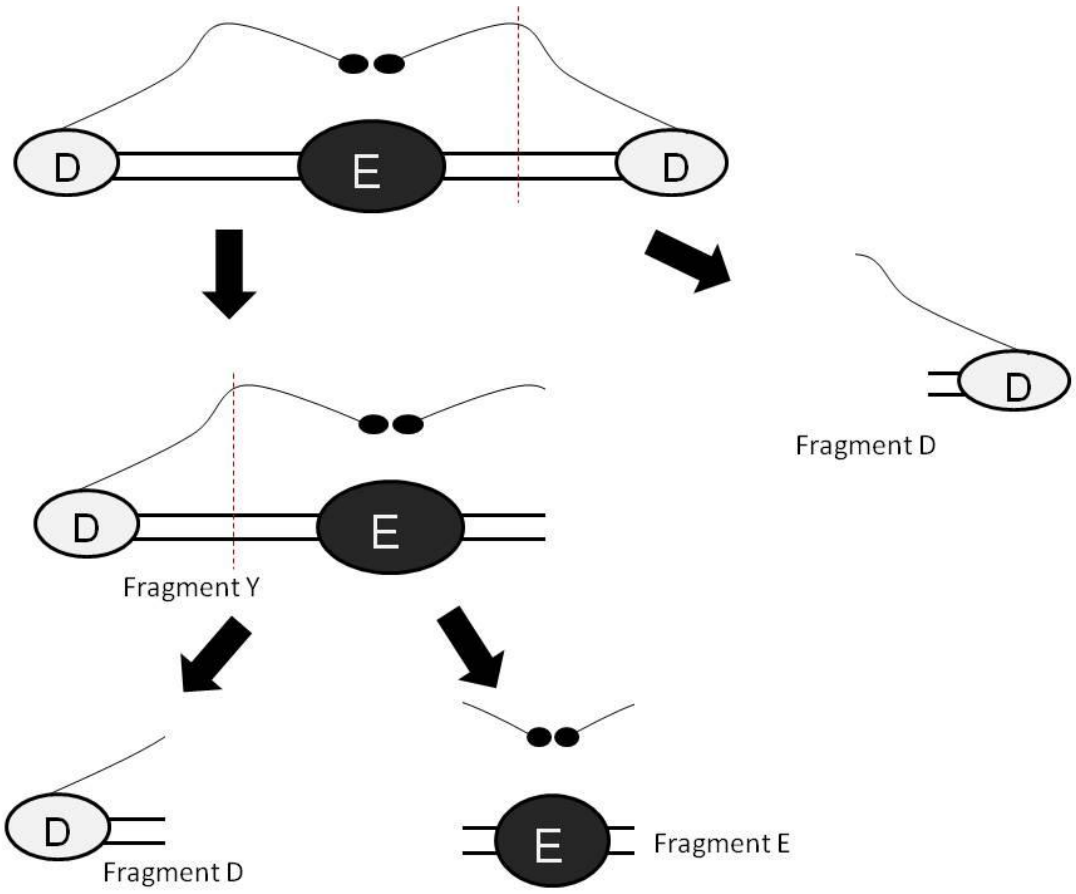

Figure 1-4 Schematic representation of fibrin degradation by plasmin.

Initial cleavage produces two fragments, Y and D. Further cleavage of fragment Y produces a second D fragment and fragment E. The dashed lines represent site of plasmin cleavage.

1.3.2.3 uPa

Urokinase plasminogen activator (uPA) is another serine protease and like tPA is also able to activate plasminogen to produce plasmin but to a lesser degree. It is synthesised in the lung, kidney and EC and is also converted from an inactive single chain protein to an active 2 chain serine protease by the actions of plasmin and FXIIa causing cleavage of the Lys158-Ile159 bond[36]. It has a larger role in the proteolysis of the extracellular matrix and is involved in wound healing as well as new vessel formation[7].

1.3.3 Inhibition of fibrinolysis

Fibrinolysis is closely regulated through the action of several different proteins.

1.3.3.1 PAI-1

Plasminogen activator inhibitor-1 (PAI-1) is a 43,000Da serine protease inhibitor (serpin) and is the physiological inhibitor of tPA and uPA. It is secreted from numerous cells including EC, vascular smooth muscle cells, hepatocytes and adipocytes and circulates in the plasma at approximately 2-20ng/ml (40-400nM)[37]. It irreversibly binds to the plasminogen activators thereby reducing plasmin generation and modifying the rate of clot lysis. Plasma levels vary during the day, with highest levels observed on waking, falling to a nadir by the afternoon. Numerous factors such as plasma insulin and transforming growth factor β are known to induce transcription of PAI-1, and plasma levels can rise rapidly when stimulated. It is a relatively unstable molecule and is rapidly degraded to a latent, inactive form and has a plasma half-life of around 10-120 minutes[38-40]. Although the latent form appears to have no clinical relevance, antigen testing kits may not be sufficiently sensitive to differentiate the two forms, thus giving inaccurate plasma levels of the active and physiologically relevant PAI-1[39].

1.3.3.2 PAI-2

PAI-2 is less efficient at inhibiting tPA compared with PAI-1. It is mainly synthesised during pregnancy where it is involved in embryonic development and maintaining the placenta.

1.3.3.3 Plasmin Inhibitor

The activity of plasmin is inhibited by the actions of plasmin inhibitor (PI), also known as α_2-antiplasmin (α_2-AP), which is produced in the liver and has a molecular weight of 58,000 Da. Its actions are multiple, in that it can directly bind plasminogen and plasmin, thus reducing their ability to bind fibrin(ogen). It also competitively inhibits plasminogen by directly binding to fibrin. It may do this non-covalently to fibrin D regions and αC domains or through cross linking to Lys303 within the αC region of the α chain, a process facilitated by FXIIIa [16]. The inhibition in clot lysis is directly proportional to the circulating levels of α_2-AP, and its importance in maintaining haemostasis is emphasised by the bleeding tendency exhibited by homozygous deficiency of the PI gene[41]. More detailed description of the role of PI can be found in Chapter 5.

1.3.3.4 Thrombin activatable fibrinolysis inhibitor

A third inhibitor of fibrinolysis is thrombin activatable fibrinolysis inhibitor (TAFI), which becomes activated by thrombin/thrombomodulin to TAFIa (also known as carboxypeptidase B). This is able to cleave the c-terminal lysine residues of partially degraded fibrin fibres, which serves to greatly reduce the binding affinity, and therefore activation of plasminogen [42-44].

	Molecular weight (kDa)	Plasma concentration	Actions
PAI-1	43	2-20ng/ml	Inhibits tPA and uPA
PAI-2	47	<0.01µg/ml[36]	As above but to a lesser degree
PI	58	70µg/ml[36]	Inhibits activation of plasminogen and activity of plasmin
TAFI	56[45]	4-15µg/ml[45]	Reduces activation of plasminogen

Table 1-1 Summary of inhibitors of fibrinolysis

1.4 Factors affecting coagulation proteins

Several factors are known to affect the plasma levels of various clotting factors and also directly impact on its function without altering levels. These can be broadly divided into genetic and environmental effects.

1.4.1 Quantitative

1.4.1.1 Genetic

Several polymorphisms within genes encoding clotting factors have been described resulting in elevated or reduced plasma levels of the protein. The most recognised are those affecting TF, FVII, FXIII and fibrinogen[16]. TF polymorphisms do not appear to have major clinical implications, except for 603 A/G polymorphism in the promoter region, which may be associated with myocardial infarction (MI). However, the reason for this association is unclear and it is not related to elevated TF levels[46]. Reduced levels of FVII are associated with coding polymorphisms but the clinical relevance of this is association is unclear. Mutations within the promoter region which increase (402 G/A) or decrease (323 A1/A2) FVII plasma levels have been associated with elevated or reduced risk of MI respectively[47-49]. Increased plasma levels of fibrinogen are associated with polymorphisms within the β chain of fibrinogen, such as G to A substitutions at 455 and 854. Longitudinal studies to conclusively demonstrate a link between these polymorphisms and CVD are contradictory[50]. A relatively common coding polymorphism affecting the A subunit of FXIII (FXIIIVal34Leu) appears to affect its activity rather than directly alter plasma levels and the clinical consequences of such a polymorphism is variable[16]. In some individuals the increased activity appears protective against

CVD, possibly due to depletion of the protein or through disordered clot formation caused by early cross linking. However, in vitro work demonstrates that this polymorphism generates clots with thinner fibres which are classically more difficult to lyse[51] and may help explain reduced efficacy of thrombolytic therapy following MI and stroke in these individuals[52;53].

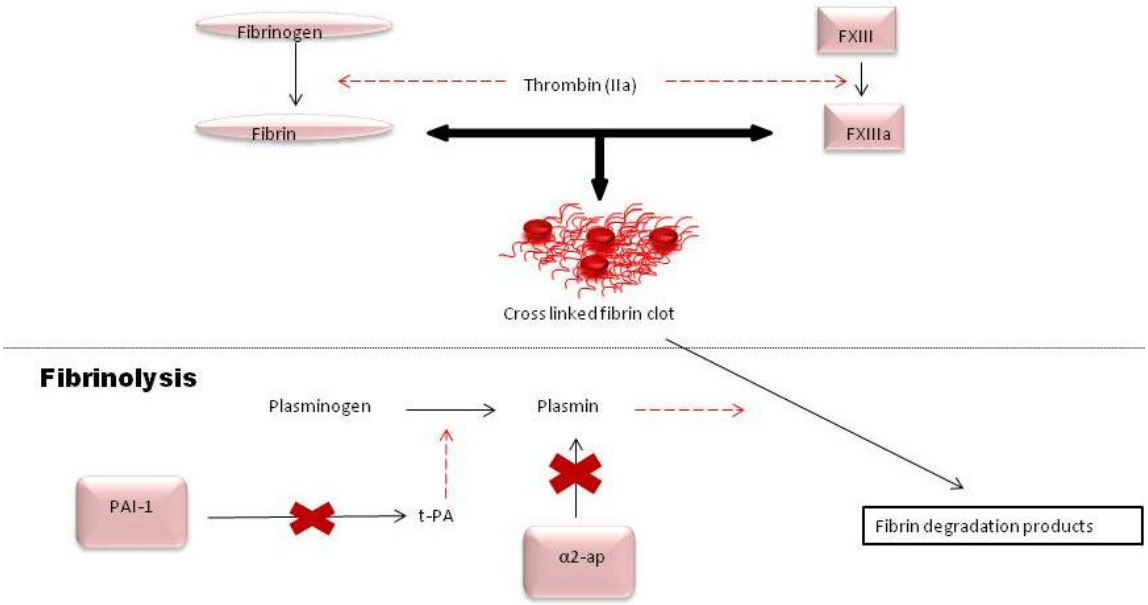

Figure 1-5 Formation of fibrin clot and proteins involved in fibrinolysis.

Thrombin cleaves fibrinopeptides A and B from fibrinogen to form fibrin and also activates FXIII which causes cross linking of the fibrin chains. The insoluble clot is broken down by plasmin (produced following cleavage of plasminogen by t-PA). The anti-fibrinolytic proteins PAI-1 and α2-ap inhibit clot lysis by inhibiting t-PA and plasmin.

To date it has proven difficult to confidently ascertain the role each polymorphism affecting coagulation factors has upon the development of CVD. This is due to the difficulties encountered in recruiting large number of individuals into longitudinal studies, necessary in studies investigating genetic polymorphisms. Also, the multifactorial pathogenic mechanisms involved in

atherothrombosis question the role and the clinical relevance of a single polymorphism. Finally, concrete conclusions are usually difficult to achieve with such studies, given the complexity involved in gene-gene as well as gene-environment interactions.

1.4.1.2 Environmental

Elevated levels of coagulation factors such as fibrinogen, FXIII, FVII, TF, and PAI-1 are associated with environmental factors such as diabetes, obesity, smoking, and hyperlipidaemia[16]. This leads to a pro-thrombotic environment and an increased risk of CVD. Medical intervention can ameliorate this thrombotic tendency. Improving glycaemic control lowers TF activity and reduces levels of fibrinogen, the latter being less consistent with insulin therapy however[35;37]. Treatment with the oral hypoglycaemic agent, metformin also lowers PAI-1 and FVII levels, the former effect being over and above its glucose lowering capabilities[37]. Lowering cholesterol and triglyceride levels through the use of statin and fibrate medications can reduce levels of TF, PAI-1, and FVII[16]. Adopting a healthy lifestyle through regular exercise, cessation of smoking, moderate alcohol consumption and losing weight can all lower levels of fibrinogen, PAI-1 and FVII and help reduce cardiovascular risk[54].

1.4.2 Qualitative

The formation of certain coagulation factors can be affected to various degrees whilst having no affect on the overall plasma levels. These qualitative changes can also alter the individuals' thrombotic potential as outlined below.

1.4.2.1 Genetic

Although less numerous than quantitative changes to plasma levels of coagulation factors, polymorphisms that affect clot structure are also clinically significant and adversely affect CVD risk. Two polymorphisms of the fibrinogen molecule are well recognised affecting the α chain (312 A/T) and β chain (488 G/A) produce clots with increased stiffness and in the latter, increased clot density, thinner fibrin fibres and decreased permeability. Substitution of arginine to lysine at position 488 is associated with more severe CAD and is seen with increasing frequency in patients with triple vessels coronary artery disease.

As mentioned in section 1.4.4.1.1, a polymorphism within the A subunit of FXIII, (FXIIIVal34Leu) in which valine is substituted for leucine at position 34, increases the activation of FXIII by thrombin. This single amino acid substitution alters the structure of the clot as cross linking of the fibrin molecule occurs earlier, reducing the lateral aggregation and forming clots with thinner fibres[51].

1.4.2.2 Environmental

Coagulation proteins can be affected qualitatively after synthesis by various post-translational modifications (PTM) which covalently attach functional groups to amino acid side chains. The effect of PTMs on fibrinogen molecules has been investigated most extensively and thus is the main focus of the following section.

1.4.2.2.1 Glycation

Several proteins are known to undergo glycation and fibrinogen is one example. Glycation occurs through non-enzymatic formation of fructosamines and advanced glycation endproducts (AGE) following an interaction between amino

groups (typically lysine) on the target protein and an aldehyde group from glucose[55]. In diabetes elevated levels of plasma glucose increases the glycation of fibrinogen and ex-vivo clots made with this fibrinogen form a tighter, rigid clot network compared to fibrinogen from patients without diabetes. Plasminogen is also increasingly glycated in diabetes patients which appears to decrease plasmin generation and also reduce the efficacy of the plasmin produced[56].

1.4.2.2.2 Oxidation

Many circulating plasma proteins can be oxidised by compounds such as reactive oxygen species (ROS) and AGEs, both of which are increased as a results of oxidative stress, inflammation and hyperglycaemia[57]. Following oxidation, fibrinogen may be more readily transformed to fibrin, and along with other consequences of oxidation such as enhanced platelet aggregation and decreased plasminogen activation, contribute to a pro-thrombotic environment[58;59].

1.4.2.2.3 Acetylation

Aspirin is capable of acetylating lysine residues on the α-chain of fibrinogen, resulting in clots composed of thick fibres and large pores with reduced rigidity which are less resistant to lysis. Changes in charge distribution and fibrin conformation as a result of acetylation may explain these alterations to clot structure[16]. It has been demonstrated that Lys539, 429, 224 and 208 are sites of acetylation on fibrinogen, and interestingly all are involved in FXIIIa crosslinking of fibrin fibres[60].

1.4.2.2.4 Phosphorylation

Phosphorylation of fibrinogen readily occurs, affecting the Aα-chain at Ser3 and Ser345 leading to fibrinogen circulating in its phosphorylated form in plasma. Ser3 lies within FpA and shows increased phosphorylation under conditions of acute stress which is thought to modulate FpA release by enhancing thrombin binding. The degree of protein phosphorylation is also associated with an increase in resistance to plasmin-induced fibrinolysis[61].

1.4.2.2.5 Homocysteinylation

Certain lysine residues within fibrinogen can also be modulated by the introduction of sulfhydryl groups following the action of homocysteine thiolactone, a metabolite of homocysteine. Fibrinolytic proteins bind at lysine residues, which are also the sites of plasmin cleavage and thus modification of lysine residues in this manner leads to fibrin that is unable to support plasminogen activation by tPA and generates clots that are resistant to lysis[62].

1.4.2.2.6 Nitration

The *in-vivo* nitration of fibrinogen molecules is thought to occur on tyrosine residues 292 and 422 within the β chain. This PTM leads to accelerated clot formation with an altered ultrastructure, increased fibrin stiffness as a result of increased cross linking by FXIII and ultimately hypofibrinolysis[63].

1.5 Inflammation and atherothrombosis

Inflammatory molecules, such as tissue necrosis factor α (TNFα) and interleukin- 6 (IL6) are instrumental in the development of atherosclerotic plaques contributing to endothelial cell damage, the formation of lipid dense

macrophages, termed foam cells, plaque formation and eventually plaque rupture. This is discussed in greater detail in section 1.8. Several components of the complement system are also thought to contribute to this process, given their presence within atherosclerotic plaques and also the fibrin network. The key protein, complement C3 is independently associated with the development of myocardial infarction (MI) and has been implemented in the formation of fibrin clots with an increased resistance to fibrinolysis[6,64].

1.5.1 The complement system

1.5.1.1 Overview

Our knowledge and understanding of the complement system has developed through the study of its role in providing a defence against invading pathogens and was named following the discovery that it is able to "complement" host antibodies in their efforts of killing bacteria[65]. It contains greater than 30 proteins and cell surface receptors, that form vital components in mammalian immunity and inflammation[66]. Evidence is emerging that it also plays a pivotal role in the homeostasis of cells and tissues[67] and that activation of the pathway and subsequent inflammation in damaged tissue may be detrimental rather than protective; tissue ischemia that follows myocardial infarction (MI) is one example[68].

The complement system is activated by three distinct pathways, all of which share the same three physiological goals: 1) defending the host against invading pathogens, 2) providing a link between the innate and adaptive immune responses, and 3) removing the resultant immune complexes and apoptotic cells[69]. The pathways involved are termed, classical, alternative and

mannose binding lectin (MBL) pathways. In a similar manner to the coagulation and fibrinolytic pathways, each involves a series of cascading enzymatic reactions, and although they are triggered by differing methods, they all share the common feature of activating complement protein C3 (Figure 1-6)

1.5.2 Classical pathway

1.5.2.1 Activation

The classical pathway contains proteins labelled C1 – C9 and is activated following antibody/antigen attachment to cell surface. C1 binds the antibody-antigen complex and is activated to C1r which in turn activates C1s. This allows cleavage of C4 to C4a and C4b, and C2 to C2a and C2b.

1.5.2.2 Action

On the surface of invading bacteria, C4b and C2a form a complex which acts as a serine protease, termed C3 convertase, and this converts C3 into C3a, a potent anaphylatoxin, and the larger protein C3b, which promotes opsonisation by binding to the cell surface of pathogens. C3b also has a key role in continuation of the complement cascade, forming, together with C4b and C2a, C5 convertase (C3bC4bC2a), and releasing C5b from C5. On cell surfaces, C5b forms the membrane attack complex (MAC), a transmembrane pore or channel consisting of C5b, C6, C7, C8, C9, which causes membrane perforation and cell lysis.

1.5.2.3 Amplification

Amplification of the pathway occurs following the attachment of C3b molecules to a pathogen, and subsequent binding of factor B, which in turn is activated to

another C3 convertase (C3bBb) through the action of the enzyme factor D, thus generating further C3b and amplifying the cascade[65].

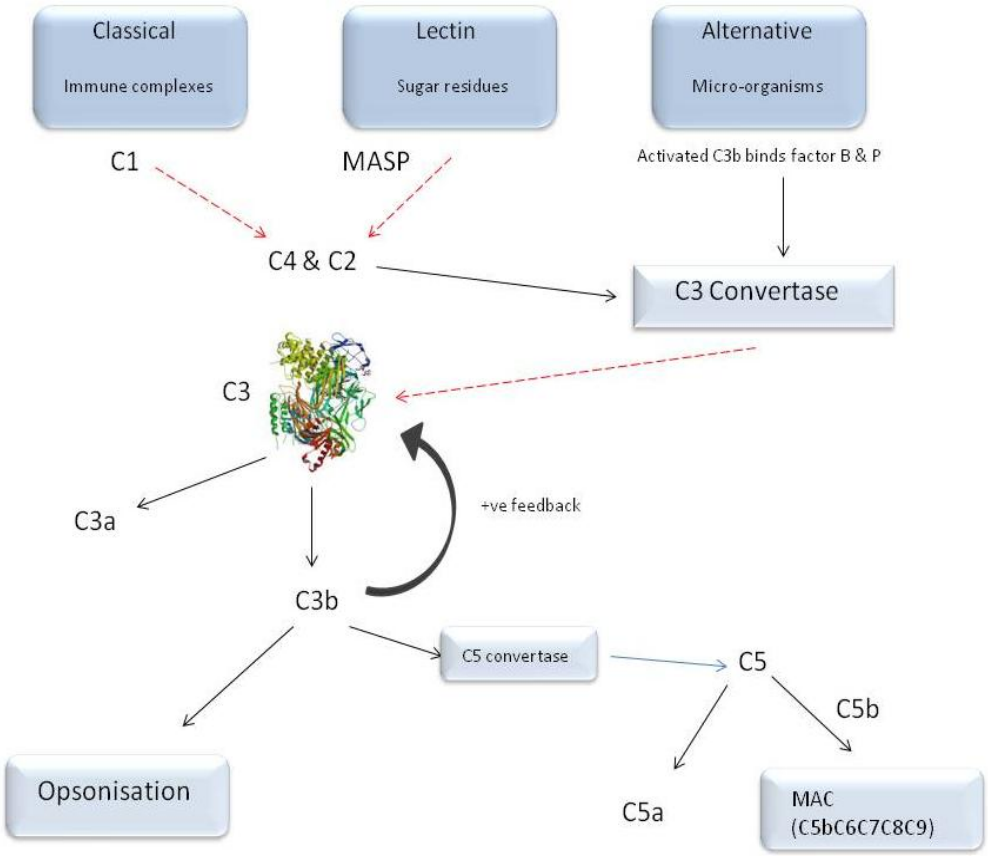

***Figure 1-6 Simplified illustration of complement activation*.**

The C3 convertase (C4bC2a) is formed following activation of C1 and MASP by the classical and lectin pathways respectively whereas a modified version (C3bBbP) is formed by the alternative pathway. Both enzymes act upon C3 producing C3a and C3b. The latter of these molecules can form further C3 convertase and produce more C3b or may form C5 convertase enzymes (C3bC4bC2a and C3bBbC3bP), generating C5a and C5b, which is involved in the terminal pathway, namely the production of the membrane attack complex (C5bC6C7C8C9)

.

1.5.2.4 Regulation

Regulation of the cascade is achieved through interaction with other complement proteins [70]. For example, C1 inhibitor protein inactivates C1r and C1s, which can modulate survival rates in animal models of septic shock. Physiological breaks can also be applied to the amplification stages. The formation of C3b activates an internal thioester bond, allowing it to covalently attach to hydroxyl groups on carbohydrates and proteins. Once bound to a cell surface it may combine with factor B or factor H and a host cell would favour C3b to bind factor H much more readily, allowing the formation of the inactive compound iC3b following the action of factor I[65]. Other membrane and plasma proteins such as C4-binding protein (C4bp), decay accelerating factor (CD55) and membrane cofactor protein (CD46) are also able to control amplification. The MAC can be inactivated in plasma before it is inserted into the membrane of a cell by S-protein binding to the C5b-7 complex and by the protein CD59 (protectin), which is able to bind C8 and C9. Anaphylatoxins such as C3a, C4a and C5a can be inactivated by the removal of the carboxyl-terminal arginyl residues by carboxypeptidase-N and R[71].

1.5.3 Mannose-binding Lectin pathway

This pathway is similar to the classical pathway, albeit with slightly different personnel[72]. Antibodies are substituted for lectin proteins, such as MBL which is structurally similar to C1q. They are able to bind sugar residues found on pathogens but are unable to bind the sugars found naturally on mammalian cells[73]. The pathogen's sugars, such as mannose, therefore act as the antigens in this variation of the classical pathway.

Activity	Complement protein involved
1) Defending host against pathogens	
Opsonisation	C3b, C4b
Chemotaxis	C3a, C4a, C5a (anaphylatoxins)
Lysis of bacteria	C5b-C9 (MAC)
2) Link between innate and adaptive immune response	
Arrange antibody response	C3b, C4b, C3 receptors on B cells
3) Removal of debris	
Clearing immune complexes	
Clearing dead cells	C1q, C3b, C4b
	C1q, C3b, C4b

Table 1-2 **The three main activities of the complement system and the proteins responsible**

In order for the cascade to be activated, one further player is required, namely MBL – associated serine proteases (MASP). These enzymes form complexes with MBL, thus replacing C1s[74]. The cascade of events that follows is identical to that of the classical pathway.

1.5.4 Alternative pathway

The alternative pathway differs from above by the ability to initiate the cascade without contact with proteins[75;76]. The labile thioester bond of C3 produces a small but constant amount of auto activated C3 (iC3) which marshals the surrounding environment. Upon contact with bacteria or foreign organisms,

activated C3 binds factor B as described previously, which in turn is cleaved by factor D producing Bb and Ba[77]. The C3 convertase C3bBb is formed and is stabilised by properdin forming the complex C3bBbP, which produces more C3b from C3 cleavage and is deposited onto foreign cells. This results in amplification of the alternative pathway and exponential production of C3b molecules. A further C3b molecule attaches to this C3 convertase, forming a C5 convertase, C3bBbC3bP. The pathway beyond the cleavage of C5 follows the same pattern as the classical and MBL pathways, resulting in the assembly of the MAC. The C3b proteins produced by the classical and MBL pathway are also able to form the same C3 convertase (C3bBbP) and thus benefit from this positive feedback loop.

1.5.5 Other pathways of activation

A fourth pathway may exist involving other serine proteases from the coagulation and fibrinolytic cascades. Studies have demonstrated the generation of biologically active C3a and C5a by FXa, plasmin, thrombin, FIXa and FXIa independently from the classical, alternative and MBL pathways [78-80]. The acute phase protein, C-reactive protein (CRP) is also able to activate the classical complement pathway by binding the C1q region of complement protein C1 which in turn activates C1r and C1s[81](Figure 1-7). Oxidative stress following tissue hypoxia is also able to activate the complement pathway and increase iC3b deposition on vascular endothelial cells through activation of the MBL pathway[82;83].

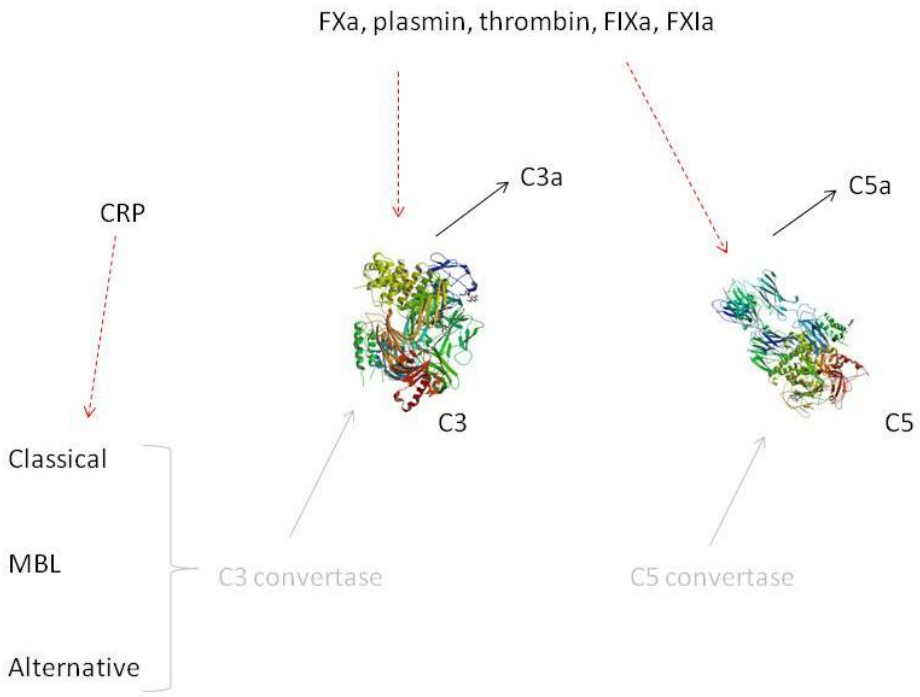

Figure 1-7 Other pathways of complement activation.

The coagulation proteins FXa, plasmin, thrombin, FIXa, FXIa are able to produce C3a and C5a independently of the classical, alternative and MBL pathways. The acute phase protein CRP is able to activate the classical pathway through its action on C1 The shaded text represents the conventional activation pathways.

1.5.6 Complement C3

1.5.6.1 Structure of C3

Activation of complement protein C3 (187,000Da) is a key step in each of the three pathways that make up the complement system. It is synthesised mainly in the liver, and, to a lesser degree, in adipose tissue and is present in plasma at 550-1200 µg/ml. The crystal structure of C3 has been determined[66] (as shown in Figure 1-8) and contains 1641 residues consisting of two chains, ß (residues 1-645) and α (residues 650-164). These chains combine to form 13

domains which are termed macroglobulin domains (MG) 1-8, with the 5 remaining domains appearing to be either inserted between domains or extending from an existing domain. The β chain is composed of domains MG1-5 and part of MG6 which is shared with residues from the α-chain. These 6 domains form the core, stable portion of the protein, onto which is formed the flexible α chain[66, 86].

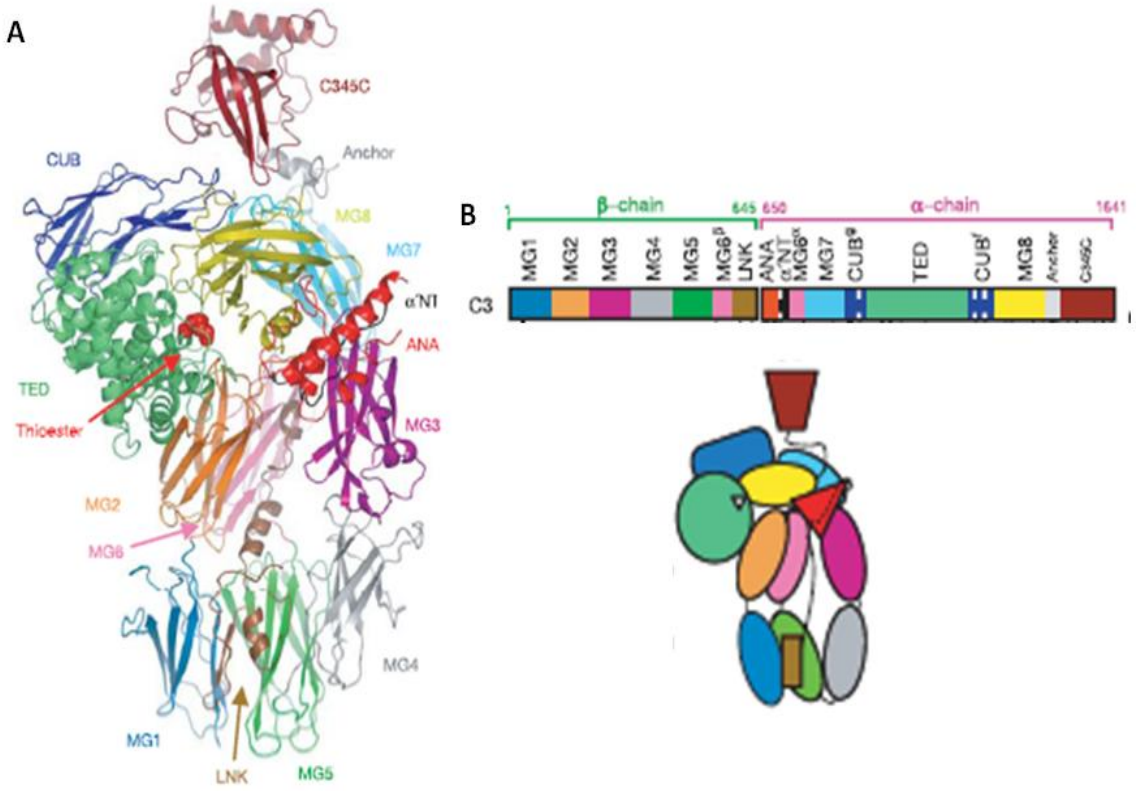

Figure 1-8 Structure of complement component C3

Ribbon representation of C3 (A) and the domain sequences and arrangements (B). Reprinted by permission from Macmillan Publishers Ltd: Nature Janssen BJC et al, copyright 2005.

1.5.6.2 C3 interactions

Complement C3 is able to interact with numerous components of the complement pathway, along with non-complement proteins such as pathogens. Cleavage by different convertase enzymes produces conformational changes within mature C3 that exposes cryptic binding sites and alters its binding affinity for different complement proteins and receptors. The first major conformational change occurs following cleavage by C3 convertase, resulting in the generation of the anaphylatoxin C3a (9kDa) and C3b (177kDa)[84]. The action of C3 convertase not only produces large movements of domains, thus altering the structure of native C3, but also appears to alter the structures of the domains themselves. As detailed above, this triggers a cascade of events, generating numerous proteins, which act as anaphylatoxins (C3a, C4a, C5a), inducing inflammation; opsonins (C3b, C4b), aiding antigen clearance through binding to macrophages, lymphocytes, erythrocytes and other peripheral blood cells[85], as well as culminating in terminal complement complexes (TCCs) such as the membrane attack complex (MAC) which promotes cell lysis[67]. The different conformation of C3b exposes hidden binding sites along with a thioester bond, the latter enables C3b to bind to cell surfaces. The thioester containing domain (TED), which forms part of the α-chain, rotates vertically and rests in greater proximity to MG1 of the β chain, thus exposing the thioester moiety (Figure 1-9). The hidden binding sites promote interaction with proteins such as factor B producing C3 convertase C3bBb and subsequent amplification of the alternative complement pathway. This activity is regulated by largely through the action of factor I which mediates cleavages within the α chain and generates iC3b which is further cleaved to produce C3f (2kDa), the main proteolytic component C3c

(135kDa) and C3dg (40kDa)[84]. The binding of these proteins is thought to largely occur in the flexible α-chain[86].

1.5.7 Factors affecting complement system

Alterations in the levels of numerous complement proteins or in their function are strongly associated with several disease states. These effects can be divided into those caused by environmental pressures and from genetic defects.

1.5.7.1 Quantitative changes

1.5.7.1.1 Genetic

Numerous inherited deficiencies of proteins, enzymes and receptors of the complement pathway have been described with varying clinical courses. These include C3 and mannose binding lectin deficiency which increases susceptibility to pyogenic infections[65] A deficiency in any of the components required to form the MAC is associated with Neisserial infection as extracellular lysis of the pathogen through the formation of a lytic channel within the pathogen is essential to kill the organism which is capable of surviving intracelluarly[87]. Total deficiency of a complement protein causes predisposition to autoimmune conditions, the most commonly occurring of which is systemic lupus erythematosus (SLE)[65]. The autosomal dominant disease, C1 inhibitor deficiency is also known as hereditary angiodema which can cause recurrent and potentially life threatening attacks of angiodema. This serine protease inhibitor inactivates C1r and C1s along with kallikrein and factors XI and XII of the coagulation system. The angiodema is a result of unregulated action of kallikrein which is generally triggered by plasmin activation which consumes the small amount of C1 inhibitor that is present in these patients[88]. Elevated levels

of C3 have been demonstrated with single nucleotide polymorphisms (SNP) affecting the C3 gene[89].

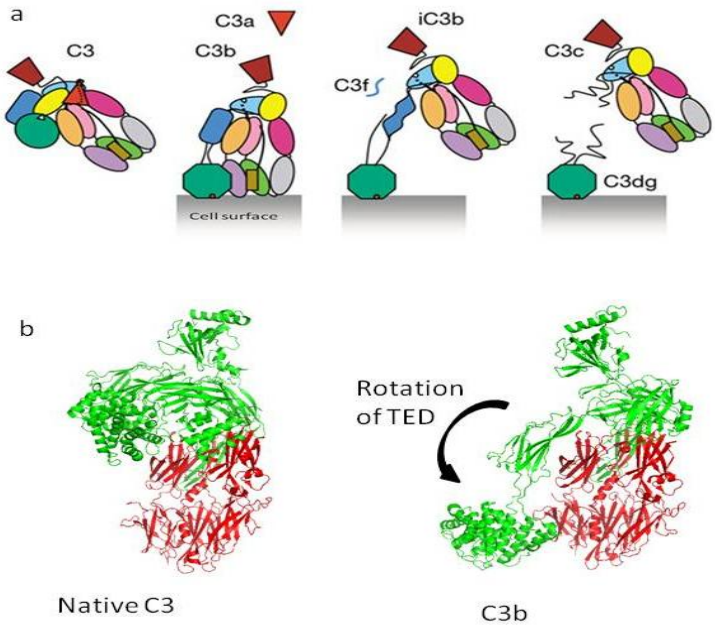

Figure 1-9 **Conformational changes of C3**

a) Hypothesised conformational changes of C3 following attachment to a cell surface (reprinted by permission from Macmillan Publishers Ltd: Nature Janssen BJC et al, copyright 2006). b) Ribbon representation of native C3 and C3b illustrating the vertical rotation of the thioester containing domain (TED) which exposed the thioester moiety to the cell surface. The α chain is in green and β chain in red. The colour scheme in a) matches that in Figure1-8.

1.5.7.1.2 Environmental

Elevated levels of complement proteins such as C3 and C4 are associated with inflammatory diseases and acute infections as a result of increased hepatic synthesis[90;91]. Elevated levels of C3 are associated with type 1 and type 2 diabetes and are also seen in patients with coronary artery disease. In addition to increased hepatic synthesis, C3 levels are also increase in obese individuals

due to increases adipose tissue production of C3[6]. Many diseases associated with circulating immune complexes of autoantibodies of IgG or IgM class may cause acquired complement deficiency through complement consumption. These conditions include SLE, Sjogrens disease, anticardiolipin and others [90].

Complement deficiency may also be due to reduced hepatic synthesis, and is typically seen in advanced chronic liver failure, and also by loss of complement proteins in the urine which can occur with severe forms of nephritic syndrome. The latter mechanism is a rare cause as only factor D (the smallest complement protein) is lost in large quantities, and does not appear to be associated with adverse clinical outcome.

1.5.7.2 Qualitative changes

Qualitative changes to numerous complement proteins have been described which lead to significant clinical consequences

1.5.7.2.1 Genetic

The function of the C3 convertase C3bBb is altered by an autoantibody, C3 nephritic factor. This antibody stabilises the enzyme leading to increased cleavage of C3. The clinical manifestations are membranoproliferative glomerluonephritis and partial lipodystophy. The loss of fat is linked to adipose cell production of factor D, suggesting increased levels of the C3 convertase within adipose tissue which is then stabilised by C3 nephritic factor generating sufficient components of the MAC which subsequently lyses adipose cells[65].

SNP within the C3 gene has been associated with ischaemic stroke, independent of risk factor such as diabetes, hypertension and smoking, and with increased risk of developing metabolic syndrome [89;92]. Alterations to genes

encoding complement receptors also manifest clinically with SNP of the C3a receptor gene associated with increased severity of childhood bronchial asthma[93].

1.5.7.2.2 Environmental

Studies investigating qualitative changes in complement proteins are limited and focus mainly on post-translational modifications such as glycation. In vitro and in vivo glycation of lysine residues on complement proteins C3, CD59 and factor B has been demonstrated with some attempts made to correlate this glycation to clinical findings.[94-98]. Glycation of C3 may alter its structure and thus interaction with bacteria, reducing opsonisation of pathogens such as Staphylococcus aureus, partly explaining increased susceptibility to infections in diabetes patients[96]. The function of C3 may also be altered through glycation of the concealed thioester bond which is exposed upon activation of C3 to C3b[99]. In vivo glycation of CD59 appears to inactivate this complement regulatory protein thus increasing the formation of MAC and cell lysis. This phenomenon may play a role in the pathogenesis of the vascular complications associated with diabetes, such as nephropathy, neuropathy and retinopathy given that MACs are increasingly deposited in renal, nerve and retinal cells of diabetes patients compared to control subjects[100;101]. Unregulated complement lysis of erythrocytes may also contribute to anaemia associated with diabetes[97].

1.6 Diabetes and cardiovascular disease

1.6.1 Incidence and mortality

The link between diabetes and CVD is irrefutable, with a two to 3 fold increase in the incidence of coronary artery disease and stroke compared to non-

diabetes sufferers [1;102]. Those with diabetes also have a worse outcome than non-diabetics following a myocardial infarction (MI) or stroke, with studies demonstrating a 2-4 fold increase in death from CVD[103;104]. The use of advanced treatments such as primary percutaneous intervention following acute myocardial infarction appears unable to buck this trend[4]. The apparent strength of this association has led many to conclude that diabetes sufferers without a prior history of MI, are at the same risk of coronary artery disease (CAD) as a non-diabetic who has a history of MI[105-108]. Recent treatment of diabetes has therefore encompassed more than glucose homeostasis alone with increasing emphasis being placed on targeting other modifiable cardiovascular risk factors such as hypertension, elevated lipid profile and obesity, and using anti-platelet medication as if they had pre-existing CAD. Several studies have shed doubt on this concept of diabetes as a coronary risk equivalent, and that treatment should be based on the individuals calculated risk of CAD rather than a one size fits all approach[1;109-112].With the introduction of modern treatment regimens following MI, the mortality rate has fallen significantly for non-diabetes subjects, however this does not appear to be the case with diabetes patients[5], highlighting the increased risk of CVD in these individuals and the multifactorial approach required to improve outcomes.

1.6.2 Development of atherosclerosis

The acute, clinical entities of myocardial infarction, unstable angina, stroke and lower limb ischaemia are the consequence of longstanding atherosclerotic plaque formation. These plaques are the result of chronic inflammation and develop over several years, influenced by factors such as insulin resistance, shearing stress and oxidation which cause endothelial dysfunction. They consist

of macrophages, lymphocytes, collagen and proliferated smooth muscle cells and are protected by a fibrous cap consisting of collagen, elastin and proteoglycans[11]. The plaques will often remain quiescent for several years, with the clinical complications described above occurring following rupture of the plaque and subsequent thrombus formation. Plaque rupture is followed by platelet adhesion, activation and aggregation at the site of injury and the generation of a cross linked fibrin clot. Further platelet adherence forms an occlusive fibrin mesh, with subsequent vascular occlusion and tissue necrosis.

Insulin resistance and hyperglycaemia alter the composition of these plaques by altering endothelial function, which together with a more lipid rich, macrophage laden plaque seen in diabetes, increases their susceptibility to rupture[35]. When combined with the hypercoagulable environment that is associated with diabetes, this leads to a perfect storm, and increased risk of CVD. It is also likely that the complement pathway is activated during myocardial ischaemia as numerous components of the complement pathway such as C3, C5a and the membrane attack complex (MAC), along with CRP and macrophages have been identified within atheromatous plaques of patients with unstable angina compared with stable disease[113;114].

1.6.2.1 Diabetes and endothelial dysfunction

It is thought that chronic, low grade inflammation seen in diabetes leads to endothelial dysfunction and is the initial step in the development of atherothrombosis. EC dysfunction results in a reduction in the bioavailability of NO, and subsequently increased platelet activation[11], increased smooth muscle cell proliferation and increased expression of adhesion molecules[32]. The latter leads to an increase in inflammatory cells such as macrophages and T-

lymphocytes within the vessel wall, the formation of a fatty streak and eventually the atherosclerotic plaque as well as releasing cytokines including interleukin-6 (IL-6) and tumour necrosis factor (TNF) α, which increase the release of tissue factor (TF) from endothelial cells[115-117]. Obesity contributes in a similar manner to this inflammation as adipose tissue releases pro-inflammatory cytokines from macrophages and T-lymphocytes[118-122]. Hyperglycaemia is likely to contribute significantly to the inflammation process, but insulin resistance, which leads to hyperinsulinaemia, is also of importance. Studies have demonstrated endothelial dysfunction, through impaired vasodilation, in T2DM patients[123], healthy individuals with insulin resistance[124;125] and first degree relatives of patients with T2DM[126]. Weight loss is associated with increased insulin sensitivity and improvement in endothelial function[127;128]. EC dysfunction may also be characterised by increased levels of vWf[35] which have been demonstrated to correlate with diabetes[129;129]. This link may be explained by the development of insulin resistance following loss of EC function [130;131]. Numerous mechanisms therefore contribute to endothelial dysfunction and the resulting disruption to vascular homeostasis is the beginning of a pro-atherothrombotic state.

1.7 Effect of Diabetes on coagulation and CVD risk

Diabetes affects haemostasis in a variety of ways resulting ultimately in thrombosis and hypofibrinolysis. This occurs through increased levels of numerous key elements of coagulation and fibrinolysis along with post-translational modifications and alteration of the clot structure itself leading to a pro-thrombotic state. Many of the changes seen are also associated with increased risk of cardiovascular disease. The changes associated with diabetes

outlined below contribute to the formation of dense clot with thinner fibres and fewer pores ex vivo, that prove more resistant to lysis by plasmin[132;133]. Improving glycaemic control can favourably alter the clot structure [134].

1.7.1 Quantitative changes in coagulation and fibrinolysis proteins

Both diabetes and CVD are independently associated with alterations in the level of coagulation and fibrinolysis proteins.

1.7.1.1 Thrombin

Hyperglycaemia is linked with increased thrombin production and elevated levels are seen in T1DM and T2DM. Reducing glucose levels has been shown to reduce thrombin production[135-137]. Thrombin levels are also involved in clot structure and enhanced concentrations play a role in the dense clots described above[37].

1.7.1.2 Fibrinogen

Elevated levels of fibrinogen are associated with increased risk for CVD and have been used as a marker of clinical and subclinical disease [138-141]. Both T1DM and T2DM are associated with high levels of fibrinogen[142] which may be a result of inflammation causing hepatocyte stimulation[9]. Elevated insulin levels associated with insulin resistance is also likely to play a part in T2DM through increased hepatic production of fibrinogen [143;144]. This is supported by the finding of higher levels of fibrinogen in healthy first degree relatives of T2DM patients[145] and that plasma levels were able to predict development of T2DM in healthy individuals which implies that hyperinsulinaemia rather than hyperglycaemia alone is important [146]. This finding may also explain why improving glucose levels through the use of insulin does not correlate with a

reduction in fibrinogen levels[147;148] whereas metformin use has been associated with lowering plasma levels[149]. Higher levels of fibrinogen also alter final clot structure and, in part at least, help explain the differences seen in clot structure in CVD and DM as above.

1.7.1.3 Tissue Factor

The levels of TF have been shown to be elevated in patients with CAD, with highest levels seen in those with unstable angina or MI compared with stable angina[9]. Diabetes is also associated with elevated levels of TF [137], possibly as a result from diminished inhibition of TF synthesis by insulin in diabetes compared with non-diabetes subjects [150].

1.7.1.4 Plasminogen activator inhibitor-1

Elevated levels are seen in CAD, and levels are strongly correlated with risk factors for the MetS, namely BMI, blood pressure, plasma triglycerides and insulin levels[35]. In diabetes subjects there are increased levels of PAI-1, which is positively correlated with glycaemic control[151], and elevated levels are also thought to be an independent risk factor for the development of T2DM[146], adding further weight to the suggestion that insulin resistance and hyperinsulinaemia, in the face of normoglycaemia, is sufficient to trigger hypofibrinolysis[152]. A combination of inflammatory cytokines, insulin, free fatty acids and very low density lipoproteins are likely to cause increased release of PAI-1 from the liver and also adipocytes[11;153-157]. It has not been clearly demonstrated that elevated PAI-1 levels are a risk factor for CVD[158], however tPA/PAI-1complexes, which correlates with PAI-1 activity, has been shown to

be a risk factor for MI, supporting the role of PAI-1 in reduced fibrinolysis and CVD[159;160].

1.7.1.5 Factor VII

Factor VII forms a complex with TF and initiates the formation of thrombin. Studies investigating the effect of coagulant activity of FVII (FVII:c) on cardiovascular events have yielded conflicting results although there is a suggestion that elevated FVII:c levels are associated with fatal cardiovascular events[35;161;162]. Levels of FVII:c are raised in T2DM and also in healthy first degree relatives of T2DM and those with the MetS[163-165], again suggesting that observed changes precede the development of T2DM and hyperglycaemia. Possible explanations for this observation is that triglycerides (TG), which are associated with elevated FVII:c[163;164;166] along with poor glycaemic control and MetS, are involved in activation of FVII[167]. FVII is also thought to bind to TG rich particles and reduced post-prandial breakdown of these fatty proteins lead to increased plasma FVII levels[35].

1.7.1.6 Factor VIII and vWf

The clotting factor VIII circulates in the plasma bound to vWf, which offers stability and increases half life of the protein [11]. Elevated levels of FVIII and vWf have been associated with CVD but as with other clotting factors, this association is lost following adjustment for traditional CVS risk factors [168;169]. Levels are elevated in diabetes and in these individuals the correlation with CVD remains after adjustment for cardiovascular risk factors [129;170;171]. It is likely that raised levels of FVIII and vWf are an indication of EC damage resulting from inflammation and insulin resistance [130;131].

Haemostatic component	Function	Changes in DM	Effect in DM
TF	Initiates clotting cascade	↑levels	↑ thrombosis
FVII	Forms complex with TF	↑levels	↑thrombosis
FVIII & vWf complex	Adherence of platelets to endothelial cell wall	↑levels	↑platelet activation
Thrombin	Converts fibrinogen to fibrin	↑ levels	Altered clot structure
Fibrinogen	Forms fibrin clot	↑levels ↑glycation	Altered clot structure ↓fibrinolysis
Plasmin	Breaks down fibrin clot	↓levels	↓fibrinolysis
PAI-1	Inhibits production of plasmin	↑levels	↓fibrinolysis
Platelets	Activation of coagulation factors and forms fibrin mesh	↑activation	↑thrombosis

Table 1-3 Summary of changes to haemostatic components in diabetes

1.7.1.7 Plasmin

As described in section 1.4.2.2.1, plasminogen conversion to plasmin is reduced in diabetes as a result of increased glycation, along with the efficacy of plasmin itself once generated. Lower levels of plasmin are also expected due to increased activity of PAI-1 and cross linking of PI in diabetes, all of which contributes to the hypofibrinolytic environment associated with diabetes.

1.7.2 Qualitative changes in coagulation and fibrinolysis proteins

1.7.2.1 Fibrinogen

In vivo hyperglycaemia in poorly controlled diabetes patients in known to cause glycation of fibrinogen[172;173] which causes an alteration in the structure and function of the molecule and contributes to the development of the rigid clot architecture seen in diabetes[132;174] which is associated with premature CVD[175;176]. Improving blood glucose control can modulate fibrinogen glycation [134;172].

1.7.2.2 Plasminogen

Increased glycation of plasminogen has been observed in diabetes compared with healthy controls, with lysine residues 107 and 557 preferentially glycated. These sites are involved in fibrin binding and cleavage of plasminogen to form plasmin which may explain the reduction in plasmin generation and activity[56].

1.7.2.3 Plasmin inhibitor

The glycation of PI in diabetes has not been reported, but its anti-fibrinolytic properties may be enhanced in these patients by increased FXIII mediated cross linking to fibrinogen during clot formation[133]. The mechanism underpinning this has not been elucidated but it may be related to structural changes that occur within the fibrinogen molecule as a result of hyperglycaemia or through increased activation of FXIII by thrombin[133].

1.8 The Role of inflammation in Diabetes

A tantalising glimpse of the association between inflammation and diabetes was first noted around the turn of the last century, with evidence that sodium

salicylate lowered levels of glycosuria in patients with “mild” forms of diabetes. This concept failed to fully capture the imagination of the scientific world at the time, and it was not until the end of the 1950s that further evidence emerged. High dose aspirin was found to improve blood glucose control in T2DM patients such that insulin treatment could be withdrawn[177]. However, researchers attempted to link their observations to the secretion of insulin rather than resistance to its action, secondary to an inflammatory milieu, and therefore the association between inflammation and diabetes was once again dismissed. The discovery in the 1990s that adipose cells were capable of secreting the pro-inflammatory cytokine, TNF-α, which was subsequently able to induce insulin resistance, revolutionised thinking at the time [178]. There was now direct evidence that adipose cells were metabolically active, rather than mere energy storage cells with the potential to impair cellular responses to insulin and therefore ultimately lead to the hyperinsulinaemic hyperglycaemic state characteristic of T2DM.

Current theories linking diabetes and inflammation revolve around the nuclear factor NF-κB which once activated by IκB kinase (IKK), promotes and maintains inflammation, and that this pathway is stimulated by hyperglycaemia[179]. Elevated levels of circulating pro-inflammatory cytokines exert varied effects within different tissue cells. Within hepatocytes and adipocytes there is increased monocyte recruitment and macrophage formation leading to further cytokine secretion. This is associated with insulin resistance through impairment of adipocyte differentiation and insulin signalling along with hepatic and skeletal muscle lipid accumulation[180]. Insulin resistance and obesity are not associated with T1DM and so it is likely that other mechanisms are involved. However,

insulin-treated T1DM subjects can acquire later in life a type 2 phenotype, and therefore distinguishing pathogenic mechanisms in T1DM and T2DM is not that straightforward.

Inflammation has been linked to both T1DM and T2DM through elevated plasma levels of inflammatory markers such as CRP, C3 and IL-6[6;181-183]. In addition both CRP and C3 levels have been demonstrated to predict the development of T2DM [184-186]. Both proteins have also been closely linked with features of the MetS [187-189], which may suggest that obesity and insulin resistance may account for much of this observed inflammation in T2DM subjects. Inflammatory cytokines, released from macrophages and adipocytes induce CRP release from the liver. CRP is able to activate the complement pathway, and may also play a functional role in inducing further inflammation, atheromatous formation and haemostasis as described previously. Complement C3 is secreted by adipocytes which may explain the elevated plasma levels observed in obesity, and reductions in C3 levels following weight loss via diet and lifestyle modifications or through bariatric surgery in non-diabetes individuals [190-192]. Hyperglycaemia, common to all forms of diabetes, is likely to induce inflammation through increased mitochondrial reactive oxygen species (ROS) formation[193].

Studies have shown that improving glycaemic control can reduce plasma C3 levels and other inflammatory proteins [6;194]. The hypoglycaemic agent, rosiglitazone, has been shown to reduce C3 levels[195], whereas treatment with the biguanide, metformin appears to lower CRP and not C3[196]. Both medications improve insulin sensitivity and so the differences observed may be related to the direct effect of rosiglitazone on adipose cells. The use of bariatric

surgery to treat poorly controlled diabetes in obese T2DM patients, demonstrated a reduction in highly sensitive CRP compared with intensive medical treatment. Major differences were seen in the degree of weight loss and reduction in glycated haemoglobin between the medical and surgical groups, along with reduced markers of insulin resistance, but not for lipid indices[197]. A summary of the contributing factors that lead to increased risk of CVD in diabetes can be seen in Figure 1-10.

1.8.1 Inflammation and CVD

The link between inflammatory markers such as C-reactive protein (CRP) and CVD is not a new concept, with elevated levels known to be associated with CVD and also predicting the risk of developing the condition [198;199]. Other inflammatory molecules such as C3, C4, C5a and IL-6 have been demonstrated to be elevated in coronary artery disease[200]. Of these, C3 appears most strongly and independently associated with MI, and is thought to be a more specific marker for inflammation than CRP [64;188;201-206]. As well as elevated plasma levels of complement proteins, elevated levels of C3 and C5a, CRP, MAC and macrophages have also been found in atherosclerotic plaques of patients with unstable angina compared with stable disease, indicating activation of the complement pathway during myocardial ischaemia[113;114;207;208]. The complement proteins C1q, C3, C4 and C5b-9 have also been found within the fibrin clots[209]. These proteins can directly influence the composition of atheromatous plaques and induce a pro-thrombotic state by activating the clotting cascade. The uptake of oxidised low density lipoprotein (LDL) molecules into macrophages (producing foam cells with the vessel wall) is enhanced by CRP[210]. These macrophages produce matrix metalloproteinases

which reduce plaque stability and make them increasingly prone to rupture[211], triggering acute cardiovascular events. Cytokines released from macrophages, along with CRP, C3a and C5a are able to induce TF expression within EC, initiating the generation of the fibrin clot[115;117;212;213]. Within the ischaemic myocardium the activated complement pathway is thought to be the major mediator of immune and inflammatory responses. Further ischemia ensues due to activation of leucocytes, EC and up-regulation of genes involved in cytokine production along with reduced NO production[203]

1.8.2 Effect of Diabetes on the complement system

The effect of diabetes on the components of the complement system is much less understood and studied in comparison to the coagulation system.

1.8.2.1 Quantitative changes

As described in 1.3.7.1.2, levels of the key protein C3 are elevated in patients with T1DM and T2DM and improving glycaemic control can lower these levels. The incorporation of C3 into a fibrin clot slows its subsequent lysis ex vivo, and this incorporation appears to be increased in clots prepared from diabetes patients. The reason for this increased incorporation is unclear but may be related to enhanced interaction between C3 and fibrin(ogen) in diabetes as a results of glycation of one or both molecules[6;214]. Oxidative stress and increased ROS, are associated with chronic hyperglycaemia and both play a role in the activation of the MBL pathway within the EC[11;215].

1.8.2.2 Qualitative changes

The glycation of complement proteins CD59 and C3 have been associated with an increased susceptibility to infection as described in 1.3.7.2.2 and may also

be implicated in the microvascular complication of diabetes in the case of CD59 due to dysregulation of the MAC. Moreover, a recent study suggests that diabetes-induced post-translational modifications in C3 may increase its antifibrinolytic activity[6], although further work is required to conclusively prove these preliminary findings.

1.9 Interaction between coagulation and complement

The enzymes responsible for the cascade of events within the complement cascade are serine proteases, and many of the proteins involved in coagulation and fibrinolysis also belong to this family. In vitro production of biologically active C3a and C5a have been demonstrated by factors Xa and XIa as well as thrombin and plasmin, suggesting that proteins involved in haemostasis are also able to activate the complement pathway independently of the recognised and established pathways[78-80]. Complement proteins may also activate and inhibit the formation of a fibrin clot as C3a and C5a have been shown to induce expression of TF within the EC and C1 esterase inhibitor is able to inhibit the action of FXIa. This indicates that both haemostasis and inflammation are interlinked and may combine to create a prothrombotic environment within certain at risk individuals.

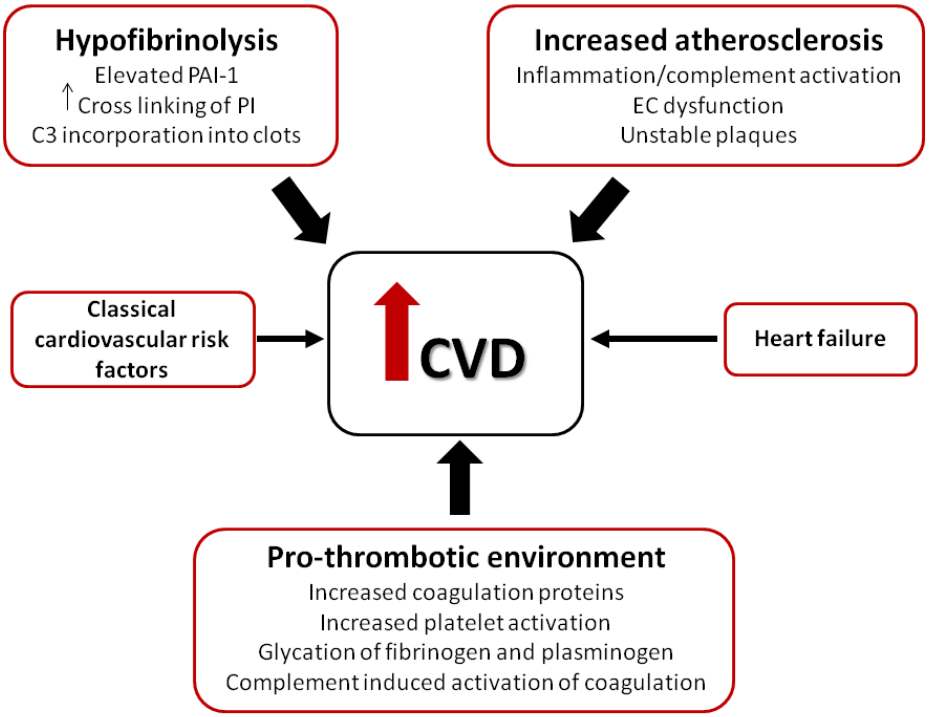

Figure 1-10 Summary of the contributing factors to increased CVD in diabetes.

There is a pro-thrombotic, hypofibrinolytic environment due to alterations in numerous proteins involved in coagulation and fibrinolysis and also platelets. Chronic, low level inflammation and activation of the complement system contributes to this and increases the atherosclerotic burden predominantly through EC dysfunction.

1.10 Complement inhibition to treat CVD

Given the evidence that activated complement proteins can activate the coagulation cascade and have further deleterious effects on an already damaged and ischaemic myocardium, its inhibition as a treatment for CVD would seem a logical approach. Trials using pexelizumab, an anti-C5 monoclonal antibody, to block the action of C5, following MI and post coronary artery bypass grafting have had mixed results with inconsistent reductions in

mortality and major cardiac events[216-218]. The use of a C1 esterase inhibitor, TP10, has also been trialled. Given as a bolus at least 6 hours following the onset of acute myocardial infarction symptoms, and subsequent infusion for 48 hours it demonstrated reductions in the release of myocardial enzymes troponin T and creatinine kinase, elevated levels of C1 inhibitor activity and reduced levels of C4[219;220]. Its use following emergency reperfusion with coronary artery bypass grafting demonstrated improved cardiac function compared to placebo but with no improvement on early mortality[221]. The mixed results of these trials in the acute setting may indicate that inhibition of complement may be better directed at preventing the progression of unstable atherosclerotic plaques[222].

Activation of the complement system following oxidative stress has been demonstrated in animal studies in vivo, as a result of myocardial reperfusion, and is a possible mechanism for increased tissue injury observed as a result of reperfusion following ischaemia. Inhibition of the MBL pathway through the use of anti-MBL monoclonal antibodies has been shown to significantly reduce deposition of MBL and C3b on human endothelial cells in vitro and may form the basis of novel therapeutic strategies to prevent further tissue injury following MI[82].

Another option for preventing activation of the MBL pathway would be inhibition of MASP-2. Mice deficient in MASP-2 demonstrated significantly smaller volumes of myocardial infarction in a model of post-ischaemia reperfusion injury. The generation of a similar monoclonal antibody to MASP-2 may inhibit its function in-vivo and thus activation of the MBL pathway[223].

It is likely that mechanisms involved in inflammation in diabetes are a complex interplay between hyperglycaemia, insulin resistance and obesity. Activation of

the complement pathway results in communication with the coagulation pathway and vice versa. Limiting their activation may lead to a lower incidence of CVD and improved outcomes in these high risk individuals. Increased incorporation of C3 into the fibrin clot occurs in diabetes, resulting in prolonged lysis time. A better understanding of the mechanisms involved in this interaction may lead to the generation of novel therapeutic targets.

Hypothesis and aims of the work

It is clear that delayed or inadequate breakdown of fibrin clots occurring within the arterial tree increases an individual's risk of developing CVD. Up until the last decade or so, the most effective treatment of an acute myocardial infarction was the intravenous administration of recombinant fibrinolytic agents, such as tPA, in order to restore blood flow within an occluded coronary artery. This practice has largely been superseded by primary percutaneous coronary angioplasty, however fibrinolytic therapy is still administered to acute ischaemic strokes with proven efficacy[224]. Patients with diabetes are at increased risk of CVD as outlined previously, and hypofibrinolysis is one of several factors implicated. This pathological resistance to clot breakdown is explained in part by increased incorporation of anti-fibrinolytic proteins C3 and PI into the fibrin network. Interference with the incorporation of these two proteins into the clot may be a potential, diabetes specific, target for the development of novel therapeutic agents to reduce the thrombosis potential and cardiovascular risk in this population. This leads to my hypothesis that the incorporation of C3 into the clot is an important factor in hypofibrinolysis associated with T1DM and T2DM and that interfering with C3-fibrniogen interactions will modulate clot lysis.

My work investigates new pathways responsible for hypofibrinolysis in diabetes and employs alternative methodologies in an attempt to modulate the fibrinolytic process in this condition. The thesis is therefore largely presented in two distinct parts. In the first, I examine the role of complement C3 in hypofibrinolysis in diabetes and investigate the mechanisms involved. In the second part, I attempt to target modulation of fibrin clot lysis through disruption of the fibrinogen-PI and fibrinogen-C3 interactions using novel approaches.

The aims of the study are as follows:

A. Study the role of C3 in hypofibrinolysis in diabetes:

1. Investigate the interaction between C3 plasma levels and clot lysis in a large cohort of type 2 diabetes patients.

2. Analyse potential differences in the anti-fibrinolytic effects of C3 comparing diabetes with control protein and investigate post-translational protein modifications as a potential mechanism.

3. Elucidate potential binding sites between fibrinogen and C3.

B. Modulate fibrin clot lysis using a targeted approach:

1. Analyse the effects of interfering with fibrinogen-PI interaction on fibrin clot lysis

2. Investigate the role of interference with fibrinogen-C3 interaction on fibrin clot lysis.

Chapter 2

General Methods

Introduction

In order to accomplish the aims of the work, a number of different methodologies were employed many of which are well validated and accepted such as protein purification, turbidimetric analysis, ELISA and mass spectrometry and these are described in this chapter. Other, known methods that were used in a novel way, such as phage display and molecular modelling, are also described here. More specific methodologies, that are relevant to each of my aims, are further detailed in the appropriate Chapter.

2.1 Claus method for plasma fibrinogen level determination

Plasma was diluted 1:10 in veronal buffer pH 7.35 (0.026M sodium acetate trihydrate, 0.026M sodium 5,5 diethylbarbiturate, 0.11M NaCl, 0.016M HCl in H_2O) and incubated at 37°C with 5U/ml bovine thrombin in a KC10 Amelung Coagulometer (Amelung, UK) with stirring. The fibrinogen concentration of each plasma sample was deduced by comparing the time taken for the clot to form against a calibration curve of reference plasma (Biomereux, Hampshire, UK) with known concentration of fibrinogen.

2.2 C3 ELISA

A NUNC ELISA plate was coated with 100µl (2µg/ml) of chicken-anti-human C3 antibody (GenWay Biotech. Inc, San Diego, USA) for 1 hour after which the wells were washed 3 times with 200µl of wash buffer (0.05% [v/v] Tween 20 in phosphate buffered saline [PBS]) and then blocked with 200µl of 1% (w/v) bovine serum albumin (BSA) for 1 hour at room temperature. The washing step was repeated, followed by the addition of 100µl of plasma samples (diluted x10,000 in 50mM Tris, 0.14M NaCl, 1% BSA, 0.05% Tween 20) and incubated for 2 hours at room temperature. The wells were washed again before adding

100µl (20ng/ml) of the secondary, HRP conjugated, chicken anti-human C3 antibody (GenWay) for 1 hour. After a final washing step, the plate was developed by the addition of 100µl of stabilised chromogen substrate, tetramethylbenzidine (TMB). The reaction was stopped by the addition of 1.5M H_2SO_4, and absorbance was measured at 450nm using a MRX Microplate Reader (Dynex Technologies, Ashford, Middlesex, UK). A standard curve containing known concentrations of C3 was included with each plate to determine the C3 levels.

2.3 Plasminogen activator inhibitor-1 ELISA

Plasma levels of plasminogen activator inhibitor-1 (PAI-1) were measured using a PAI-1 ELISA kit (Life Technologies, Paisley, UK) as per the manufacturer's instructions. ELISA plates were pre-coated with PAI-1 antibody to which 100µl of plasma samples (diluted 1 in 2 in sample diluent provided) were incubated for 2 hours at room temperature. The wells were washed four times with 200µl of wash buffer provided, after which, 100µl of biotin labelled anti-PAI-1 was added to each well for a further 2 hours at room temperature. The wells were washed again and 100µl of streptavidin-HRP conjugate added for 30 minutes at room temperature. Following the final wash step, 100µl of stabilised chromogen, TMB was added for 30 minutes and the reaction terminated by the addition of 1M H_2SO_4 and the plate read at 450nm using the plate reader as above. A standard curve containing known concentrations of PAI-1 was included with each plate to determine the PAI-1 levels.

2.4 Fibrinogen purification

2.4.1 Affinity chromatography

Purification of fibrinogen was performed by affinity chromatography using calcium dependent IF-1 monoclonal antibody (Kamiya Biomedical, Seattle, WA, USA) and an automated chromatography system (Biocad sprint, Applied Biosystems, Warrington, UK). Affinity chromatography exploits the reversible binding of certain ligands to their "receptor". In this case, fibrinogen binds with high affinity to a monoclonal antibody (that is constrained in a resin column) in the presence of calcium, whilst other, unwanted plasma proteins run through the column. Following the addition of wash buffers an elution buffer containing EDTA is introduced which causes chelation of calcium and thus fibrinogen is released from the antibody and can be collected.

To prepare the citrated plasma samples for purification on the IF-1 column, 1.2ml plasma was added to a final volume of 5ml of 100U/ml heparin and 0.02M $CaCl_2$ in equilibration buffer (0.02 M Tris, 0.3 M NaCl, 1 mM CaCl2, pH 7.4). After loading the sample, the column underwent washing using 6 column volumes (CV) of wash buffer 1 (0.02M Tris,1M NaCl, 1mM $CaCl_2$, pH 7.4) and 6 CV of wash buffer 2 (0.05M sodium acetate, 0.3M NaCl, 1mM $CaCl_2$, pH 6).

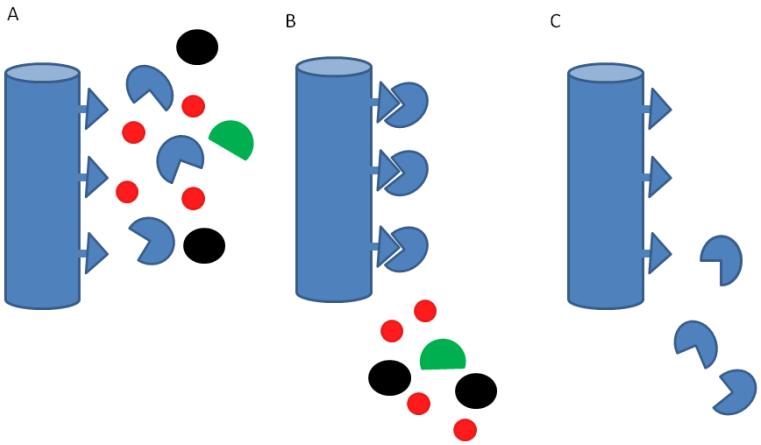

Figure 2-1 Illustration of the concept of affinity chromatography

The column containing the monoclonal antibody is equilibrated and the plasma sample is injected (A). The antigen of interest (fibrinogen) binds to the antibody and the remaining unwanted plasma proteins are washed through (B). The addition of an elution buffer alters the environment to allow the antigen to dissociate from the antibody (C) and is collected.

Elution of fibrinogen was achieved by adding 7 column volume of elution buffer (0.02M Tris, 0.3M NaCl, 5mM EDTA, pH7.4). The chromatograph produced was used to estimate a peak of the fibrinogen eluted. Those tubes of elutant within this peak were tested by spectrophotometry. All those fragments with a protein concentration >0.02mg/ml, assessed by a ND-1000 spectrophotomer (Nanodrop, Wilmington, DE, USA) measuring absorbance at 280nm and applying mass extinction coefficient 15.1 for a 10mg/ml solution of fibrinogen. Samples were pooled and stored at -80˚C.

2.4.2 Concentration

The pooled fibrinogen eluted from the BioCad was transferred to 100,000 MWCO vivaspin20 concentrator tubes (Generon, Maidenhead, Berkshire, UK) and spun at 3000rpm until the volume in the filter was approximately 1ml.

2.4.3 Dialysis

Dialysis tubing (Sigma) was prepared by boiling the tubing in 1M EDTA. The tubing was rinsed with ddH_2O and each of the fibrinogen samples dialysed against 50mmol Tris, 100mmol NaCl pH 7.4 at 4°C for 2 consecutive hours and overnight with gentle stirring.

2.4.4 Quantifying fibrinogen concentration

The final protein concentration was determined using a ND-1000 spectrophotomer (Nanodrop, Wilmington, DE, USA), applying mass extinction coefficient, 15.1 as above.

2.5 C3 purification

The purification from individual plasma samples was performed in three separate steps, namely precipitation, ion exchange chromatography and finally gel filtration

2.5.1 Precipitation

The first process involves precipitation of the plasma sample using polyethyleneglycol 4000 (PEG). From a stock concentration of 15% PEG 4000 , 3ml is added to 6ml of plasma along with 90µl of 100mM PMSF and stirred for 30 minutes at 4^oC, followed by centrifugation for 30minutes at 4^oC and 10,000g in a Beckmann centrifuge. The pellet is discarded and 8.4ml of the supernatant is added to 4.2ml of 26% PEG 4000 and 42µl of PMSF and stirred again for 30 minutes at 4^oC and then spun at 10,000g at 4^oC for 30minutes. The supernatant is then discarded and the pellet is resuspended in 6ml of starting buffer (20mM Tris, 5mM EDTA, pH 8.7) and the protein concentration measured using a Nanodrop as described previously.

2.5.2 Ion exchange chromatography

The technique of protein separation using ion exchange chromatography is dependent on the reversible interaction of a charged protein with an oppositely charged chromatography medium. Altering the pH of the surrounding environment will alter the net surface charge of a protein and thereby disrupt this interaction. In this instance, a stepwise increase in NaCl concentration causes elution of differently charged plasma proteins at differing time points.

To separate C3 from the precipitated plasma proteins the samples were loaded into a MonoQ 5/50 GL column (GE Healthcare) which was equilibrated with five column volumes (CV) of starting buffer (SB). Approximately 25mg of protein was injected onto the column followed by 15 CV of SB to wash the column. The C3 was eluted from the column by adding increasing concentrations of elution buffer (EB: 20mM Tris, 5nM EDTA, 1M NaCl) in 10 sequential steps each of 10 CV, starting at 90%SB and 10%EB and finishing with 100% EB. The C3 elution peak was observed at around the fourth elution step. Finally the column was equilibrated with 10 CV of SB. The presence of C3 within the fractions was confirmed by Nanodrop, by measuring absorbance at 280nm and applying mass extinction coefficient of 18.2 for a 10mg/ml solution of C3. The samples were kept at 4^{o}C overnight and the buffer exchanged for Tris bufferd saline (50mM Tris, 100mM Nacl) using disposable PD-10 desalting columns (GE Healthcare) and the samples concentrated to around 500µl using Vivaspin 20 concentrating tubes (MWCO: 3000)

2.5.3 Gel filtration

The final step in purification of C3 is gel filtration, also known as size exclusion chromatography. It is a relatively simple technique that will separate substances

based on size by allowing them to pass through a chromatography column containing pores of different sizes. Smaller molecules are able to enter the small pores within the beads of the column and thus take longer to traverse the length of the column compared to larger molecules that are unable to enter the pores and so have a much smaller surface area through which to navigate (Figure 2-2). A Superose 12 (10/300) GL column (GE Healthcare) was used which was loaded with 500µl of sample and eluted with 60ml tris buffered saline. The peak was collected and concentrated using Vivaspin 20 concentrating tubes (MWCO: 3000) and stored at -80^{o}C until required.

2.5.4 Quantifying C3 concentration

Protein concentration was determined using a ND-1000 spectrophotomer (Nanodrop, Wilmington, DE, USA) measuring absorbance at 280nm and applying mass extinction coefficient of 18.2 for a 10mg/ml solution of C3.

2.5.5 SDS Page

A sodium dodecyl sulphate (SDS) polyacrylamide gel (Invitrogen) was run to determine the quality and purity of the purified fibrinogen or C3. 20µl samples of fibrinogen or C3 were prepared in 10µl sample buffer and 4µl and incubated at 70°C for 10 minutes. The samples were run on a 4-12% NuPAGE Novex Bis-Tris gel (Invitrogen) at 200V for 35 minutes using MES SDS running buffer in a Novex mini-cell tank (Invitrogen). 5µl of Perfect Protein marker (Novagen, Madison, WI, USA) was added to one well as a molecular weight marker.

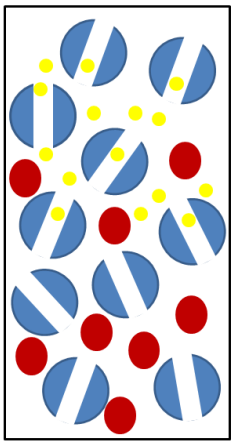

Figure 2-2 Illustration of gel filtration.

Larger molecules (red) are unable to enter the pores and so are eluted first as they have a smaller surface area to traverse compared to molecules that are small enough (yellow) to enter the pores.

2.5.6 GelCode staining

After electrophoresis, the gel was rinsed with dH_2O for 15 minutes and then stained with GelCode Blue Stain Reagent (ThermoScientific) solution for 1 hour with shaking. The gel was again rinsed in dH_2O for 15 minutes and visualised using Chemi-imager software (Alpha Innotech, San Leandro, CA, USA) and a trans-white light. Three bands were expected for fibrinogen (corresponding to the α, β and γ chains), and two for C3 (α and β chains) and correct sizes verified using an appropriate ladder, 66, 56 and 48 kDa respectively for the fibrinogen chains and approximately 115 and 70kDa for C3, as illustrated in Figure 2-3.

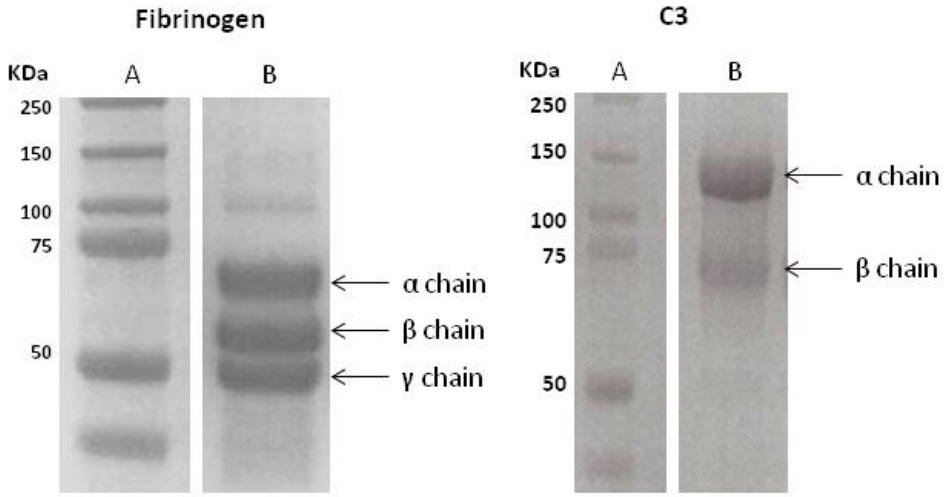

Figure 2-3 Purified human fibrinogen and C3

SDS PAGE of purified human fibrinogen with the α (66kDa), β (56kDa) and γ chains (48kDa) and human complement C3 with α (115kDa) and β chains (70kDa). Lane A in each image indicates the lane marker and lane B indicates the purified protein.

2.6 Turbidimetric analysis of clot formation and lysis

2.6.1 Turbidity

Turbidity analysis dynamically measures the formation of a clot following the addition of an activation mix (AM) containing thrombin and $CaCl_2$ to plasma or fibrinogen. As the fibrin clot begins to form, following lateral aggregation, the optical density (OD) of the reaction mixture increases which can be measured with a plate reader. The amount of light reflected by the clot when light of a set wavelength is directed through it is estimated and by taking measurements at set time points, clot formation can be expressed graphically.

2.6.2 Turbidity and lysis

Turbidity and lysis experiments are more representative of physiological conditions. The addition of a tpA and plasminogen to the reaction mix allows fibrinolysis to occur alongside clot formation. Fibrin polymerisation occurs as with turbidity only experiments, causing an increase in OD and as the clot is broken down, OD and absorbance decrease, finally reaching a baseline at which point the clot is fully dissolved.

2.6.3 Turbidimetric parameters

Several clot formation and lysis parameters can be calculated from the turbidity and lysis curves generated.

The Lag phase corresponds to the initial stages of clot formation, during which time fibrinogen is converted to fibrin monomers and subsequently protofibrils. Given that lateral aggregation of protofibrils has not occurred during this phase, optical density remains at baseline. The end of lag phase is marked by the exponential rise in optical density above baseline as lateral aggregation occurs. Short lag times may be associated with thrombogenic clots ex vivo[225-227].

Final turbidity (FT) equates to the maximum absorbance of the reaction mix and represents the stage at which the clot is fully formed. FT is recorded as the highest reading of optical density from the turbidity and lysis trace. In plasma experiments this measurement reflects both the thickness of the fibrin fibres and clot density. In a purified system, FT only reflects fibre thickness. In terms of clinical relevance, higher FT of clots prepared from plasma samples results in more thrombogenic clots[225;226;228].

Clot formation time (CFT) represents the time between the end of the lag phase and the point at which FT is reached. It is an indication of the rate of fibrin fibre

formation, with long CFT indicating the formation of clots with higher final turbidity or ineffective fibrinolysis. Prolonged CFT is associated with previous ischaemic heart disease in men and women[229].

Lysis time (LT) represents the time from FT to the point at which 50% of the clot has lysed (i.e. 50% FT on the descending limb of the turbidity and lysis curve). A prolonged LT is an indicator of clot resistance to fibrinolysis, and is associated with an increased risk of CVD[229;230].

The lysis area is complex and combines clot formation time, clot final turbidity and lysis times and so it is unsurprising that a larger lysis area is associated with more thrombotic clots.

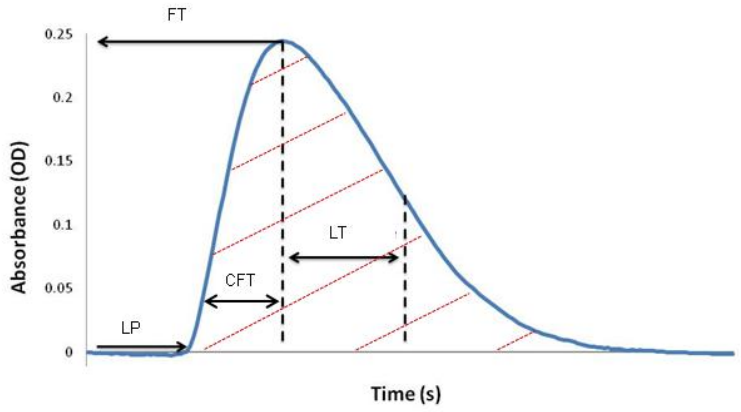

Figure 2-4 Illustration of turbidity and lysis curve and parameters

FT, final turbidity, LP, lag phase, LT, lysis time, CFT, clot formation time. The lysis area is indicated by dashed red lines

2.6.4 Clots made from plasma samples

A total of 25µl of each plasma sample was mixed with 75µl lysis mix (LM) containing 0.1M NaCl, 0.05M Tris, and 2.4nM tissue plasminogen activator (tPA) (Tecnoclone, Vienna, Austria), at room temperature in a 96 well clear polystyrene flat bottom microplate (Grenier Bio-one, Gloucester, UK).

Polymerisation was initiated by addition of 50µl of activation mix (AM) containing 0.1M NaCl, 0.05M Tris, 22.5mM $CaCl_2$ and 0.09U/ml human thrombin (Calbiochem, Nottingham, UK). The increase in turbidity at 340nm was continuously monitored every 12 seconds on a ELx-808 IU ultramicroplate reader (BIO-TEK Instruments INC, Winooski, VT, USA) over a period of 60 minutes.

2.6.5 Clots made from plasma purified or commercial protein

A total of 50µl of purified fibrinogen or commercial fibrinogen (Calbiochem, Merck, Germany), at 1mg/ml (2.94µM), was mixed with 50µl LM, containing 0.2µM plasminogen (Enzyme Research Laboratories, Swansea, UK) in 0.1M NaCl, 0.05M Tris, pH 7.4 (permeation buffer [PB]), in a Greiner 96 well clear polystyrene flat bottom microplate in duplicate. To ensure adequate cross-linking, 5µl of FXIII (20µg/ml, 1.56nM) was added. Polymerisation and lysis were initiated by the addition of 50 µl AM containing 0.37U/ml thrombin, 3.3nM tPA and 7.5mM $CaCl_2$ in 0.1M NaCl, 0.05M Tris, pH 7.4. Increase in turbidity at 340nm was continuously monitored every 12 seconds on ELx-808 IU ultramicroplate reader over a period of 60 minutes.

2.7 Laser scanning confocal microscopy (LSCM)

Confocal microscopy was used to visualise hydrated clots. This technique is advantageous because the samples do not need special preparation such as fixing and dehydration, therefore the clots are imaged in a physiological and fully hydrated status

2.7.1 Clot preparation

For plasma clots, 7.5µl plasma and 1.5µl 448 Alexa labelled fibrinogen (0.5mg/ml) (Invitrogen) were mixed with 21µl PB. 15µl purified/recombinant fibrinogen (1mg/ml) and 1.5µl 448 Alexa labelled fibrinogen (0.5mg/ml) were mixed with 13.5µl of PB. An AM of 35mM $CaCl_2$ and 0.35U/ml thrombin was prepared in PB. 5µl AM was then added to 30µl of plasma/fibrinogen mix, mixed thoroughly and 30µl added to a well of an Ibidi confocal slide (Applied Biophysics, New York, USA). A cover was placed on to the slide which was stored in a humid dark box until imaging

2.7.2 Imaging fluorescent samples

The slide was visualised with LSM510 software on the LSM 510META microscope from Zeiss (Oberkochen, Germany), using the 488 Argon laser, green reflector and 40x 1.3 oil ph3 objective lens. The slide was loaded onto the specimen stage. Scan mode was then selected (LSM) and single track 'CY2 alexa 448' chosen. The master gain was set to 820,digital offset to 0.1 and pinhole to 94µm. To view the same section of each clot the focus wheel was used to find the top of the clot using the 'fast xy' scanning mode and then the view moved 1.5 fine focus turns into the clot. For static images a 1024^2 image size was used and the resolution set to 8. For static Z stacks the resolution was reduced to 4. 3 Z-stacks of each clot were taken, with a range of 20µM at intervals of 2µM.

2.7.3 LSCM clot lysis

Time series function was used with 400 scans with 1 second between scans. The gain, offset and pinhole were set as above, with resolution at 8 pixels and picture size at 512^2. 20µl LM (1.1µM plasminogen, 0.14µM tPA in PB) was

added to the Ibidi well and the slide tapped gently for 30 seconds to encourage the LM into the clot channel. The slide was then left for 30 seconds in a vertical position before loading onto the stage and clot visualisation. The lysis front was identified using fast xy scanning mode and a time series taken at 4minutes after addition of LM so that the lysis front just approached the edge of the first frame of the time series. The lysis time in this instance represents the time taken for the clot within the viewing filed to be dissolved completely. Images were viewed, edited and analysed using LSM image browser (Zeiss).

2.8 Scanning electron microscopy

In order to form a clot for electron microscopy (EM) a lid from a 0.5ml eppendorf tube was cut off and pierced with a needle several times before covering the bottom of the lid with parafilm in order to cover the holes.

2.8.1 Clot preparation

For purified experiments, 45µl of fibrinogen (0.5mg/ml) was mixed with 5µl of AM (0.025M $CaCl_2$, 5U/ml thrombin in PB). For plasma clots, 50µl of plasma was diluted 1:2 with PB and 5µl AM (0.05M $CaCl_2$, 11U/ml thrombin in PB) added. The mixture was mixed with a 200µl tip that had the end removed to ensure even distribution of the AM. The clots were left to form in the lids for 2 hours in a humidity chamber.

2.8.2 Fixing and dehydration

After removing the parafilm, the clots were washed by placing the lids containing the clots in a beaker of sodium cacodylate buffer (0.078M Cacodylic acid (Sigma), pH7.4) for 10 minutes, which was then replaced with fresh buffer and left for a further 10 minutes. The clots were then fixed by transferring the

lids to 2% Glutaraldehyde (Sigma) in sodium cacodylate buffer for 30 minutes followed by washing steps as above. Once fixed, the clots were dehydrated by placing them in acetone at increasing concentrations, 30%, 50%, 70%, 80%, 90% and 95% for 10 minutes each, after which they were transferred to 100% acetone 3 times for 10 minutes.

2.8.3 Critical point drying

I was unable to perform critical point drying (CPD) which was carried out by Martin Fuller at the University of Leeds using a E3000 critical point dryer (Quorum Technologies Ltd, UK). This involves dehydrating the fibrin structure in order for it to be coated with platinum and be detected under the EM. It is crucial not to disturb the surface of the fibrin network during the drying process as EM relies on analysing the surface morphology of biological specimens. Air drying is therefore unsuitable as it may lead to deformation and collapse of protein structures as water has a high surface tension to air, and so as liquid in the sample evaporates the specimen is subjected to large forces at the boundary between the water in the sample and surrounding air. By replacing the water in the sample with liquid $CO_{2,}$ this surface tension is reduced in CPD, and the surface tension between the liquid in the sample and surrounding air is progressively lowered. Liquid CO_2 then passes from liquid to gas without an abrupt change in state and avoids damaging the surface of the sample.

2.8.4 Sputter coating

In order to prevent the clots from charging under the electron beam the samples were covered in a thin layer of conducting metal. The clots were mounted onto aluminium specimen mounts that were covered in a carbon film. They were then

coated with a 7nm thick layer of platinum palladium applied in a 208HR high resolution sputter coater (Cressington, UK).

2.8.5 Clot imaging

Samples were viewed and photographed using a Quanta 200F FEGESEM field-emission scanning electron microscope (FEI, Oregon, USA) in 3 different areas of each clot at magnifications of x5000, x1000 and x30000. Fibre diameters of all clots were measured with image analysis software package ImageJ ,23y (National Institutes of Health, Bethesda, MD, USA).

2.9 Phage display

Phage display technology is being increasingly used in translational research as a tool for validating novel therapeutic targets and drug design. It has multiple uses in studying for example, protein-protein interactions, enzyme specificity, antibody-antigen interaction and epitope mapping[231]. The principle is centred on the construction of a phage library, approximately $3x10^{10}$ in size, consisting usually of random peptides. Insertion of a foreign DNA fragment into the coat protein gene of a phagemid vector causes fusion of the peptide with the N-terminus of the coat protein and is thus expressed externally on the phage particle. The genetic information encoding the peptide is held within the phage which can be sequenced and therefore connects genotype and phenotype. Incubation of the phage library with a target molecule selects the phage that bind which can be sequenced to identify the peptide residues involved. This is achieved by a process called biopanning which involves immobilising a target molecule to a 96 well microtiter plate and adding the phage library to the wells. Unbound phage are removed by washing steps followed by elution of the target

bound phage which are subsequently amplified and a second library (containing increasingly specific phage) produced ready for a second round of biopanning. This process is repeated around 3-6 times, after which the phage particles can be anaylsed by DNA sequencing.

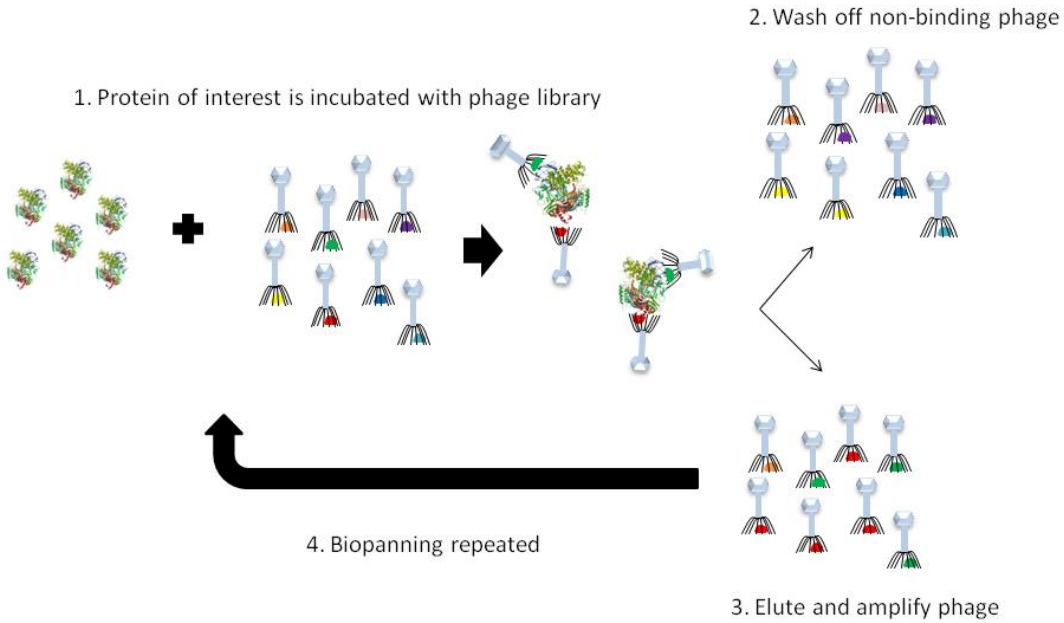

Figure 2-5 Illustration of steps involved in phage display screening

The protein of interest is incubated with the phage library and the unbound phage is washed off. The bound phage is eluted and amplified within E.coli cells and the addition of helper phage. A second phage library is produced and the biopanning process is repeated.

Two separate phage display libraries were used during my research to screen for binders to C3 and fibrinogen and the production of these will be described in more detail in Chapters 4 and 5. For both libraries the peptide sequences were inserted into the coat protein 3 gene (pIII) within a phagemid vector. A phagemid is similar to a plasmid but also contains the f1 phage origin of replication. The vector was electroporated into electrocompetent E.Coli cells containing an F' pilus. In order for single stranded DNA replication to occur, it is

necessary to superinfect the E.coli cells with helper phage (e.g. VCSM13 or M13K07), which contain the necessary genes for phage assembly. The advantage of infection of a bacterial host cell with a phagemid and helper phage in this manner allows the display of larger peptides with fewer copies of the coat protein.

2.9.1 Fibrinogen α chain cDNA phage library

A phage library consisting of fragments of the α chain of fibrinogen was constructed (as detailed in chapter 4) to identify interaction sites between the α chain and C3.

2.9.1.1 Biopanning and enrichment

Commercial C3 (MyBioSource, CA, USA) was immobilised on a 96-well micro-titre plate and left overnight at 4^{o}C. Following removal of the coating buffer the well was blocked by the addition of 400µl of 3% (w/v) BSA for 1 hour followed by washing with TBS/0.5% Tween three times. After suitable washing, 70µl of phage was added and incubated at room temperature for 1 hour, followed by 5 cycles of washing as above in order to remove any unbound phage. To elute the bound phage, 30µl of glycine (pH 2.2) was added and the whole mixture transferred to a clean eppendorf tube along with 6µl unadjusted 2M Tris to neutralise the glycine. To this mixture, 1ml of prepared E.coli cells, XL1-Blue MRF' (Stratagene, La Jolla, CA, USA) was added and incubated at room temperature for 15minutes. In order to estimate the number of phage particles eluted from round 1 of panning, 10µl was plated on agar containing ampicillin. To generate a new, enriched cDNA phage library for a second round of biopanning, the infected E.coli cells were superinfected with 10^{12} plaque forming units of VCSM13 (Stratagene) helper phage at room temperature for 30

minutes. The culture was transferred to 100ml of LB medium containing 50ug/ml ampicillin, 10ug/ml tetracycline and 10ug/ml kanamycin and incubated at 37^{o}C overnight. The phage was precipitated by centrifuging the culture followed by the addition of 0.2 volumes of 40% polyethylene glycol 4000/2.5M NaCl. It was then resuspended in 2-3ml of PBS. In total 4 rounds of panning were performed after which the phage DNA was extracted using a Wizard Minipreps DNA purification system (Promega, Southampton, UK) and sent for sequencing.

2.9.2 Non-antibody scaffold protein (Adhiron) phage library

Collaborators from the BioScreening Technology group at the University of Leeds have developed a phage library containing a non-antibody scaffold protein, termed Adhiron. The protein scaffold is based on a consensus sequence of plant-derived phytocystatins, a cysteine protease inhibitor. The inhibitory sequences within two loops of the protein have been replaced with nine randomised amino acid positions in each loop, producing a library size or 1.3 x 10^{10} clones. The presence of the peptides within the scaffold maintains the conformation of the peptide which is an important consideration when investigating protein-protein interactions.

2.9.2.1 Biopanning and enrichment

The Adhiron library was used to identify binders to both fibrinogen and C3 and the biopanning was similar to that employed with the fibrinogen α chain library. There was, however, a difference in the method used to immobilise the target protein. Commercial fibrinogen (Calbiochem) or C3 (MyBioSource) were initially biotinylated and added to streptavidin coated wells for 1 hour. The Adhiron phage library was incubated with fibrinogen or C3 for 2.5 hours, after which the

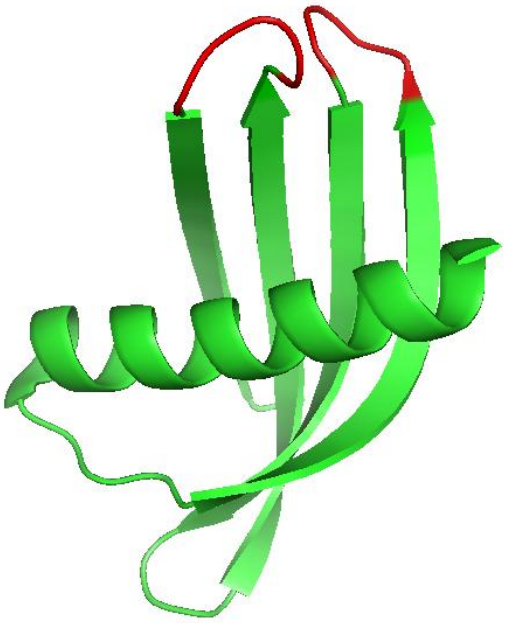

Figure 2-6 Crystal structure of Adhiron (PDB code 46NT)

The crystal structure of an Adhiron scaffold protein with the loop regions containing the random sequence of 9 amino acids shown in red.

wells were washed 10 times and the bound phage were eluted by the addition of glycine and used to infect 1ml of E. coli ER2738 electrocompetent cells (Lucigen, Wisconsin ,USA). Colonies of the ER2738 cells were grown on LB agar plates overnight followed by inoculation with M13K07 helper phage in order to generate a new library for the second round of panning as described in 2.9.1.1. In total, five rounds of panning were completed, after which individual ER2738 colonies were selected for DNA sequencing.

2.10 Molecular modelling

The crystal structures of fibrinogen, C3 and C3b (Protein Data Bank [PDB] ID codes 3GHG, 2A73 and 2I07 respectively) were visualised using, Pymol (Schrödinger, Inc.), which is free for academic groups. There is little information currently available regarding the interaction sites of C3(b) and fibrinogen and

given that both are relatively large molecules, current molecular modelling software is not able to identify sites of protein-protein interaction due to the vast computational memory required. By concentrating on the C3 molecule it was possible to identify potential sites of protein interaction through the use of the molecular modelling software, AutoLigand (Scripps Research Institute). This is an automated predictor of ligand binding sites in proteins which works by analysing the geometry of a protein surface. The majority of binding sites on a protein are within pockets or clefts and this method is able to search the entire surface of a protein structure and identify areas of maximal affinity for ligand binding sites[232]. Once suitable areas of binding are identified, the electronic high throughput screening software, eHiTS (SimBioSys, Inc.) will be used to target these regions. eHiTS is an automated docking tool which allows the rapid evaluation of large compound libraries in order to identify ligands which can bind to the protein of interest. This algorithm utilises a database of small molecules, in this case, the small molecule screening library from Peakdale Molecular. It divides the 3-dimensional ligand structures into rigid fragments and flexible chains and each rigid fragment is docked into pockets, or receptor sites, identified on the surface of the protein. Different orientations or “poses” of these fragments that are compatible with chemistry and steric constraints are tested within the receptor site and a score is determined based on interacting surface points between the ligand and cavity. The flexible chains that connect the rigid fragments are fitted to reform the ligand using the lowest energy chain configuration possible[233]. Validation studies of this docking programme has demonstrated its ability to replicate ligand binding compared to known crystal

structures of bound ligands and it has been used previously as a screening tool to identify novel drug targets[234-236].

Protein-peptide interactions are a subset of protein-protein interactions and are known to be able to regulate certain biological processes and may be manipulated therapeutically given that the interaction surface is small in relative terms[237]. This approach of studying protein-peptide interactions was a logical step in order to identify sites of interaction between C3 and fibrinogen through the use of peptide sequences, identified from techniques such as phage display and microarray screening that bind C3 and fibrinogen.

Modelling of the predicted binding sites of these peptides with C3 and fibrinogen was achieved through the use of freely available software, PepSite, (www.pepsite2.russelllab.org) that is capable of predicting the binding sites of peptides, up to 10 aa in length, to a known protein surface (using the PDB ID code). PepSite is available via a web server and the initial version has been superseded by PepSite2[237]. The original method studied 405 different protein-peptide interactions that had a known 3-dimensional structure. From this data every peptide residue was allocated a spatial position-specific scoring matrix (S-PSSM), which essentially encodes the calculated preferred binding environment for each peptide residue. PepSite2 utilises these S-PSSMs and scans them against the protein structure of interest and predicts preferred sites of amino acid binding within the protein, termed hotspots. These hotspots are subsequently matched against every residue within the peptide sequence to be analysed. The resulting pairs of matched residues are subjected to distance constraints so that they lie within a range typically seen in peptide structures[237]. The result is an approximation of the peptide structure bound to the protein

surface and is presented visually using a Java viewer, Jmol, and in a table in order of statistical significance. The results can also be downloaded as a PDB file and visualised using Pymol.

Molecular modelling technique	Description
Autoligand	Searches a known protein structure for potential ligand binding sites
eHiTS	Uses a library of small molecules to identify ligands to a known protein structure
Pepsite2	Determines predicted binding sites of amino acids within a peptide sequence (up to 10 amino acids in length) to a known protein structure
AutoDock	Predicts the docking poses of 3-dimensional peptide structures to a known protein structure.

Table 2-1 Summary of molecular modelling techniques used

Brief description of the molecular modelling techniques utilised and the information generated as a result

Another method of identifying protein-peptide interaction sites is to use the docking program, AutoDock (Scripps Research Institute). AutoDock can be used when the structure of both the ligand and target molecule are known, but the site of interaction is not[238]. It attempts to dock the peptide or ligand to the protein of interest using a grid based method that permits identification of the binding energy between the two molecules. A grid is placed over the surface of the target protein and a probe atom from the ligand is placed at each grid point which then stores the calculated interaction energy between the protein and probe. The software also generates different “trial” conformations of the ligand based on the types of bonds present and thus generates a number of potential

docking sites based on the binding energies produced by the trial conformations[239]. This technique will be employed using peptide sequences from fibrinogen identified from the microarray and phage display screening and also using Adhirons of interest. A summary of the different molecular modelling techniques employed can be seen in Table1-1. The Autoligand and AutoDock screening were performed by Dr Katie Simmons, School of Chemistry, University of Leeds.

2.11 Mass spectrometry

All mass spectrometry laboratory work and data analysis was performed by James Ault of the Faculty of Biological Sciences, University of Leeds.

2.12 Principles of mass spectrometry

A mass spectrometer consists of three components: an ion source, which converts the sample into gas phase ions, a mass analyzer which separates the ions according to their mass-to-charge ratio, and finally a detector, which calculates the abundances of each ion present. The data generated are displayed as a spectrum of the relative abundance of ions as a function of their mass-to-charge ratio (m/z).

Prior to MS analysis, the C3 protein was reduced in order for the α and β chains to be separated and analysed separately. The two chains were separated by SDS-PAGE and then fragmented further following enzyme digestion with trypsin, which cleaves proteins on the C-terminal side of lysine and arginine residues, unless they are immediately followed by proline. As a consequence, the treatment of proteins with trypsin generates peptides with an average length

of approximately ten to fifteen amino acid residues which is well suited for MS detection.

Following fragmentation of the protein, the peptide fragments are subjected to tandem MS analysis (MS/MS). The masses of the sample fragments are determined in a MS survey scan after which peptide ions of interest are isolated according to their mass-to-charge ratio value. Further fragmentation of the selected peptide ion species occurs by the addition of an inert gas such as Argon that imparts internal energy to the ions as they collide. The fragmented ions pass through the mass analyser which determines the m/z values. The molecular mass of amino acids that have been affected by PTM is altered and can be detected by increments or deficits in mass on the MS/MS spectra.

This technique very useful for studying changes as a result of PTM as it is highly sensitive, and requires very little sample. It is able to detect changes in molecular mass that correspond to modifications at both the peptide and protein level and can therefore identify the type and location of the PTM.

This technique does however have limitations as the processes of digestion, ionization and MS/MS activation can lead to dissociation of the PTM from the protein. This tends to occur if the covalent bond between the amino acid and the PTM is chemically unstable and so the PTM may not be detected. An added complication is that the presence of PTM may affect the efficiency of trypsin in cleaving the protein, leading to larger peptide fragments that are less suitable for analysis.

2.12.1 Gel processing and tryptic digestion

25 µL of each sample was buffer exchanged into 50 mM ammonium bicarbonate using Vivaspin 500 3000 kDa MWCO centrifugal concentrators

(Sartorius Stedim). 3 x 25 µL washes with ammonium acetate were performed. Samples were then reduced by addition of DTT to 5 mM with heating to 56 °C for 20 min and then alkylated by addition of iodoacetamide to 15 mM with incubation in the dark at room temperature for 15 min. Trypsin in 50 mM ammonium acetate was then added to the samples in a protein:protease ration of 1:50 w/w and incubated for 18 hours at 37 °C with shaking. After briefly vortexing and centrifuging, the trypsin reaction was quenched by addition of 5 µL of formic acid. Digested samples were stored at -80 °C until LC-MS analysis.

2.12.2 Liquid chromatography-mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS/MS) analysis of the peptide mixtures was performed on an Ultimate 3000 nano LC system (Dionex, Amsterdam, Netherlands). 1 µl of each sample was loaded onto a C18 guard column and washed with 2% acetonitrile/0.1% formic acid for 5 min at 25 µl/min. After valve switching, the peptides were then separated on a PepMap C18, 100µm i.d.x15 cm analytical column (Dionex, Amsterdam, NL) by gradient elution of 2-60% solvent B (0.05% formic acid in 20% water/80% acetonitrile) in solvent A (0.05% formic acid in 98% water/2% acetonitrile) over 60 minutes at 0.35 µl/min. The column eluant was directly interfaced to a quadrupole-ion mobility-orthogonal time of flight mass spectrometer (Synapt G2S, Waters UK, Manchester) via a Z-spray nanoflow electrospray source. The MS was operated in positive TOF mode using a capillary voltage of 3.8 kV, cone voltage of 20 V, backing pressure of 7.91 mbar and a trap bias of 4.1 V. The source temperature was 80 °C. Argon was used as the buffer gas at a pressure of 9.1×10-3 mbar in the trap and transfer regions. Mass calibration was performed by a separate injection of sodium iodide at a concentration of 2 µg/µl. [Glu1]-Fibrinopeptide B

at 2 µM was infused as a lock mass calibrant with a one second lock spray scan taken every 30 seconds during acquisition. Ten scans were averaged to determine the lock mass correction factor. Data acquisition was using data dependent analysis with a one second MS over an m/z of 350-2000 being followed by three 1 second MS/MS taken of the three most intense ions in the MS spectrum above a threshold of 1000 counts per second. Collision energy applied was dependent upon charge state and mass of the ion selected. Dynamic exclusion of 60 seconds was used. Data processing was performed using the MassLynx v4.1 suite of software supplied with the mass spectrometer. Peptide MS/MS data were processed with ProteinLynx Global Server (Waters) and searched against UniProtKB/SwissProt database (release 2011/12). Search was limited to Homo sapiens species. Precursor mass accuracy was 10 ppm and fragments ion tolerance was 0.1 Da. Trypsin was specified as the protease with up to two missed cleavages. Carbamidomethylation was used as a fixed modification and variable modifiactions were specified as acetylation, glycation, deamidation and oxidation.

2.13 Statistical analysis

Analysis of the data was done using Graphpad 6 and statistical significance accepted at $p<0.05$. Between group comparisons of normally distributed variables were carried out using independent samples student t-test. Comparisons of non-normally distributed variables were carried out using Mann-Whitney U tests. More advanced statistical analyses were carried out according to type of work conducted and these are further detailed in each Chapter as appropriate. Data are presented as mean±SEM unless otherwise stated.

Chapter 3

The effects of diabetes on the fibrinolytic properties of complement C3

Introduction

Using *ex vivo* experiments, C3 has shown anti-fibrinolytic effects in individuals with and without diabetes[6;240]. Diabetes studies to date have concentrated predominantly on T1DM patients in an attempt to avoid confounding factors associated with T2DM that may impact on clot lysis. Experiments have largely been conducted using pooled purified C3 from healthy subjects added to pooled healthy control fibrinogen or purified fibrinogen from diabetes patients. From the limited experiments using pooled purified C3 from diabetes patients, the effect on lysis prolongation appears to be exaggerated[6]. Given the heterogeneity associated with T2DM patients, it is likely that a variation in the extent of C3 induced prolongation of lysis exists between these individuals. Reasons for this variation may be related to glycation, or other post-translational modifications of C3, glycaemic control, medications and co-morbid conditions such as CVD, obesity and dyslipidaemia. Ex-vivo glycation of C3 has been demonstrated to affect protein function with regards to its role in killing bacteria[94], and thus it may be that glycation also affects its incorporation into the fibrin network.

Although the anti-fibrinolytic effects of C3 appear to be exaggerated in diabetes, only individuals with T1DM have been investigated and little is known about the relationship between C3 and clot lysis in type 2 diabetes.

The aim of this Chapter was firstly to study the role of C3 in type 2 diabetes by analysing the interaction between C3 plasma levels and lysis time in a large cohort of patients. Secondly, I set out to investigate the inter-individual variability in C3 induced prolongation of lysis in diabetes by studying the effects of purified C3 on fibrinolysis using purified fibrinogen and purified C3 from the same individual. Cohorts of T1DM, T2DM and age matched healthy controls

were analysed in this section. Thirdly, potential mechanisms for differences in the effect of C3 on lysis were examined by investigating potential diabetes-specific post translational modifications in the C3 protein.

3.1 Methods

3.1.1 Sample collection

A large cohort of T2DM samples (n=837), provided from collaborators in Edinburgh working on the Edinburgh Type 2 Diabetes study (ET2DS)[241], was available for analysis. The collection of these samples along with clinical data was done entirely in Edinburgh as was measurement of plasma proteins such as C3, PAI-1 and fibrinogen. I was involved in helping to perform turbidimetric analysis on the samples along with other members of the research laboratory and therefore only part of this work, relevant to my thesis, will be included here.

For the purified experiments, the aim was to recruit twenty patients with T1DM and T2DM along with twenty age and sex matched, healthy controls for both T1DM and T2DM subjects. Data gathered include blood pressure, height, weight and body mass index (BMI). Collected data and blood results are stored in paper format in a locked office at the Leeds Institute for Genetics, Health and Therapeutics (LIGHT) laboratory, and also electronically on a password protected computer. Informed consent was obtained from participants in accordance with the Declaration of Helsinki (Appendix). Ethical approval for the study was provided by the Leeds East, National Research Ethics Service (NRES) committee. My role included identification of patients, obtaining consent and help research nurses with blood sample collection.

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3.1.2 Sample preparation and storage

Fasting blood samples were collected without a tourniquet and separated into 9ml EDTA, 3ml lithium heparin and 5ml and 10ml citrate bottles. The EDTA and 10ml citrate tubes were kept on ice prior to and following sample collection.

Plasma was separated within 2 hours by centrifugation at 4^{o}C, snap frozen in liquid nitrogen and stored at -40^{o}C at the LIGHT laboratories. Samples were also collected for full blood count, urea and electrolytes, liver function, thyroid function, lipid profile, fasting blood glucose and glycated haemoglobin (Hb_{A1c}) for each subject and these were sent to the Biochemistry department at Leeds Teaching Hospital NHS Trust to be analysed.

3.1.3 Plasma protein analysis

Plasma levels of fibrinogen, PAI-1 and C3 were measured by the Clauss method and ELISA respectively as described in Chapter 2.

3.1.4 Fibrinogen purification

Fibrinogen was purified from 15 T1DM patients and controls, along with 20 T2DM patients and 15 controls. Affinity chromatography was used using an IF-1 monoclonal antibody as described in Chapter 2.

3.1.5 C3 purification

Complement C3 was purified from the same number of individuals as described above using the methods outlined in Chapter 2. Collaborators at the University Hospital Aachen contributed to C3 purification. More specifically, C3 from all type 1 diabetes and matched controls were purified in Aachen.

3.1.6 Turbidimetric analysis

Turbidimetric analysis was performed on plasma samples from T1DM, T2DM and age/sex matched control individuals along with the Edinburgh Type 2 Diabetes samples as described in detail in Chapter 2. The clot parameters, lysis time, lag phase and final turbidity were measured for each subject.

Experiments were also performed using the purified fibrinogen and C3 from each individual. A mixture containing 1µM of fibrinogen and 0.9µM C3 from the same individual, 0.01µM plasminogen and 0.01µM FXIII was prepared to 100µl with Tris buffered saline and added to a well of a 96 well Greiner plate. After 30 minutes of incubation, 50µl of an activation 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 previously described.

3.1.7 Mass Spectrometry

In order to identify post-translational modifications of diabetes C3 compared to control, 2 separate pools of purified C3 were prepared from 6 diabetes and 6 control subjects. In order to account for inter-individual variability in C3 glycation, C3 purified from individuals were also analysed separately (6 from each of the following groups, T1DM, T1DM controls, T2DM, T2DM controls). Following tryptic digestion of C3, the fragments were analysed using liquid chromatography mass spectrometry as described in Chapter 2.

3.1.8 Statistical analysis

Between-group comparisons of normally and non-normally distributed variables were carried out using T-test and Mann-Whitney test, respectively. Associations were tested using Spearman's correlations (presented as $r_{s)}$. The R environment for statistical computing was used to fit regression models, to determine independent predictors of clot lysis time and complement C3 plasma levels, with both univariable and multivariable models fitted to study the effects of multicollinearity. The robustness of multivariable regression models was assessed using examination of model residuals by QQ plots, scale location

plots and a plot of Cook's distance. The effect of each of the predictors in a regression model was characterised using 95% confidence intervals for the regression coefficients.

Between group comparisons of normally distributed variables in the in-vitro studies were carried out using independent samples student t-test. Comparisons of non-normally distributed variables were carried out using Mann-Whitney U tests. Data are presented as mean±SEM unless otherwise stated.

3.2 Results

3.2.1 Association between C3 and LT in T2DM

The patient and clinical characteristics of the Edinburgh Type 2 Diabetes study (ET2DS) samples (n=837) can be seen in Table 3-1. Analysis of the results indicated that LT again correlated with C3, fibrinogen and PAI-1 plasma levels (r=0.23, p<0.0001 and r=0.20, p<0.0001; respectively). This association remained for C3 and PAI-1 following multivariable regression analysis involving age, sex, BMI, C3, PAI-1, CRP and fibrinogen. This, however, was not the case for fibrinogen and CRP. There was no correlation observed within the cohort between C3 and PAI-1 levels (r=-0.006, p>0.1), which is similar to previous studies and suggests that C3 plasma levels are independently associated with LT in T2DM patients and occurs via a different pathway to that of PAI-1.

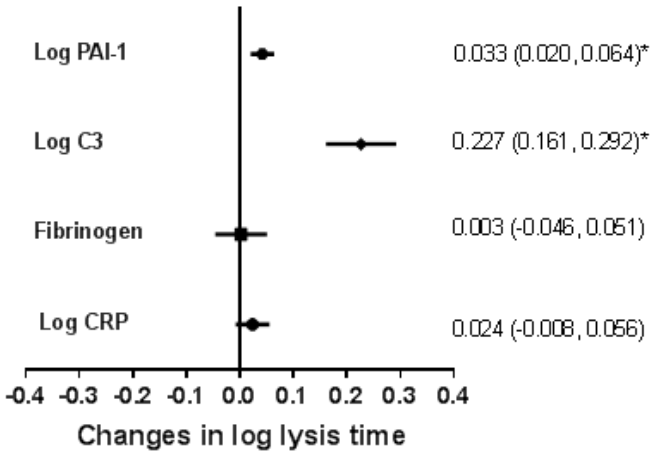


Figure 3-1 Predictors of clot lysis time in 837 type 2 diabetes subjects in multivariable analysis adjusted for age, gender and BMI

*Results are presented as regression coefficient for changes in plasma levels of C3, C-reactive protein (CRP), fibrinogen and plasminogen activator inhibitor-1 (PAI-1), *p<0.01. The 95% confidence interval is indicated in brackets.*

Variable	Values
Sex (m/f)	421/402
Age (range)	67.8 (60-76)
Duration of DM (years)	8.0±0.2
Inflammatory parameters	
CRP (mg/l)	3.7±0.2
C3 (mg/ml)	1.34±0.01
Fibrinogen (mg/ml)	3.6±0.02
PAI-1 (pg/ml)	924±33
Vascular markers	
Systolic BP (mmHg)	133±0.56
Diastolic BP (mmHg)	69±0.31
Anthropometrics	
BMI (kg/m^2, range)	31.37 (18-55)
Cardiometabolic factors	
HbA1c, mmol/mol	57.0±0.3
Plasma glucose, mmol/l	7.5±0.07
Total Cholesterol, mmol/l	4.3±0.03
HDL cholesterol mmol/l	1.2±0.01

Table 3-1 Clinical characteristics of ET2DS subjects

3.2.2 Clinical characteristics of subjects

The clinical characteristics of the subjects used for analysis of purified proteins are presented in Table 3-2 and Table 3-4. Regarding the T1DM patients and controls, there was no difference in age or sex and no difference in markers of cardiometabolic disease namely BMI, and blood pressure. As would be expected there was a significant difference in HbA1c and fasting plasma glucose, but in no other biochemical markers analysed.

With the T2DM patients and controls there was again no difference in age although there was a larger proportion of females in the control group (30% vs 53%). There was a trend toward higher systolic blood pressure in the T2DM group (128±2.41 vs 120±3.4, p=0.056) and a definite increase in BMI compared

to the control cohort (31±1.1 vs 25.4±0.9, $p<0.001$), along with the expected elevated HbA1c and fasting plasma glucose. The lipid profile was also different between the T2DM group and controls, with significantly lower total and LDL cholesterol, and higher HDL cholesterol compared to the controls, but lower triglyceride levels observed in the control group. It is worth noting that all of the T2DM group were on cholesterol lowering statin medication and 95% were taking at least one anti-hypertensive agent. Almost half (45%) of the T2DM group had a diagnosis of ischaemic heart disease and 30% had retinopathy. Only one of the T1DM suffered from any complications of diabetes (retinopathy) and none took any medication in addition to their subcutaneous insulin. Both sets of control groups were healthy with no significant medical problems and took no regular medications.

3.2.3 Plasma protein levels

There was no significant difference between measured plasma levels of fibrinogen, C3 and PAI-1 between the T1DM group and controls (Table 3-3). In contrast, with the T2DM cohort, the plasma levels of all 3 proteins were elevated compared to the controls (Table 3-5).

3.2.4 Changes in plasma clot parameters with T1DM and T2DM

The effect of diabetes on clot formation and lysis was studied using plasma from the above cohorts and also in a purified system using purified fibrinogen and C3 from a selection of subjects in both the T1DM and T2DM groups along with corresponding controls.

Variable	T1DM (n=15)	T1DM Control (n=15)	p value
Age (range)	24.4(18-39)	26.6(21-34)	0.24
Sex (M/F)	10/5	10/5	
Duration of diabetes, months	99.9(16.4)	-	
Current smokers, n (%)	3(20)	0(0)	
Vascular parameters			
Systolic BP, mmHg	117(3.8)	118(3.3)	0.85
Diastolic BP, mmHg	76(3.1)	75(2.6)	0.81
Anthropometrics			
BMI, kg/m^2	24.8(0.8)	23.8(0.5)	0.32
Metabolic			
HbA1c, mmol/mol	78.4(4.6)	35(1.1)	<0.0001
Plasma glucose, mmol/l	12.5(0.9)	4.37(0.2)	<0.0001
Total Cholesterol, mmol/l	4.6(0.3)	4.71(0.3)	0.79
Triglycerides mmol/l	1.17(0.1)	1(0.1)	0.33
LDL cholesterol mmol/l	2.61(0.1)	2.66(0.2)	0.82
HDL cholesterol mmol/l	1.46(0.1)	1.63(0.1)	0.23
TSH mIU/l	2.33(0.3)	2.34(0.3)	0.98
Free T4 pmol/l	14.8(0.4)	15.01(0.4)	

Table 3-2 Clinical characteristics of T1DM cohort and controls

Variable	T1DM (n=15)	T1DM Control (n=15)	p value
Clot Structure parameters			
Lag phase, seconds	682(±45)	659(±17)	0.63
Final turbidity, au	0.176(±0.02)	0.195(±0.01)	0.35
Lysis time, seconds	430(±11)	426(±13)	0.82
Plasma protein levels			
Fibrinogen, mg/ml	2.01(±0.09)	2.16(±0.07)	0.19
C3, mg/ml	1.34(±0.05)	1.4(±0.06)	0.43
PAI-1, pg/ml	664.5(±154)	905.5(±162)	0.29

Table 3-3 Plasma clot structure parameters and protein levels in T1DM cohort and controls.

3.2.4.1 Plasma turbidimetric assays

There was very little difference between the LT of the T1DM and control groups which is contrary to previously published work and may reflect the relative good health enjoyed by the T1DM cohort and the relatively small sample size. Although numerical differences were evident in fibrinogen, C3 and PAI-1 levels comparing patients with controls (higher in patients), none reach statistical significance. There was also no difference in the lag phase and final turbidity between both sets (Table 3-3). There was however a significant increase in LT between the T2DM and control groups, with elevated plasma levels of C3, PAI-1 and fibrinogen (Table 3-5).

Variable	T2DM (n=20)	T2DM Control (n=15)	p value
Age (range)	59(44-70)	59.7(52-73)	0.5
Sex (M/F)	(14/6)	(7/8)	
Duration of diabetes, months	130.7(19.6)	-	
Current smokers, n (%)	3(15)	1(6.6)	
Vascular parameters			
Systolic BP, mmHg	128(2.4)	120(3.4)	0.057
Diastolic BP, mmHg	80(1.7)	81(2.6)	0.74
Anthropometrics			
BMI, kg/m^2	33.1(1.1)	25.4(0.9)	<0.0001
Metabolic			
HbA1c, mmol/mol	64.5(3.6)	37.6(1.1)	<0.0001
Plasma glucose, mmol/l	9.53(0.75)	4.83(0.12)	<0.0001
Total Cholesterol, mmol/l	3.64(0.16)	5.21(0.14)	<0.0001
Triglycerides mmol/l	1.63(0.22)	0.93(0.07)	0.01
LDL cholesterol mmol/l	1.92(0.12)	2.91(0.15)	<0.0001
HDL cholesterol mmol/l	3.38(0.16)	1.9(0.14)	<0.0001
TSH miu/l	1.8(0.22)	2.12(0.26)	0.36
Free T4 pmol/l	14.67(0.46)	13.81(0.51)	
Past medical history			
Ischaemic heart disease, n (%)	9(45)	-	
Retinopathy, n (%)	6(30)	-	
Nephropathy, n (%)	1(5)	-	
Hypertension, n (%)	19(95)	-	

Table 3-4 Clinical characteristics of T2DM cohort and controls

Variable	T2DM (n=20)	T2DM Control (n=15)	p value
Clot Structure parameters			
Lag phase, seconds	634(±30)	567(±17)	0.09
Final turbidity, au	0.296(±0.02)	0.253(±0.01)	0.07
Lysis time, seconds	569(±34)	477(±15)	0.03
Plasma protein levels			
Fibrinogen, mg/ml	2.80(±0.1)	2.55(±0.09)	0.07
C3, mg/ml	1.83(±0.08)	1.19(±0.03)	<0.0001
PAI-1, pg/ml	4239(±583)	1301(±263)	0.0002

Table 3-5 Clot structure parameters and plasma protein levels in T2DM cohort and controls

3.2.4.2 Correlation of plasma protein concentrations with LT

Combined analysis of the 4 groups as a whole revealed a correlation between LT and plasma levels of C3 (r=0.3, p<0.05), fibrinogen (r=0.41, p<0.001) and PAI-1 (r=0.49, p<0.0001) as seen in Figure 3-2.

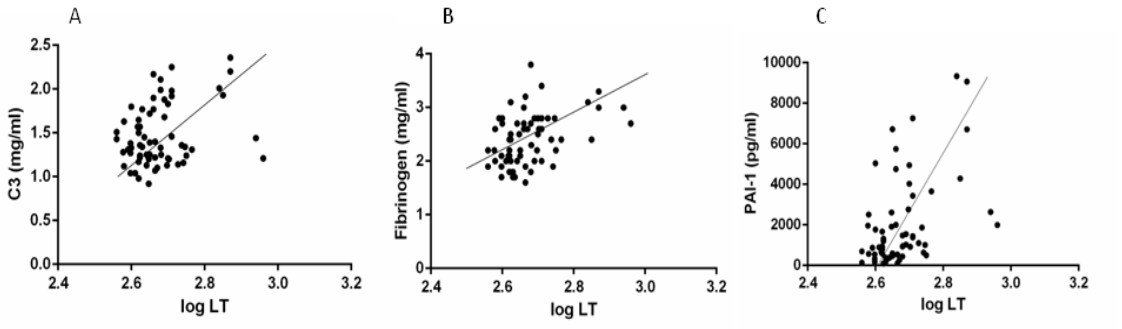


Figure 3-2 Correlation between clot lysis time and plasma levels of C3, fibrinogen and PAI-1 plasma levels

Spearman correlations between clot lysis time and (A) plasma C3 levels, (B) plasma fibrinogen levels, (C) plasma PAI-1 levels (r=0.3, p<0.05, r=0.41, p<0.001, r=0.49, p<0.0001 respectively)

3.2.5 Effect of C3 on LT in T1DM

Due to difficulties encountered with C3 purification and limited protein obtained, it was not possible to conduct the purified turbidity assays on all of the subjects. The yield of C3 was disappointingly low and thus only 9 T2DM and control fibrinogen and C3 were available for analysis and 6 from the T1DM group. The mean LT for T1DM and controls without the addition of C3 was 3238±403 seconds and 2871±533 seconds respectively (p=0.13). There was a trend towards greater difference in C3 induced prolongation of lysis comparing control and diabetes subjects (297±148 seconds vs 800±531.7 seconds prolongation, respectively; p=0.076, Figure 3-3). There was a wide variation in LT in individual samples from control and diabetes groups, particularly in diabetes subjects. In the T1DM group the prolongation in LT induced by C3 ranged from 144-1476 seconds (5-51%), compared to 108-468 seconds (5-17%) in the control group.

3.2.6 Effect of C3 on LT in T2DM

The lysis times in the T2DM cohort in the absence of C3 were longer than the T1DM cohort to the point whereby the T2DM samples did not lysed to 50% within the 2 hours. To counter this problem, the concentrations of tPA and plasminogen within the lysis mix were increased 4-fold in order to achieve full lysis of the clots. The outcome of this change was noticeably shortened LT in the T2DM and control subjects compared to the T1DM cohort, being1104±219 seconds for the controls and 1501±733 seconds for the T2DM subjects. Given these results it was perhaps not too surprising to find that the increase in LT following the addition of C3 was less significant. The mean prolongation in LT for the control subjects was 45.3±138 seconds and 136±249 seconds for the diabetes group (p=0.65, Figure 3-3). There was a wide variation observed again

between the T2DM and control individuals but unlike the T1DM group, the addition of C3 appeared to augment fibrinolysis in a number of samples. The ranges in LT prolongation were less dramatic and may be a reflection of the overall quicker LT observed. For the T2DM cohort LT ranged from -144 to 696 seconds (-8 to 22%) and from -96 to 336 seconds (-10 to 38%) in the controls.

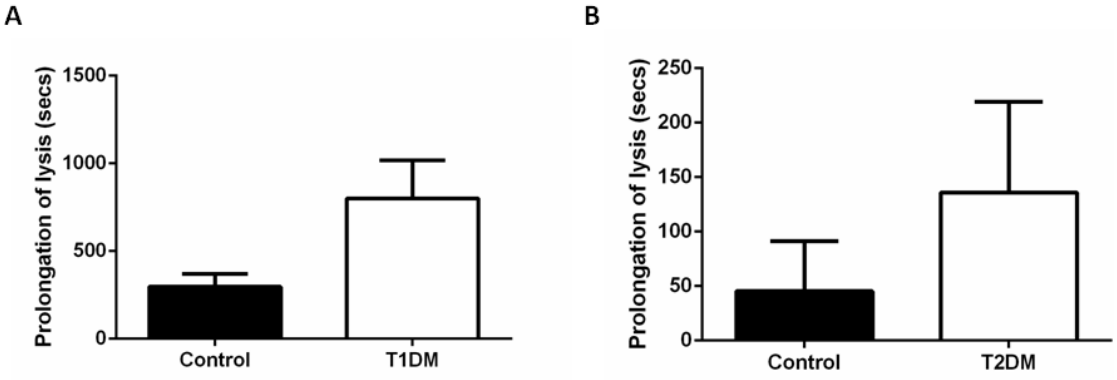


Figure 3-3 The effects of C3 on lysis time during clot formation

The mean±SEM of C3 induced prolongation of lysis in (A) T1DM cohort and controls and (B) T2DM cohort and controls. Experiments were conducted by addition of C3 purified from patient or control plasma added to fibrinogen purified from the same individual. The prolongation in lysis was measured by subtracting the LT in absence of C3 from LT in the presence of C3.

3.2.7 Glycation of C3 as a potential mechanism for inter-individual variation in lysis prolongation

3.2.7.1 Sequence coverage of C3 by mass spectrometry

Sequence coverage of the diabetes pool and control pool was 80% and 75% respectively. Both samples contained albumin, possibly due to its ubiquitous nature within plasma and its high affinity for binding to circulating plasma proteins.

3.2.7.2 Post-translational modifications

No areas of glycation on C3 were identified in the DM and control pools. Given the inter-individual variability in the C3 induced prolongation of lysis in both T1DM and T2DM cohorts it was decided to also analyse individual samples of C3 in case there is also a variation in glycation between individuals. Again there were no areas of glycation identified within any of the 12 healthy controls studied (6 matching T1DM and the other 6 matching T2DM patients). However, when analysed individually, several Lysine residues in the T1DM and T2DM C3

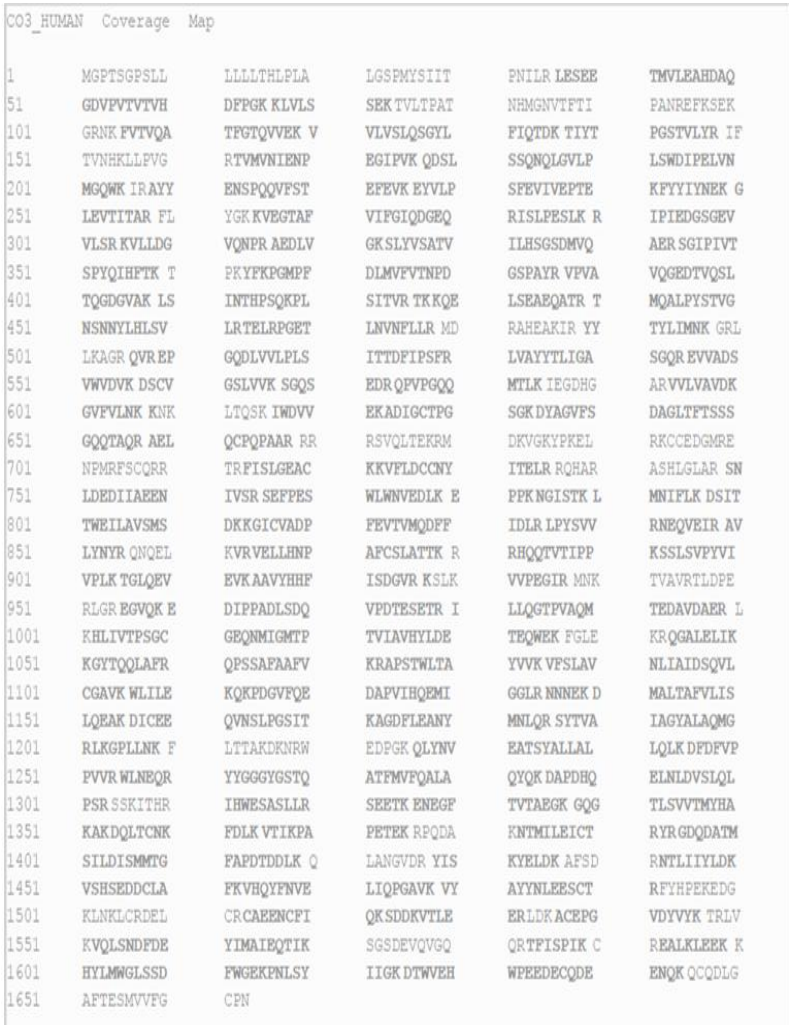


Figure 3-4 LC-MS sequence coverage of pooled diabetes C3 samples

The amino acid residues in bold were covered by the LC-MS analysis

samples were found to be glycated by an increase in mass of 162Da, indicating the presence of a hexose molecule. In total, 11 separate Lys residues were glycated, 2 occurred only in the T1 samples, 8 occurred only in the T2 samples and 3 occurred in both groups. More than one glycation site was noted in each sample (range 2-4) with a mean of 3±0.9 modifications in the T1DM samples and 2.3±0.5 modifications in the T2DM samples. The locations of the modified Lys residues are shown in Table 3-6 and their positions within the C3 molecule are illustrated in Figure 3-5.

Glycation site	Number of samples containing PTM
K 1181 & 1187	4
K 1187	3
K 665	4
K 1117	4
K 1505	2
K 464	2
K 1359	2
K 242	1
K 699 & 700	1
K 839	1
K 909	1
K 1049	1
K 1342	1

Table 3-6 Sites of glycation identified by MS in individual C3 samples

The Lys residues (K) that are glycated and their position within the C3 sequence are shown along with the number of samples analysed that contained the glycation. The light grey row indicates which residues were glycated in T1DM only, white row those sequences identified in T1 and T2DM samples, and dark grey row the sequences identified in T2DM samples only.

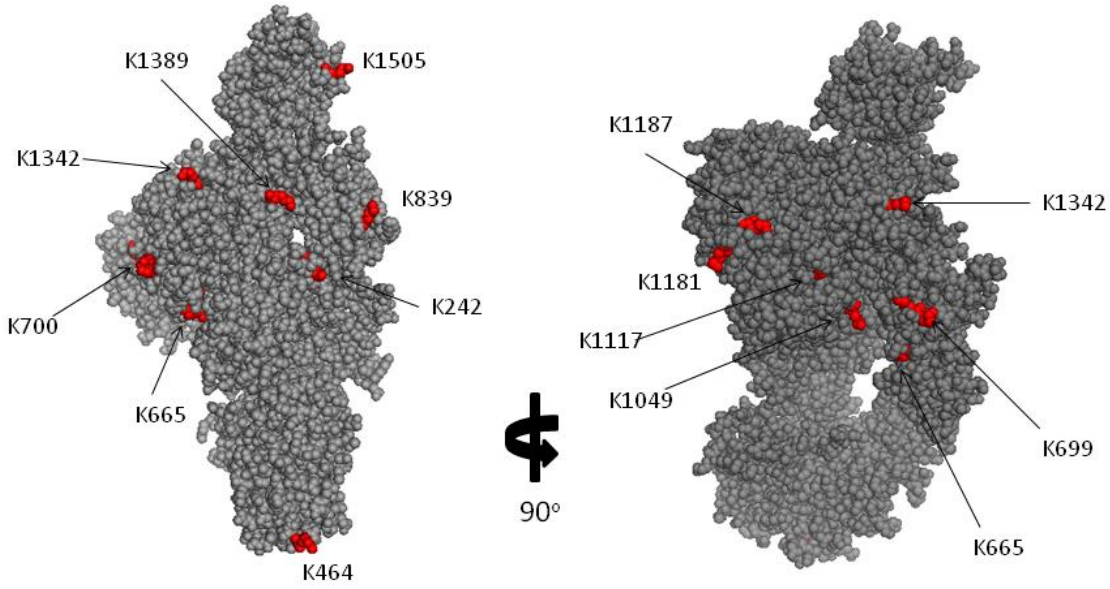


Figure 3-5 Glycation sites within C3 identified by mass spectrometry

The crystal structure of C3 (PDB ID 2A73) is shown with the glycated Lysine (K) residues indicated in red along with corresponding position with the sequence of C3.

3.3 Discussion

The work in this Chapter was aiming to build on the known association between fibrinolysis and C3 plasma levels in T1DM by i) studying the relationship between plasma C3 levels and fibrinolysis in a cohort of T2DM patients, ii) examining the inter-individual variability in the effects of C3 on fibrinolysis in T1DM and T2DM patients and iii) relating the observed differences to the degree of incorporation of C3 into the fibrin network and to post-translational modifications of C3, in particular glycation.

In the T2DM cohort there was a strong correlation between C3 plasma levels and fibrin clot lysis, which was at least as pronounced as the association between PAI-1 and fibrinolysis in this population. Furthermore, there was no correlation between C3 and PAI-1 plasma levels, consistent with the observations that the two molecules affect separate pathways in the fibrinolytic system[6]. PAI-1 inhibits fibrinolysis by interfering with plasmin generation, whereas C3 modulates clot lysis through incorporation into the clot. This is thought to increase mechanical resistance to lysis, and may compromise plasmin activity[6;80]. Clinically, this work suggests that measuring PAI-1 plasma levels only addresses part of the fibrinolytic potential in diabetes and C3 offers an additional assessment tool. Importantly, these results, together with previous work on T1DM, indicate that interference with C3-fibrin interactions may offer a novel therapeutic target to improve fibrin clot lysis and reduce cardiovascular events in individuals with diabetes.

Regarding the subjects recruited specifically for my PhD, there was a correlation between the plasma levels of C3, fibrinogen and PAI-1 and LT in both the DM and control groups, which is consistent with previous work.

In terms of the T1DM group, the clinical characteristics were very well matched to that of the control group apart from HbA1c and fasting plasma glucose as would be expected. Somewhat surprisingly, and in contrast to previous studies, plasma levels of fibrinogen, C3 and PAI-1 were also similar between the two groups and may, in part at least, explain the similarities observed in the clot structure parameters, most notably similar LT. Given the relatively small numbers in this study, these were not specific aims of my research, and thus it is important not to draw too many conclusions. If similar results were obtained in a much larger cohort, this may suggest that chronic hyperglycaemia alone is not sufficient to prolong fibrinolysis ex-vivo and that the sequeale of diabetes, such as inflammation and alterations in haemostatic proteins are more important contributors to increased thrombosis potential. The T1DM cohort analysed in this work had no complications from diabetes and therefore are arguably a "healthy" group of diabetes patients. The study by Hess et al (2012) analysed fibrinolysis in a much younger cohort of T1DM (mean age 14.5±0.3 years) and although there was a mean duration of 6.32 years since diagnosis in these individuals, it is possible that they had higher residual, autoimmune processes than this current cohort, which could affect LT and offer some explanation as to the differences between these two T1DM groups.

The T2DM group were reasonably matched for age; however there was a greater percentage of females in the control group (30% vs 53%), a significant increase in BMI and trend towards an increase in systolic blood pressure in the T2DM group. During the recruitment process there was a difficulty in recruiting suitable males to act as controls. This was due to the fact that a large proportion of men in the target age group were taking some form of medication thus

excluding them from the study. Again as would be expected there was significant differences in HbA1c and plasma glucose levels between the two groups, and a difference was also observed in the lipid profiles. The diabetes subjects had a significantly elevated triglyceride levels, commonly seen in T2DM, however the control subjects had significantly lower HDL levels and higher total and LDL cholesterol. These results are likely to reflect the fact that all of the diabetes subjects were taking cholesterol lowering, statin medication. In contrast to the T1DM group, and in keeping with previous studies, the plasma levels of C3, fibrinogen and PAI-1 were all significantly elevated in the diabetes group compared to the control. Unsurprisingly this was associated with significant differences in clot structure parameters and lysis, with increased FT in the diabetes group and prolonged LT. The mean LT of the control group was also significantly longer than that of the T1DM cohorts and this is possibly due to the increased age of the T2DM controls but also due to the higher proportion of females in this group, given that female sex is associated with prolonged LT in T2DM[242].

Effect of C3 on LT in T1DM

In the purified experiments, pre-incubation of C3 and fibrinogen caused a prolongation in LT, compared to fibrinogen alone, and this effect was observed with both control and T1DM subjects. Although the mean increase in LT was far longer in the diabetes group (800±531sec vs 297±148 seconds) the difference between the groups failed to reach statistical significance ($p=0.076$) presumably due to low numbers and large standard deviation. There was wide variability in C3 mediated prolongation of LT between individuals in the purified experiments

which ranged from 144-1476 seconds (5-51%) in the diabetes subjects, compared to 108-468 seconds (5-17%) in the controls. Fibrinolysis was actually inhibited following the addition of C3 in one of the control subjects which was unexpected and may reflect an undiagnosed coagulopathy.

Effect of C3 on LT in T2DM

Following the plasma turbidimetric results it was somewhat surprising to find very little difference in the C3 mediated prolongation of lysis between the 9 T2DM and control subjects used in the purified experiments (136±249 seconds and 45.3±138 seconds respectively, p=0.65). Possible explanations for this observation include the relative short LT as a whole, in comparison to the T1DM group. It was necessary to increase the concentrations of tPA and plasminogen in the T2DM experiments as the conditions used for the T1DM samples failed to achieve sufficient lysis in several subjects, making analysis impossible. Shortening the process of clot formation and subsequent LT is likely to make the effect of C3 upon lysis less apparent. Another explanation for the differences observed between the T1DM and T2DM groups may relate to the heterogeneous nature of T2DM patients and thus much larger numbers are required to demonstrate the true effect of C3. A wide variation in the lysis prolongation was also observed between the T2DM and control individuals and surprisingly the addition of C3 appeared to enhance lysis in 3 control subjects and 2 T2DM subjects, which is difficult to explain. The prolongation in LT ranged from -144-696 seconds (-8-22%) in the diabetes subjects and from -96-336 seconds (-10-38%) in the controls. A possible explanation may relate to the overall reduced LT in this group as a result of the increased tPA concentration.

By speeding up the turbidity and lysis experiments, the hypofibrinolytic effect of C3 may be diminished or lost altogether due to reduced incorporation of C3 into the clot. The supposed reduction in LT observed in 5 samples following the addition of C3 may fall within the error of the assay, and in reality C3 has had no effect on LT. The inter-individual variation observed in C3 mediated lysis prolongation may relate to the degree of incorporation of C3 into the clot, which is known to be increased in diabetes. An important factor in this incorporation may relate to the glycation of C3, which was assessed using mass spectrometry. The data from the pooled diabetes and control C3 samples did not reveal any evidence of glycation and was a curious finding as *in-vitro* glycation of several lysine residues within C3 has been reported previously[94]. These results coupled with the variability in C3 mediated lysis prolongation suggested that perhaps the glycation of C3 was heterogeneous. Mass spectrometry analysis of individual C3 samples was therefore employed which identified 13 lysine residues that were glycated within the T1DM and T2DM subjects, but not controls. Although *in-vivo* glycation of C3 has been described previously[243;244], the residues involved have only been identified by *in-vitro* glycation as above. One of the thirteen residues glycated, K242, was also found to be glycated under in vitro conditions by Hair et al (2012). Within the T1DM group, each sample had slightly more glycation sites than the T2DM group (3±0.9 vs 2.3±0.5), with increased commonality of glycated lysine residues. There was a degree of overlap with the T2DM group in terms of glycation sites, but there was more heterogeneity within these samples, with glycation of some lysines occurring in only one of the six samples. These findings would explain the pooled MS results as the presence of multiple, different glycated lysine

residues within each sample may not become evident once the samples are pooled together. The amount of glycation sites, variation in frequency of occurrence and variation between T1DM and T2DM samples suggests that not all lysine residues are equal in terms of their importance in modulating C3-fibrinogen interactions. Another possible explanation may relate to changes in C3 conformation caused by elevated plasma glucose levels, altering its function[245]. Hair et al (2012) found changes in C3 structure at increasing concentrations of glucose that were sufficient to impair its interaction with staphylococcus bacteria. Several lysine residues were increasingly or decreasingly biotinylated at higher glucose concentrations, suggesting conformational changes to C3 that were sufficient to make certain lysine residues more, or less, available for protein interactions. Transferring this hypothesis to clot structure and lysis, it may be that altered conformation of C3 during conditions of hyperglycaemia, along with glycation of certain lysine residues, increases its likelihood to bind to fibrinogen and become incorporated into the clot thereby delaying fibrinolysis.

This work confirms that complement C3 plays a crucial role in delayed fibrinolysis in T2DM as well as T1DM, and that the mechanism underlying this process is different to that of PAI-1. The effect of C3 on clot lysis appears to vary between different individuals, and this may relate to differing rates of incorporation of C3 into the clot as a result of variations in glycation of the molecule. A greater understanding of the interaction sites between C3 and fibrinogen may help explain the inter-individual variability observed and shed

more light on the role of glycation and glycaemia, enabling a targeted approach to inhibit the hypofibrinolytic effects of C3.

Chapter 4

Assessing C3-fibrinogen interactions

Introduction

Despite a growing body of evidence linking complement C3 to CVD, and its anti-fibrinolytic effect ex-vivo, which is enhanced in diabetes subjects, relatively little is currently known regarding the mechanisms involved. The physical presence of C3 within the fibrin network has been repeatedly demonstrated [6;240] and a reasonable assumption would be that this is related to an interaction with fibrin(ogen). Numerous complement proteins and receptors are able to bind to C3 and the affinities of this binding alter as C3 is converted to C3b and C3c. The determination of the crystal structure of these proteins has demonstrated that C3 is a very flexible protein and subsequently undergoes major conformational changes, mainly affecting the α-chain, upon activation. Multiple hidden binding sites are exposed as C3 becomes C3b allowing key complement proteins such as factor H, properdin and CR1 to bind[84;86]. The exposure of the thioester moiety also permits covalent attachment to cell surfaces, enabling opsonisation and phagocytosis of foreign material.

Investigating potential interactions between C3 and fibrinogen may be problematic largely due to the relative size of both molecules. In such instances, the proteins in question are typically analysed in smaller more manageable fragments. Previous unpublished work from our laboratory has identified one potential interaction site on the α-chain of fibrinogen with C3 (Figure 4-1). Using surface plasmon resonance (SPR), a microarray chip containing fragments of the fibrinogen α chain was immobilised and subsequently incubated with C3. This identified a possible interaction site between residues 341 and 371 of the α-chain of fibrinogen and C3. However, this fragment was only able to partially

inhibit fibrinogen binding to C3 and at very high concentrations indicating the presence of other, more important interaction sites.

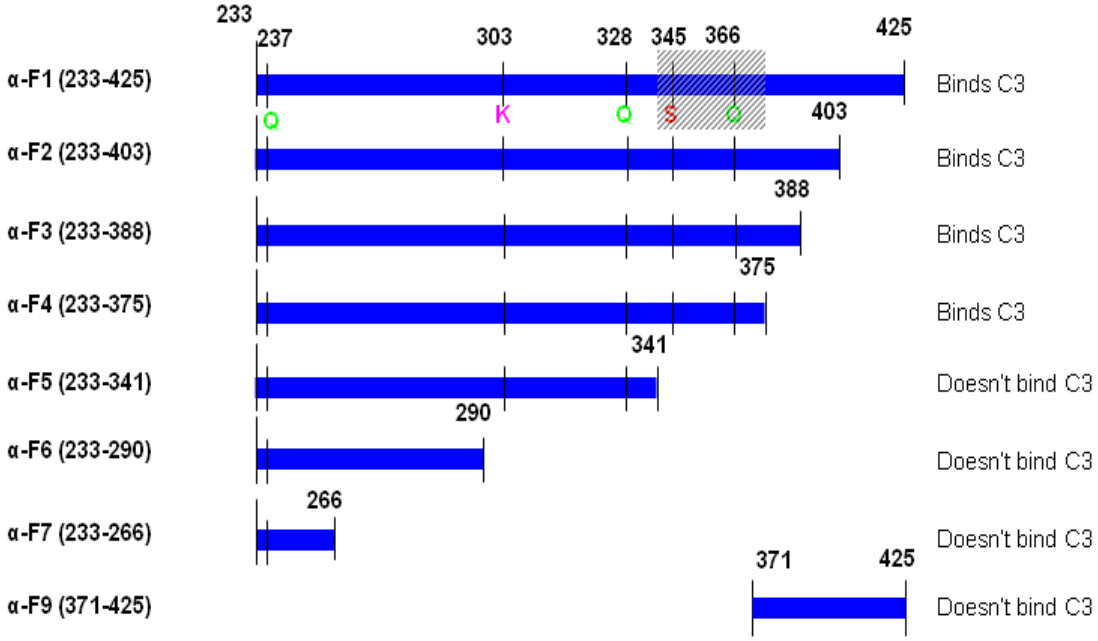


Figure 4-1 Binding of fibrinogen α chain fragments to C3

Surface plasmon resonance results of different fibrinogen α chain fragments binding to C3. The left hand side denotes the fragment and amino acid residues contained within. This data suggests that a binding site for C3 lies within residues 341-371 of the fibrinogen α chain.

Further insight into the interaction sites between fibrinogen and C3 may allow the development of novel therapeutic agents capable of inhibiting C3 incorporation into the clot, thus reducing cardiovascular risk. The aim of this chapter therefore was to further investigate the interaction between fibrinogen and C3. Firstly, to corroborate the SPR findings, a cDNA library of the α-chain of fibrinogen was produced for use in a phage display system to identify sequences that interact with C3. Secondly, a peptide microarray of the whole of the fibrinogen molecule was purchased to confirm potential α-chain interaction sites and identify any other sites of interaction within the β or γ chains. Thirdly,

the results from these methods were studied using molecular modelling software to predict potential binding sites on the crystal structure of C3 and model the docking of peptide sequences of interest to C3.

4.1 Methods

4.1.1 Fibrinogen α chain cDNA phage display library

Constructing a cDNA phage library consisting of fragments of fibrinogen α-chain should hopefully allow further identification of peptide sequences involved in fibrinogen and C3 interaction. Construction of the cDNA phage library and subsequent biopanning rounds were performed with Dr Phil Watson at the University of Sheffield.

4.1.1.1 Creating a Fibrinogen α-chain cDNA phage library

In order to create a cDNA phage library of the α chain of fibrinogen the first step was to produce a large quantity of the α chain itself. A plasmid (p584) containing the entire DNA encoding the α chain of fibrinogen was amplified by polymerase chain reaction (PCR). To 10µg of the plasmid was added 10mM dNTPs, 50mM $MgCl_2$, 2µl 10X buffer, 0.2µl Taq Polymerase (Life Technologies, Paisley, UK), 35.3µl dnaH_2O and 10µM of both forward and reverse primers.

Forward primer: CTCTCGAGTGAATTGTCG

Reverse primer: GTAAAACCTCTACAAATGTGG

Fifty separate reaction mixtures were produced and the tubes placed in a PTC-200 Peltier Thermal Cycler (MJ Research, Quebec, Canada), held at 94^oC for 3minutes, before 35 cycles of 94^oC for 45 seconds, 55^oC for 30 seconds and 72^oC for 90 seconds. This was followed by 10 minutes at 72^oC and the reaction again held at 4^oC until sample collection. The PCR products were pooled and 1µl was run on a 1% agarose gel containing ethidium bromide, along with a base pair marker, at 200V for 1hour. The gel was imaged under UV light with a band observed at 2000 base pairs (bp) as expected (Figure 4-2).

Purified and precipitated DNA was re-suspended in 90µl of water along with a digest mixture in order to randomly produce fragments of the DNA in the region of 100-200bp. The digest mixture contained 10µl of 10X buffer (0.5M Tris, 0.1M $MnCl_2$, 1mg/ml BSA) and 3µl of a diluted DNAse enzyme. The enzyme was diluted by a factor of 1:50 in an enzyme diluent which contained 50mM Tris and 1mg/ml of BSA.

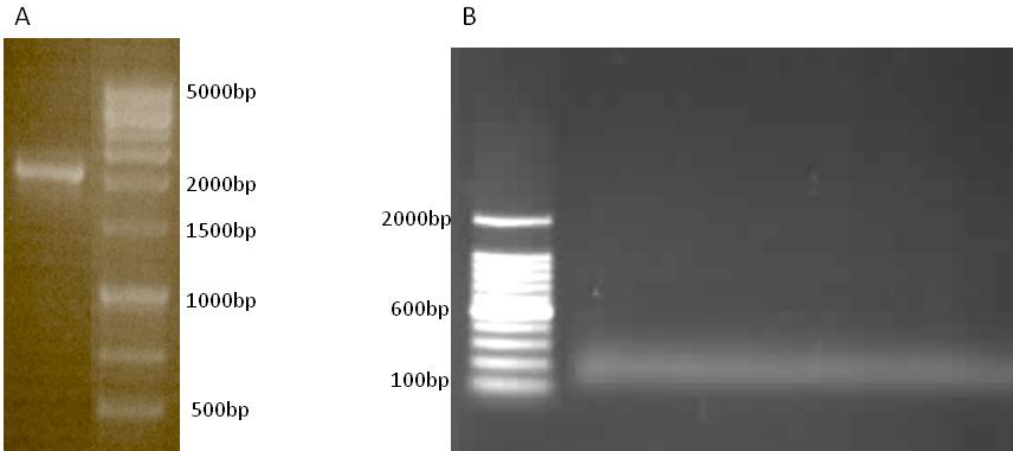


Figure 4-2 Agarose gels of α chain PCR product following digestion

A) 1% agarose gel indicating the fibrinogen α chain PCR products with a band visible under ultraviolet light at approximately 2000 base pairs as expected. B) 1% agarose slot gel of digested α chain demonstrating fragments of roughly 100-200bp produced.

The digest mixture was incubated at 37^{o}C for 15minutes to allow sufficient cutting of the fragments. Once enough suitable fragments have been produced the DNA was purified, precipitated and again re-suspended in 90µl of water along with 10µl T4 polymerase buffer, 5µl MgCl2 (25nM), 5µl 10mM dNTPS, 2µl T4 polymerase and 2µl of DNA polymerase and incubated at room temperature for 15 minutes. The mixture was run on 1% agarose slot gel and the subsequent band (Figure 4-2) was cut from the gel under UV light. The sliced

gel was transferred into a dialysis tube along with TE buffer in order to recover the DNA from the gel.

4.1.1.2 Vector Purification & Ligation

A plasmid vector (pCOMB3) was cut to produce a linear plasmid suitable for ligation of the fragmented fibrinogen α chain. This produces fragments that are around 4000bp as seen on a 1% low melting point agarose gel. The vector was then phosphorylated in preparation for ligation. A second, slot gel, was performed and as previously, the band of purified vector was cut away from the gel under UV light and recovered using a proprietary gel extraction kit (Promega). A 20µl ligation mixture was prepared containing 10µl of cut vector, 7µl of fibrinogen fragments, 2µl of ligation buffer and 1ul ligase. The mixture was incubated at 16^{o}C overnight.

4.1.1.3 Electroporation

Following heat treatment at 75^{o}C for 10 minutes, 2µl of the ligation mixture was added to 50µl of electro competent E.Coli XL1-Blue MRF' cells (Stratagene, La Jolla, CA, USA) in an electroporation cuvette and 1700V passed through. Cells are recovered in 500µl of LB medium and after 1hour, 2µl was plated onto selective medium containing 50µg/ml ampicillin and left overnight, the remainder of the mixture was left on ice. The number of colonies can be counted the following day and the cDNA library size estimated. In order to prepare the cDNA phage-display library, the electroporated cells were superinfected with 10^{12} plaque forming units of VCMS13 helper phage at room temperature for 30 minutes. The culture was transferred to 100ml of LB medium containing 50ug/ml ampicillin, 10ug/ml tetracycline and 10ug/ml kanamycin and

incubated at 37^{o}C overnight. The phage was precipitated by centrifuging the culture followed by the addition of 0.2 volumes of 40% polyethylene glycol 4000/2.5M NaCl. It was then resuspended in 2-3ml of PBS. The number of clones produced can be estimated through titration of the phage library. A random selection of colonies can be cultured and sequenced to ensure they contain fragments of the α-chain.

4.1.1.4 Panning and enrichment

In total 4 rounds of biopanning were performed as described in Chapter 2.

4.1.2 Peptide Microarray

A company from Germany, PEPperPRINT, was commissioned to study the interaction sites of fibrinogen and C3 through the use of a peptide microarray. This work would hopefully confirm any sites of interaction within the α chain identified from the phage display experiments and also cover the remainder of the molecule. In order to create the peptide chip, the α, β and γ chain of fibrinogen were linked with neutral GSGSGSG linkers and translated into 15 aa peptides with a peptide-peptide overlap of 14 aa. The resulting peptide microarrays contained 1,602 different peptides as duplicates (3,204 peptide spots) and were framed by Flag (DYKDDDDKGG) and HA (YPYDVPDYAG) control peptides (118 spots each). A pool of purified complement C3 from healthy controls was used in the experiments.

Pre-staining of the peptide array was done with biotinylated anti-C3 antibody (LifeSpan Biosciences Inc, Seattle, USA) at a dilution of 1:1000 followed by staining with streptavidin conjugated DyLight680 (Thermo Fisher Scientific) to investigate background interactions that could interfere with the subsequent

main assays. Incubation of the peptide microarray with C3 at concentrations of 0.8 µg/ml and 8 µg/ml in incubation buffer (PBS, 0.05% Tween 20 , 10% blocking buffer) was followed by incubation blocking buffer (Rockland buffer MB-070) and subsequently with the biotinylated anti-C3 antibody, staining with streptavidin conjugated DyLight680 and read-out at a scanning intensity of 7 (red). In a variant assay, 1 µg/ml Complement C3 was allowed to pre-complex with 2 µg/ml biotinylated anti-C3 antibody for 1 h to form a bivalent Complement C3-antibody complex. After centrifugation, incubation of one of the peptide arrays was done at a relative Complement C3 concentration of 8 µg/ml for 16 h at 4°C and slight shaking. Subsequently, staining was done with streptavidin conjugated DyLight680 for 15 min at a dilution of 1:1000, 4°C and slight shaking. The goal of this variant assay was the compensation of possibly high off-rates of the monovalent Complement C3 protein by formation of a bivalent complex with a higher avidity. HA and Flag control peptides framing the peptide arrays were finally stained as internal quality control to confirm the assay quality and the peptide microarray integrity

Quantification of spot intensities and peptide annotation were done with PepSlide® Analyzer and a software algorithm breaks down fluorescence intensities of each spot into raw, foreground and background signal and calculates the standard deviation of foreground median intensities. Based on averaged foreground median intensities, intensity maps were generated for each assay. The averaged spot intensities of all assays were plotted against the linked fibrinogen sequence from the N- to the C-terminus to visualize overall spot intensities and signal to noise ratios The intensity plots were correlated with peptide and intensity maps as well as with visual inspection of the

microarray scan to identify peptides and consensus motifs that interacted with C3.

4.1.3 Molecular modelling

In order to identify potential sites of ligand binding on C3, the molecular modelling software, Autoligand was employed to scan the whole of the molecule as described in Chapter 2. Briefly, Autoligand is an automated predictor of ligand binding sites in proteins which works by analysing the geometry of a protein surface and identifies areas of maximal affinity for ligand binding[232].

Molecular modelling was also used as a complimentary technique to predict the binding sites of any peptide sequences identified from the phage display and microarray screening with complement C3 and C3b using the website Pepsite2®, as described in Chapter 2. Pepsite2 predicts the binding site of each residue in a peptide (up to 10 aa in length) to a known protein surface using pre-determined preferred binding environments for each residue. The result is an approximation of the peptide structure bound to the protein surface. Images were viewed and produced using Pymol software.

4.2 Results

4.2.1 Fibrinogen α chain cDNA phage library

The final fibrinogen α chain cDNA library contained approximately 100,000 independent phage clones, with α chain fragment inserts between 100-200bp.

To ensure the cDNA library was capable of binding immobilised protein, several biopanning rounds were completed against immobilised polyclonal anti-fibrinogen antibody (abcam). Following each round there was enrichment of the phage library indicating increased selectivity of phage particles. However, biopoanning against immobilised C3 did not demonstrate enrichment and suggests that the α-chain fragments were not binding to C3.

4.2.2 Peptide microarray screening

4.2.2.1 Background interactions

To investigate for any background interactions between the biotinylated anti-C3 antibody and fibrinogen peptide microarray, the chip was incubated with the antibody at a dilution of 1:1000 followed by staining with streptavidin conjugated DyLight680. Figure 4-3 demonstrates the intensity map of the chip with no observed background signals indicating that there was no non-specific binding of the antibody or streptavidin conjugated DyLight680.

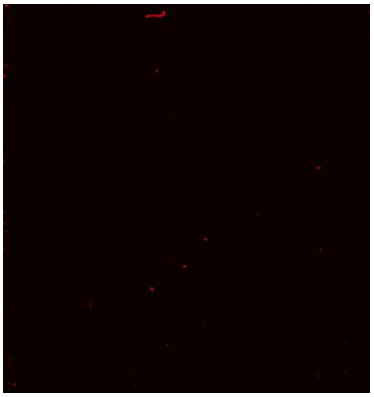


Figure 4-3 Incubation with biotinylated anti-C3 antibody followed by staining with streptavidin conjugated DyLight680 at a dilution of 1:1000

No background signalling observed following incubation with biotinylated anti-C3 antibody and streptavidin conjugated DyLight680 indicating that the anti-C3 antibody did not cross-react with the fibrinogen microarray peptides.

4.2.2.2 Complement C3 microarray scans.

Incubation of the peptide microarray with C3 at a concentration of 0.8 µg/ml followed by incubation with the biotinylated anti-C3 antibody at a dilution of 1:1000 and staining with streptavidin conjugated DyLight680 resulted in a very weak and hardly visible spot pattern. Therefore the protein concentration was increased tenfold and the assay repeated at a concentration of 8 µg/ml. After blocking with Rockland blocking buffer, there appeared to be weak but complex staining patterns (Figure 4-4). Analysis of the variant assay, which was done with a pre-formed C3-antibody complex to generate a bivalent species, demonstrated the highest signal-to-noise ratios and spot intensities.

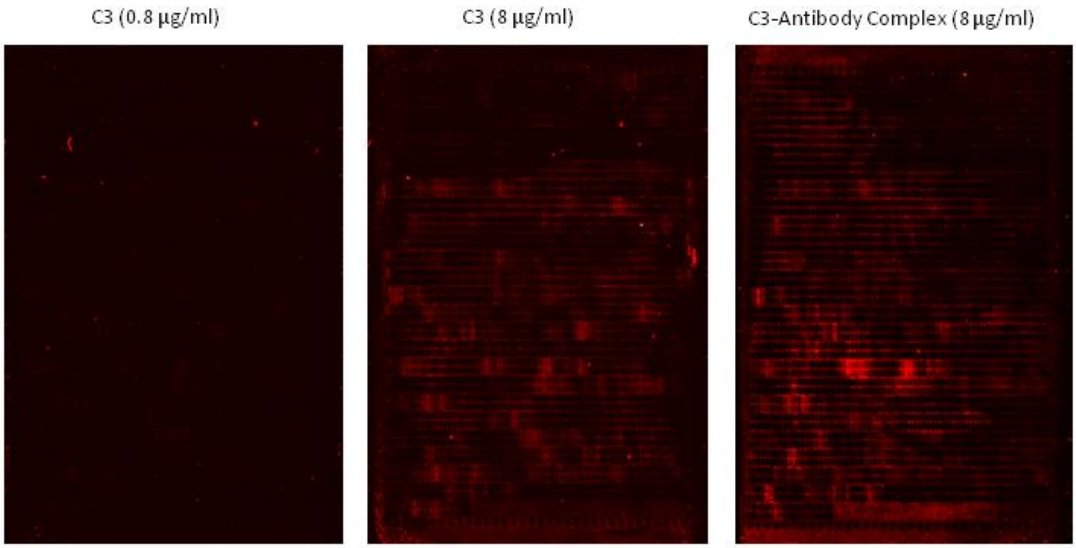


Figure 4-4 Complement C3 microarray scans

The staining patterns of the C3 microarray scans using C3 at 0.8µg/ml, 8µg/ml and in complex with biotinylated anti-C3 antibody at 8µg/ml, all incubated with the fibrinogen microarray peptides, washed and stained with streptavidin conjugated DyLight680.

4.2.2.3 Intensity plots and peptide identification

Data quantification was followed by generation of peptide and intensity maps as well as of intensity plots that were leveled to provide a better overview. In accordance with the microarray scans and the intensity maps, there were complex spot patterns in all assays with varying spot intensities, but also with some clear similarities. The brightest spot patterns in the assay with the C3-antibody complex were highlighted in the intensity plot and also identified in all other assays as distinctive features. These spot patterns were based on peptides with the consensus motifs AVSQTSSSSFQYMYL (peptide A), QCSKEDGGGWWY (peptide B) and YNRCHAANPNGRYY (peptide C), which are all located in the β chain of fibrinogen (Figure 4-5). Out of the three motifs,

the peptides with the consensus AVSQTSSSSFQYMYL, exhibited less blurry spot morphologies in all assays. Taking only the α-chain of fibrinogen into account, there were interactions with peptides with the consensus motifs FPGFFSPMLGEFV and SGNVSPGTRREY. These interactions were much weaker than the β chain peptides, as evidenced by lower spot intensities and spot patterns that were less clear.

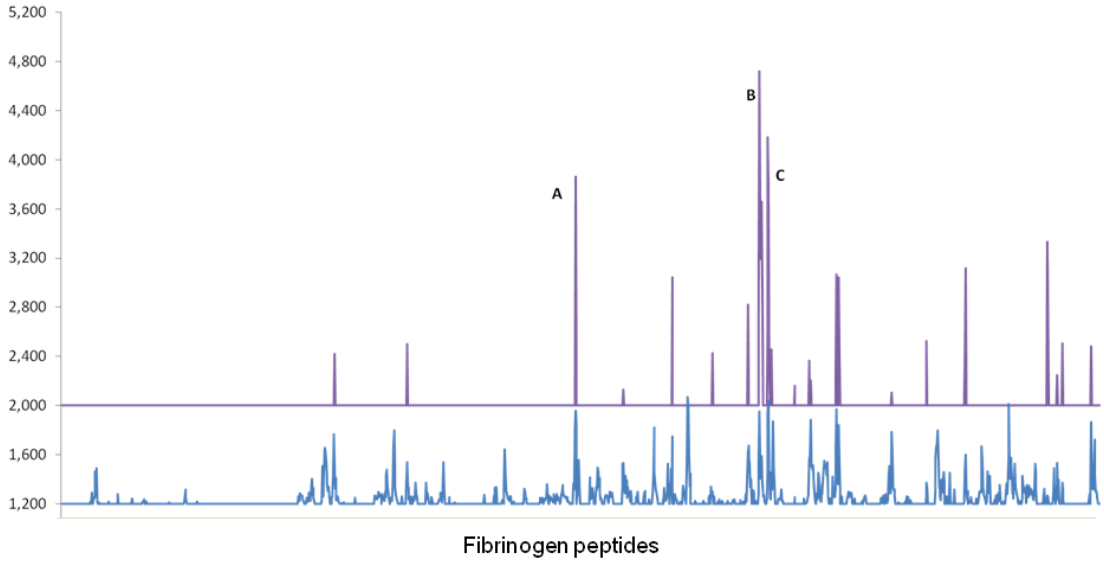


Figure 4-5 Intensity plots of the C3-antibody complex microarray screening

The peptide sequences are along the x axis with the peptide motifs AVSQTSSSSFQYMYL (A), QCSKEDGGGWWY (B) and YNRCHAANPNGRYY (C) indicated all of which are located within the β chain of fibrinogen. The intensity plot from C3 (8μg/ml) is indicated in blue and the C3-antibody complex (8μg/ml) in purple.

4.2.3 Potential binding sites within C3

Analysis of the C3 crystal structure (PDB code: 2A73)[66] using the AutoLigand software revealed 10 potential binding regions, two of which encompassed the

same region and so were classed as one (Figure 4-6). Unfortunately due to the size of the C3 molecule, it was not possible to investigate these 9 different regions using eHiTS as the computational memory required was too large to perform such an analysis. eHiTS is generally utilised for much smaller peptides and ligands and although disappointing this was not perhaps a surprise.

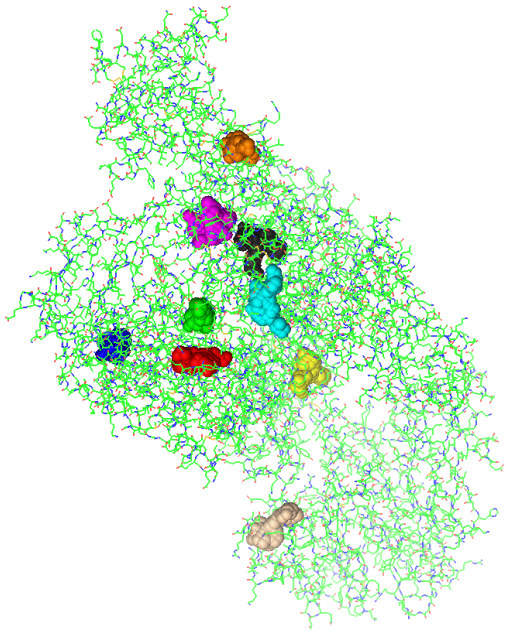


Figure 4-6 Potential binding sites on C3

The 9 different regions identified by AutoLigand are shown as coloured spheres.

4.2.3.1 Binding of fibrinogen peptides to C3 and C3b

Using the results from microarray screening the position of the peptides within the β chain and α chain were determined in terms of the sequence and then within the molecule itself. The sequence positions can be seen below, with peptides B and C being within one amino acid of each other and near the C-terminus of the β chain. Peptide A is located closer to the N-terminus. In terms of physical location within the molecule itself, this can be seen in Figure 4-7. Peptide A is close to the central E region as might be expected from the

sequence position, whilst the other two motifs are located within the coiled coil regions of the β chain as it extends towards the peripheral D regions.

α-chain

MFSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVRGPRVVERHQSACKDSDWPFCSDED
WNYKCPSGCRMKGLIDEVNQDFTNRINKLKNSLFEYQKNNKDSHSLTTNIMEILRGDFS
SANNRDNTYNRVSEDLRSRIEVLKRKVIEKVQHIQLLQKNVRAQLVDMKRLEVDIDIKI
RSCRGSCSRALAREVDLKDYEDQQKQLEQVIAKDLLPSRDRQHLPLIKMKPVPDLVPGN
FKSQLQKVPPEWKALTDMPQMRMELERPGGNEITRGGSTSYGTGSETESPRNPSSAGSW
NSGSSGPGSTGNRNPGSSGTGGTATWKPGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRP
GSTGTWNPGSSERGSAGHWTSESSVSGSTGQWHSESGSFRPDSPGSGNARPNNPDWGTF
EEVSGNVSPGTRREYHTEKLVTSKGDKELRTGKEKVTSGSTTTTRRSCSKTVTKTVIGP
DGHKEVTKEVVTSEDGSDCPEAMDLGTLSGIGTLDGFRHRHPDEAAFFDTASTGKTFPG
FFSPMLGEFVSETESRGSESGIFTNTKESSSHHPGIAEFPSRGKSSSYSKQFTSSTSYN
RGDSTFESKSYKMADEAGSEADHEGTHSTKRGHAKSRPVRGIHTSPLGKPSLSP

β-chain

MKRMVSWSFHKLKTMKHLLLLLLCVFLVKSQGVNDNEEGFFSARGHRPLDKKREEAPSL
RPAPPPISGGGYRARPAKAAATQKKVERKAPDAGGCLHADPDLGVLCPTGCQLQEALLQ
QERPIRNSVDELNNNVEA**VSQTSSSSFQ**YMYLLKDLWQKRQKQVKDNENVVNEYSSELE
KHQLYIDETVNSNIPTNLRVLRSILENLRSKIQKLESDVSAQMEYCRTPCTVSCNIPVV
SGKECEEIIRKGGETSEMYLIQPDSSVKPYRVYCDMNTENGGWTVIQNRQDGSVDFGRK
WDPYKQGFGNVATNTDGKNYCGLPGEYWLGNDKISQLTRMGPTELLIEMEDWKGDKVKA
HYGGFTVQNEANKYQISVNKYRGTAGNALMDGASQLMGENRTMTIHNGMFFSTYDRDND
GWLTSDPRKQ**CSKEDGGGWW**Y**NRCHAANPNGR**YYWGGQYTWDMAKHGTDDGVVWMNWKG
SWYSMRKMSMKIRPFFPQQ

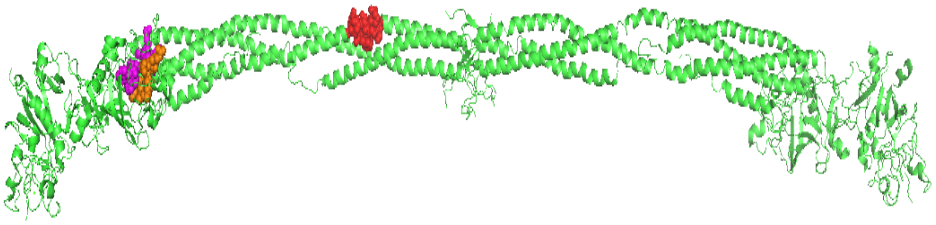


Figure 4-7 The location of the peptide sequences that bind to C3 within the fibrinogen molecule

The peptide sequences identified by the microarray screening are illustrated within the crystal structure of fibrinogen (PBD code 3GHG). The red spheres indicate peptide A (VSQTSSSSFQ), pink spheres indicate peptide B (CSKEDGGGWW) and orange spheres indicate peptide C (NRCHAANPNGR).

It was not possible to visualise the α chain peptides as the crystal structure of the α chain containing those particular peptides is not available (only part of the α-chain of fibrinogen has been crystallised). The same sequences of the β chain and α chain peptides were entered into the Pepsite2 software to predict their binding sites to C3 (PBD code 2A73). All were predicted to bind in a similar region on the α chain of C3 within the MG7 domain (Figure 4-8).

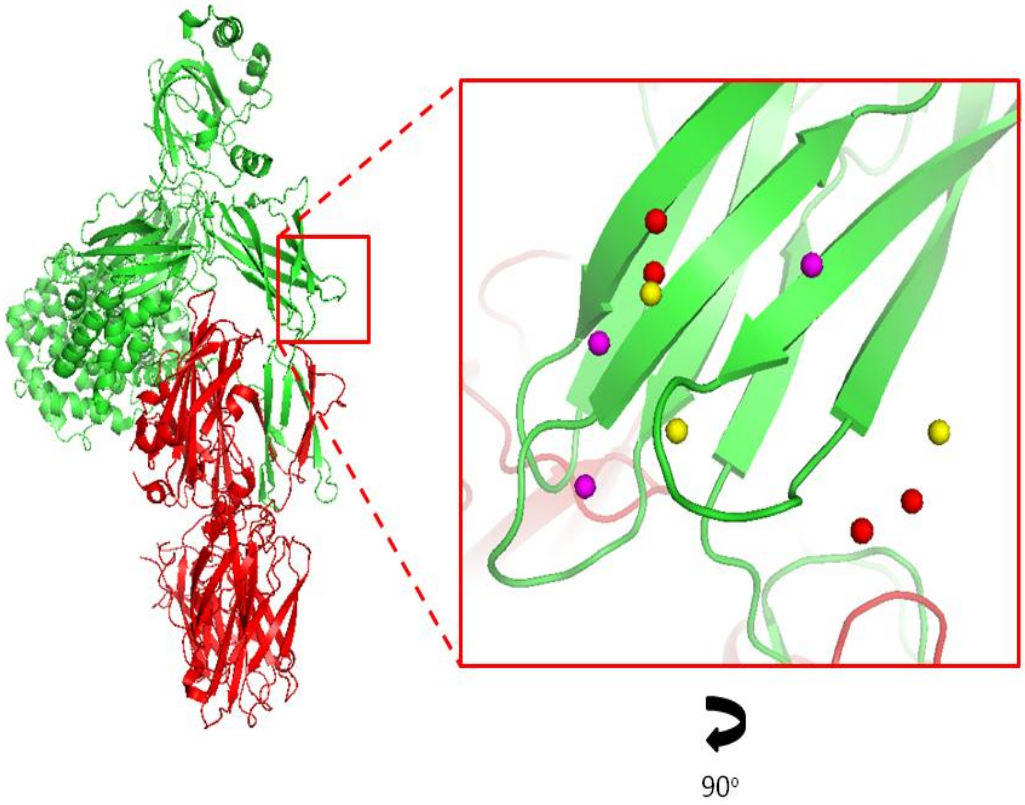


Figure 4-8 The predicted binding sites of the fibrinogen β chain peptides on C3.

Predicted binding sites of the three fibrinogen β chain peptides, identified from the microarray screening, were generated using the web server Pepsite2. The red, pink and orange dots represent residues from peptides A, B and C respectively. The α chain is shown in green and β chain in red.

The receptors within C3 that were predicted to bind to peptide B (CSKEDGGGWW) contained Lysine 839 that was noted to undergo glycation from the MS analysis of individual C3 samples (Figure 3-5). Given that complement activation is enhanced in diabetes and C3b is the active form of C3 and has a very different structure, it was decided to repeat the procedure with the crystal structure of C3b (PDB code 2I07). As may be expected the predicted binding for the same fibrinogen peptides were in a very different region, presumably due to the exposure of previously hidden binding sites (Figure 4-9).

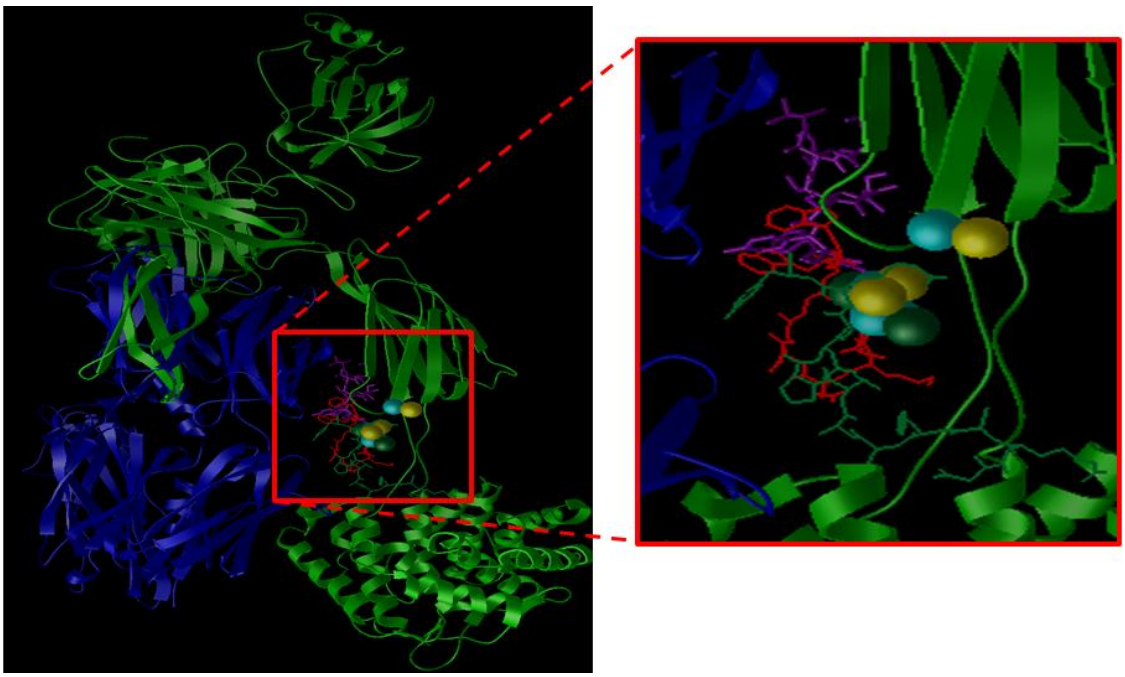


Figure 4-9 Docking of fibrinogen β chain peptides to C3b

The similarities of potential docking and binding sites of the fibrinogen β chain peptides B and C identified from the microarray screening on C3b. The results from AutoDock indicating the peptides docked to C3b are shown as sticks and predicted binding sites from Pepsite are shown as spheres.

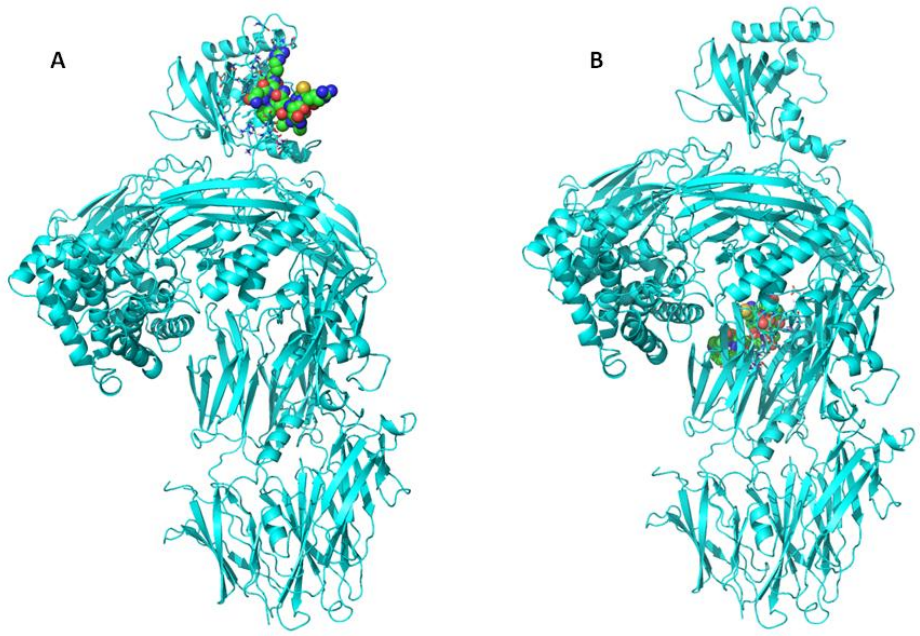


Figure 4-10 Predicted binding of peptides B and C to C3 by AutoDock

(A) Results of AutoDock predicting the binding of fibrinogen β chain peptide B (CSKEDGGGWW) and (B) results of docking peptide C (NRCHAANPNGR) to C3. The peptides are represented by coloured spheres.

4.2.3.2 Docking of fibrinogen peptides to C3 and C3b

The potential binding regions identified by pepsite2 were used to facilitate more rigorous docking of 3D structures of the β chain peptides to C3 and C3b using the automated docking suite AutoDock as described in Chapter 2 . The 3 peptide sequences (A, B, C) were “clipped” from the β chain of the original fibrinogen crystal structure (PDB ID 3GHG). The docking of peptides B and C to native C3 were in a different location to the binding sites predicted by Pepsite as can be seen in Figure 4-10.The docking poses do appear to encompass areas containing Lysine residues observed to undergo glycation, namely K665 for peptide B and K1505 for peptide C. The lowest energy poses for dockings of the same peptides to C3b actually matched the binding sites predicted by

Pepsite (Figure 4-9). The conformational changes that occur during the formation of C3b likely exposes binding sites for fibrinogen that were previously hidden in C3, and suggests that C3b may be important in fibrinogen interactions in addition to native C3.

4.3 Discussion

Attempting to identify sites of fibrinogen-C3 interaction was not straightforward due to a lack of published work on this topic and due to the relative size of both molecules. For these reasons several different methodologies were employed to gain some insight into where these proteins may interact.

Results from this chapter shed light on potential interactions between fibrinogen and C3 and factors that may influence this interaction. Firstly, the cDNA library of the α-chain together with the microarray screening suggest that the β chain of fibrinogen rather than the α-chain is in fact most important in terms of binding to C3. Secondly, molecular modelling work has identified areas within both C3 and C3b that would amenable to binding of fibrinogen sequences and raises the possibility that both C3 and its active component C3b are involved in the interaction with fibrinogen. The predicted binding locations suggest that post-translational modification of C3 by glycation in diabetes may play a role in determining the degree of interaction.

Previous work from colleagues within the research group had identified a potential area with the α chain of fibrinogen that may bind to C3 suing surface plasmon resonance (SPR). For this reason it was decided to construct a cDNA library of the α chain of fibrinogen for use in a phage display system to try and identify peptide sequences with the α chain that bind to C3, hopefully corresponding to the SPR data. Following several rounds of biopanning, which involved incubating the α chain library with immobilised C3, no enrichment of the phage particles was observed, indicating that the peptide sequences expressed by the phage were not binding to C3.

This was obviously disappointing and may indicate that the epitope sites on fibrinogen are conformational in nature rather than linear, as is presented by the phage library. This would not explain the binding observed with the SPR experiments however as this technique also relies on linear binding. A feasible explanation is that the SPR results identified only one of a number of interaction sites and that additional higher affinity binding sites exist. This was corroborated by the fact that the interaction between fibrinogen and C3 was only partly inhibited by the α-chain peptide αF1 from Figure 4-1, and using high concentrations, indicating the presence of other interaction sites, and would therefore be consistent with my findings.

Following these results it was decided to screen the entire fibrinogen molecule against C3. This service was offered by a German company, PEPperPRINT who were commissioned to generate a microarray peptide chip of the entire fibrinogen molecule and with our input, devised experiments to screen against purified human C3. This technique indicated that the β chain of fibrinogen probably contains more important sites for interaction with C3 than the α chain. Some areas within the α chain were identified but these were different to those previously noted by my colleague. Crucially, the previously identified area was not highlighted as an interaction site suggesting this part of fibrinogen is of secondary importance in C3-fibrinogen interaction. It should be stressed, however, that these methods only detect linear binding and fail to identify conformational interaction sites.

By using molecular modelling techniques it was possible to investigate these β chain peptide sequences further with regard to their possible role in binding to C3. It was encouraging that the peptides were predicted to bind in the same region of C3 using Pepsite, however AutoDock simulated the docking peptides B and C to different areas of C3 and given that this modelling relies on the conformation of the peptide sequences is likely to be the more credible binding sites. The docking poses did appear to encompass regions within C3 that contained glycated residues, K1505 for peptide B and K665 for peptide C. Given the proximity of these peptide sequences within the β chain of fibrinogen, it is likely that both are involved in binding C3. A more realistic model would therefore be to try and dock these two sequences as one to C3 rather than as separate entities. It is worth noting that K839 from C3 was predicted to be a receptor for fibrinogen peptide B, from the Pepsite results, and this same lysine was noted to be glycated in one of the T2DM samples. Furthermore, K839 was also described by Hair et al (2012) as being increasingly biotinylated, i.e. more available for protein interactions, with increasing glucose concentration. This combination of evidence supports this region of C3 as being important in C3-fibringoen interactions and further implicates the roles hyperglycaemia and glycation.

Native C3 is clearly important when discussing the link between the complement and coagulation systems as evidenced by its role as a substrate for many coagulation factors such as plasmin, thrombin and FXa. However, it is also worth considering the role of its active component C3b, given that activation of the complement system is increased in diabetes. The predicted

binding sites of the β chain peptides on C3b, generated by Pepsite, were very different to that of C3. This finding was not unexpected given the major conformational changes that occur following activation of C3, which exposes numerous cryptic binding sites. The areas within C3 and C3b were both within the α chain which is known to be more important than the stable β chain in interacting with other complement component for example. The site on C3b was within the CUB (complement C1r/C1s, Uegf, Bmp 1') domain which is thought to be involved in the binding of complement factors H and I[86]. In contrast to C3, the predicted docking poses of the β chain peptides using AutoDock were in the exact same region on C3b as predicted by Pepsite. This commonality of findings suggests that this is a genuine binding site for fibrinogen and C3b. It may be that fibrinogen is in fact capable of binding to both C3 and C3b, which may explain the hypofibrinolysis associated with diabetes, as the increased C3 activation, and subsequent elevated C3b levels, increases fibrinogen binding and consequently increases incorporation into the clot.

The results presented here indicate that the interaction between C3 and fibrinogen may occur at sites in the β chain of fibrinogen and α chain of C3. The glycation of certain lysine residues within C3 may serve to enhance this interaction. It is possible that fibrinogen is able to bind to C3b, the active form of C3, which may explain the increased incorporation into the clot in diabetes following increased complement activation. This lays the foundations for investigating methods that are capable of modulating this interaction, in an attempt to improve clot breakdown and hence reduce cardiovascular risk, in this susceptible population.

Chapter 5

Modulation of fibrinolysis by interference with plasmin inhibitor-fibrinogen interaction

Introduction

Current treatment strategies for primary and secondary prevention of cardiovascular disease are largely focussed on inhibition of platelet function, and the fibrin network is not usually targeted. However, several pieces of evidence indicate that modulating the fibrin network is also effective at reducing thrombotic risk[246-249]. Studies involving warfarin have shown promise in reducing this risk but have been largely outweighed by issues such as its non-specific mode of action, narrow therapeutic window, need for frequent testing and increased bleeding risk [250;251]. Newer, more specific agents, including coagulation factor X inhibitors, have shown a more consistent effect. However, the relatively high bleeding risk when combined with antiplatelet therapy has limited widespread use[252]. Given the effectiveness of antiplatelet therapy at inhibiting platelet function, further reduction of atherothrombotic events, without significant increase in bleeding risk, maybe obtained by targeting the fibrin network, specificaly the interaction between fibrin(ogen) and other proteins that are incorporated into the clot. Suitable candidates for this type of study include C3 as well as the more traditional plasmin inhibitor (PI). Both C3 and PI have shown increased incorporation into fibrin networks from diabetes patients [133;253]. Therefore, interfering with C3 or PI incorporation into fibrin clots represents a diabetes-specific strategy to improve the fibrinolytic process and decrease thrombosis risk in this population.

Collaborators from the Bioscreening Technology group at the University of Leeds have developed a new artificial binding protein scaffold called Adhiron, which is utilised as a novel drug target validation tool. Briefly the Adhiron comprises of a protein scaffold based on a consensus sequence of plant-

derived phytocystatins, a cysteine protease inhibitor. The inhibitory sequences within two loops of the protein have been replaced with nine randomised amino acid positions in each loop. A phage display library of this scaffold has been produced, comprising 1.3 $x10^{10}$ clones, with high thermal stability (Tm ca. $101^{o}C$) and high quality[254]. By screening this library against fibrinogen it is hoped that peptide sequences capable of modulating clot formation and lysis can be discovered. Specific protein-protein interactions can also be examined using this technique, for example that of fibrinogen and fibrinolytic inhibitors present within the clot such as C3 and PI.

In order to validate the technique, the library was initially used to investigate the interaction between fibrinogen and the classical anti-fibrinolytic agent PI. The role of PI in inhibiting fibrinolysis and its interaction with fibrinogen are much better understood than that of C3 and so the effect of these binders, if any, would be a useful foundation to future work involving C3.

5.1 Methods

The identification and production of Adhirons along with phage ELISA were performed by members of the Bioscreening Technology Group, University of Leeds.

5.1.1 Adhiron identification

Commercial human fibrinogen (Calbiochem) was biotinylated using EZ-link NHS-SS-biotin (Pierce), according to the manufacturer's instructions. The biopanning with the Adhiron phage library was performed as described in Chapter 2. Following three rounds of panning, >1000 fold amplification of phage was observed over streptavidin control wells. In order to refine the phage pool and select specific Adhirons capable of targeting fibrinogen–plasmin inhibitor interactions, competitive elution with plasmin inhibitor (50µg/ml) for 20 min at room temperature was applied in the fourth and fifth rounds of panning. Individual ER2738 colonies were selected and confirmed to be specific binders to fibrinogen by phage ELISA.

5.1.2 Phage ELISA

The selected E.coli colonies were grown in 100µl of 2TY growth medium (bacto tryptone and yeast extract) also containing 100µg/ml carbenicillin, at 37^{o}C and 900rpm for 6 hours, after which a 25µl aliquot was added to 200µl of 2TY and carbenicillin as before. After an hour, 10µl of 10^{11}/ml M13K07 helper phage and 25µg/ml kanamycin were added and left overnight at 25^{o}C and 450rpm. A streptavidin coated plate (Pierce) was blocked overnight with 2x casein blocking buffer (Sigma) and the plate was then incubated with biotinylated fibrinogen for 1 hour, followed by another hour with 45µl of growth medium containing the phage. After washing the wells extensively, the bound phage were detected by

the addition of a HRP-conjugate anti-phage antibody diluted 1:1000 (Seramun Diagnostica GmbH, Germany) for 1 hour and developed with 3,3',5,5'-Tetramthylnenzidine (Seramun) and OD measured at 610nm.

5.1.3 Adhiron production

The selected Adhirons were produced using the pET expression system, which produces large quantities of protein in a relatively short amount of time through manipulation of T7 RNA polymerase and the *lac* repressor protein. The DNA sequence encoding the protein of interest is cloned into a pET phagemid vector that contains a T7 promoter and *lac* operator at the 5' end of the gene encoding the protein. Transcription of the inserted gene only occurs in the presence of T7 RNA polymerase and therefore the vector needs to be transformed into a bacterial cell that has been engineered to contain the gene for this enzyme, usually E.coli strain BL (DE3). Transcription of T7 RNA polymerase within the host cell needs to be activated as the host RNA polymerase is blocked from binding to the gene by the presence of a *lac* promoter sequence which binds the *lac* repressor protein (Lacl). Lactose, or a structural mimic such as isopropyl β-D-1-thiogalactopyranoside (IPTG), will bind to Lacl and alter its conformation, to the extent that its affinity for DNA is greatly reduced, and thus the native E.coli RNA polymerase is no longer blocked and transcription of T7 RNA polymerase proceeds apace. This in turn causes transcription of the gene encoding the protein of interest within the phagemid vector (Figure 5-1).

The technique employed by the BioTechnology Screening group involves the amplification of the DNA coding sequence of Adhirons by PCR and subsequent restriction digestion with NheI and PstI, followed by cloning into a pET11a phagemid. This vector, containing the target protein gene, was transformed,

using heat shock, in to BL21 (DE3) E.coli cells and finally induced by the addition of 0.1mM IPTG. The cells were grown for 6 hour in LB medium and then lysed using Bugbuster (Novagen). The expressed protein was purified using Ni-NTA resin slurry (Qiagen) as per the manufacturer's instructions.

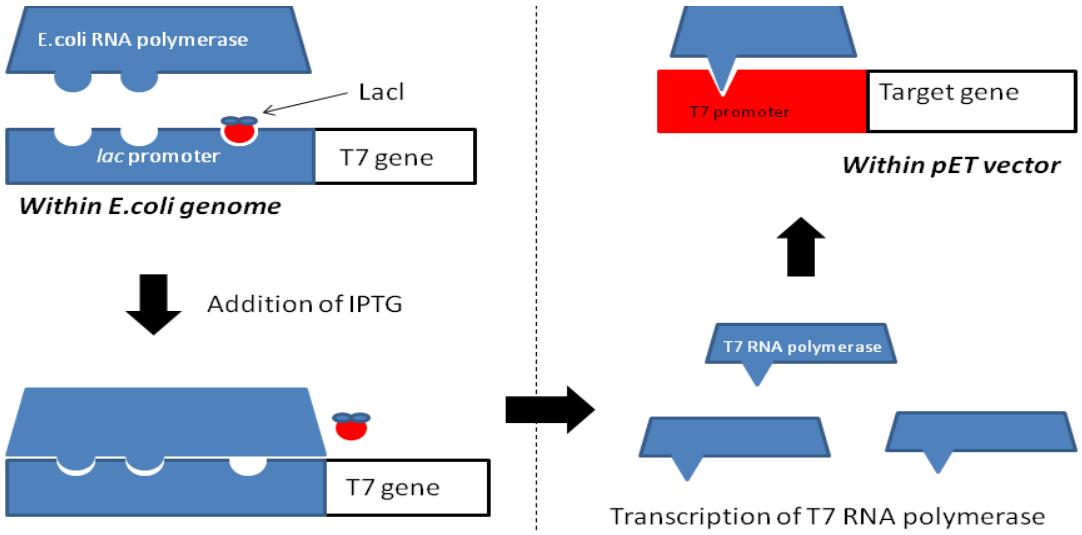


Figure 5-1 Illustration of the pET expression system

In the absence of IPTG, host RNA polymerase is unable to transcribe T7 RNA polymerase due to the presence of lac repressor protein (LacI) within the lac promoter region. The process is activated by the addition of IPTG which binds to and alters the conformation of LacI, reducing its affinity for DNA. Transcription of T7 RNA polymerase occurs followed by transcription of the target protein DNA within the pET vector.

5.1.4 Turbidimetric analysis

In order to study the effects of the selected Adhirons on clot formation, turbidimetric analysis was performed as described in Chapter 2 using both pooled plasma and purified fibrinogen. In order to incorporate the Adhirons, the protocol was altered slightly. For plasma experiments, 25µl of plasma (containing approximately 6 µM fibrinogen) was incubated with 25µl of Adhiron

(0.5mg/ml or 30µM) for 30 minutes before the addition of LM and AM. In a purified setting, 25µl of Adhiron (0.2mg/ml or 6µM) was added to fibrinogen (1mg/ml or 2.94 µM). These concentrations equated to a fibrinogen:Adhiron ratio of 1:5 in plasma and 1:2 in purified experiments. To study the effects of Adhirons on PI prolongation of lysis, commercial purified fibrinogen (2.94µM) was initially incubated with the relevant Adhiron (6µM) for 30 minutes followed by further incubation with either tris-buffered saline or, plasmin inhibitor (0.43µM). The lysis mix and activation mix were then added as previously and the absorbance at 390nm read every 12 seconds for 60 minutes.

5.1.5 Laser scanning confocal microscopy

Confocal microscopy was employed to analyse whether the Adhirons were affecting the structure of the fibrin network. Hydrated clots were prepared using either plasma or purified fibrinogen as described in Chapter 2. Adhirons were added to the clot by substituting 7.5µl of PB for the same volume of Adhiron at 0.5mg/ml for plasma and 0.2mg/ml for purified experiments, maintaining the fibrinogen:Adhiron ratio used in the turbidimetric assays. Real time lysis of these clots was also performed as described previously. All clots were prepared and viewed in duplicate.

In order to visualise Adhirons in the clots, we labelled these proteins using DyLight 488 Microscale Antibody Labelling Kit (Thermo Scientific), according to manufacturer's protocol. Briefly, 8µl of borate buffer (0.67M) was added to 100µl of Adhiron (1mg/ml) which was then added to the DyLight dye and incubated at room temperature and protected from light for 1 hour. Purification resin was added to a spin column and microcentrifgue collection tube (all provided) and spun at 1000g for 60 seconds to remove the storage solution.

The column was placed in a new collection tube and 108µl of the labelling reaction added to the column. The labelled Adhiron was collected following 60 seconds in a centrifuge at 1000g.The Labelled Adhirons at 6µM were incubated with purified human fibrinogen from healthy controls (2.94µM) in the presence of 0.105µM fluorescent labelled fibrinogen (Alexa FluorDye 594; Invitrogen) followed by the addition of an activation mix as above to form the clot and then transferring to an IBIDI slide 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 (Adhiron) and red (fibrinogen) channels which were then superimposed onto each other to allow the fluorescence intensity from each pixel to be determined.

5.1.6 Scanning electron microscopy

Clots were prepared for viewing with a scanning electron microscope in a similar manner to that described in Chapter 2 and provided information over and above the confocal microscopy images on the effect of Adhirons on clot ultrastructure. In order to incorporate the Adhirons, 50µl of plasma was incubated with 50µl of Adhiron (0.5mg/ml) and 5µl of AM was added to 45µl of the plasma/Adhiron mixture. With purified clots, 50µl of control purified fibrinogen (1mg/ml) was incubated with 50µl Adhiron (0.1mg/ml). The fibrinogen:Adhiron ratio was thus maintained at 1:5 for plasma and 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, 20 fibres were measured from at least 5 different areas of clot for each sample.

5.1.7 Enzyme Linked Immunosorbant Assay (ELISA)

ELISA was initially used to confirm the ability of the Adhirons to bind to fibrinogen outside the phage system. A Nunc ELISA plate was coated overnight with 200µl of 1µg/ml of Adhiron. A scaffold only Adhiron was used as a control along with a well with no Adhiron. After washing, the wells were blocked with 1% (w/v) BSA followed by the addition of 100µl of fibrinogen (1µg/ml; calbiochem) and incubated for 2 hours at room temperature. A goat polyclonal anti-fibrinogen HRP conjugated antibody (Dako Cytomatic, Cambridge, UK) was added following more washing and incubated for 1 hour at room temperature. HRP-conjugate antibody chromogenic substrate 1,2-phenylenediamine dihydrochloride (DaoCytomation, Dako) was added and the reaction was stopped by the addition of 1.5M H2SO4. The absorbance was measured at 490nm using a plate reader.

5.1.8 Western blot to demonstrate binding of Adhirons to different chains of fibrinogen

Initially 25 µg of purified, healthy pool fibrinogen was loaded onto the sample well of an 8% SDS-PAGE gel prepared using a prep/2-D well comb and the gel was run at 200V until good separation of the ladder (PageRuler Prestained Protein Ladder, 10 to 170 kDa, (Pierce, Biotechnology, Thermo Scientific, Loughborough,UK). The gel was transferred onto nitrocellulose using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, UK) and a Trans-Blot Turbo Mini Nitrocellulose Transfer Pack (Bio-Rad) according to the manufacturer's instructions. The membrane was then incubated overnight at 4°C in a blocking buffer of 1x Tris-buffered saline with 1% (v/v) Tween-20 (TBST) and 3% (w/v) non fat milk.

The Western blot was performed using a Mini PROTEAN II Multiscreen Apparatus (Bio-Rad). A two-fold serial dilution of the phage using blocking buffer was performed (1:37.5 to 1:2400) along with a control well with just blocking buffer and another control with no phage. The samples were loaded into the channels of the multiscreen apparatus and incubated for 1 hour at room temperature and then aspirated off and the channels washed 3 times with TBST, incubating for 15 min per wash. The antibody, Anti-Fd-Bacteriophage-HRP (Seramun, Heidesee, Germany) was diluted 1:1000 in blocking buffer and 600 μl added per channel and incubated for 1 hour at room temperature followed by washing as before. The membrane was washed for a further 15 min in TBST and the blot was developed using Luminata Forte Western HRP Substrate (Merck Millipore, Germany).

The western blot was performed by Anna Tang from the Bioscreening Technology group, University of Leeds.

5.1.9 SPR

Surface plasmon resonance was performed using a Biacore 3000 platform and data evaluated using BIAcore 3000 BIAevaluation 4.1 software (GE Healthcare, UK). For each Adhiron tested; 2000 RU of purified fibrinogen (Calbiochem) was immobilised directly onto a CM5 sensor chip (Fc2) by amine coupling according to the manufacturer's instructions. Immobilisation of fibrinogen was performed using immobilisation buffer; 10mM Hepes, 140mM NaCl, 1.5mM $CaCl_2$, 0.005% (v/v) surfactant P20, pH 7.4. A blank reference surface (Fc1) was activated and deactivated using a 1:1 ratio of 0.4M EDC and 0.1M NHS at a flow rate of 20μl/min for 7 minutes, followed by 7 min of 1M Ethanalomine pH 8.0 to block remaining active groups. Due to the high binding affinities of the Adhirons for

fibrinogen, complete regeneration of the fibrinogen surface was unsuccessful. Therefore, a Kinetic titration method was employed as described by Karlsson et al 2006[255], which removes the need for regeneration. Adhirons of interest were reconstituted into SPR running buffer (50mM Tris, 100mM NaCl, 0.005% (v/v) surfactant P20, pH 7.4). A 2-fold serial dilution of each Adhiron: A2 (6-100nM) and G2, G4 (0.63-10nM) were injected sequentially in order of increasing concentrations over the fibrinogen surface. Each Adhiron was injected within a single binding cycle over a fresh fibrinogen surface each time. 2 cycles of 5x buffer injections were performed prior to the Adhiron binding cycle to generate 'blank' responses for double referencing the Adhiron binding data. The binding titration data was analysed using the double-referenced data in a 1:1 kinetic titration model as defined by Karlsson *et al* 2006. For each Adhiron; rate constants [*k*a (M^{-1} s^{-1}) and *kd* (s^{-1})] and affinities [K_D (nM)] were obtained. Validity of the kinetic model fitting to the experimental data is given as χ^2.

All SPR experiments were performed by Dr. Kerrie Smith from the Leeds Institute for Genetics Health and Therapeutics, University of Leeds.

5.1.10 Molecular modelling

In an attempt to more accurately identify the site of Adhiron-fibrinogen interaction, predicted binding sites of the random peptide sequences constrained within the two loops of Adhirons of interest were generated using the website Pepsite2®, as described in Chapter 2. Images were viewed and produced using Pymol software. The potential binding regions were used to facilitate more rigorous docking of modelled 3D structures of the Adhirons using the automated docking suite AutoDock again as described in Chapter 2.

5.1.11 Statistical analysis

Between group comparisons of normally distributed variables in the in-vitro studies were carried out using independent samples student t-test. Comparisons of non-normally distributed variables were carried out using Mann-Whitney U tests. Data are presented as mean±SEM unless otherwise stated

5.2 Results

5.2.1 Fibrinogen binding Adhirons released by PI

From the phage display screening, 48 phage colonies eluted from fibrinogen by the addition of excess PI were tested in an ELISA to confirm binding to fibrinogen. As can be seen in Figure 5-2, non-specific binding of the phage was observed and these phage colonies were discarded. It was not possible to sequence all 48 colonies due to financial constraints and so following the phage ELISA 24 high affinity fibrinogen-binding phage colonies, that were released by PI, were selected at random for sequencing (denoted by the red line below the well number in Figure 5-2). This identified 8 different Adhirons as detailed in Table 5-1, which were subsequently produced as individual proteins. Due to potential future patent considerations of the Adhirons only the first amino acid is indicated for each loop sequence. Experiments were designed to investigate the interaction between fibrinogen and these Adhirons, and their effects on clot structure and lysis time.

5.2.1.1 Effect on clot lysis in plasma

The ability of Adhirons to alter plasma clot lysis was assessed using turbidimetric assays. Time to 50% clot lysis was 780±25sec with 5 Adhirons increasing this by a mean of 727 sec (range 108-1512, Table 5-1). Two Adhirons (G4 and G6) resulted in a small reduction in clot lysis by 48 and 36 secs, although these were not statistically significant ($p>0.1$), whereas G2 had no effect (LT 774±69 secs).

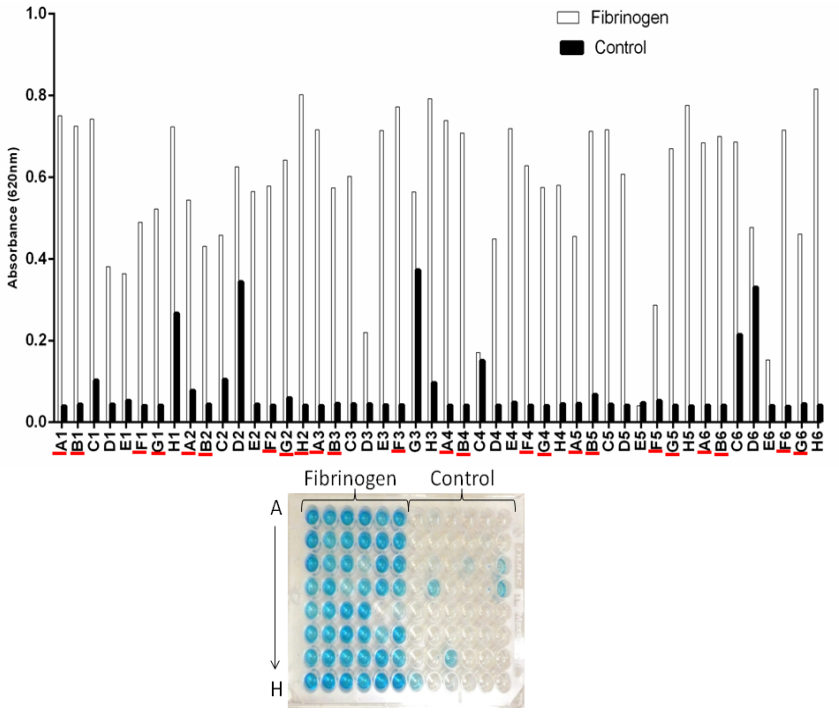


Figure 5-2. Fibrinogen binding Adhirons released by excess PI.

Top image, phage ELISA results with absorbance of phage to biotinylated fibrinogen immobilised on streptavidin coated wells in grey and empty wells in black. 48 phage colonies were added to fibrinogen coated wells and to empty, control well, extensively washed and developed by the addition of a HRP conjugated anti-phage antibody. The red lines indicate which colony was chosen for sequencing. Bottom image, ELISA plate of the same phage ELISA with fibrinogen coated wells on the left and empty, controls, wells on the right. A failure to turn the reaction mixture blue in the fibrinogen coated wells indicated the phage was not binding to fibrinogen, and non-specific binding of the phage was demonstrated by blue in the control wells.

5.2.2 More detailed study of Adhirons

Given the time constraint, detailed analysis of these Adhirons was carried out in only a subset. I chose this subset according to: i) sequence homology with PI and, ii) effects on plasma clot lysis.

None of the Adhirons described above shared sequence homology with PI, suggesting that interaction sites are conformational. Therefore, I decided to test one Adhiron that prolonged LT, one that reduced LT and one that had little or no effect. Previous work has shown that as little as 6% change in clot lysis time may be important clinically[256] and therefore a cut off of 5% change in clot lysis time by any of the 8 Adhirons was regarded as significant. All 5 Adhirons that increased clot lysis did so by more than 5%, with the percentage increase in prolongation ranging between 14-194%; (Table 5-1). Only one Adhiron (G4) decreased plasma clot lysis by more than 5%, 48±62seconds (6%), and so two Adhirons (G2 and G6) essentially had no effect.

Adhiron	Loop 1	Loop 2	Mean LT (secs)	Percentage difference in LT compared to scaffold
Scaffold	**-**	**-**	**780±50**	**-**
A2	**I**********	**Y**********	**1056±223**	**35**
B3	**L**********	**W**********	**1608±506**	**106**
F1	**Y**********	**K**********	**888±183**	**14**
F2	**W**********	**W**********	**2292±552**	**194**
F5	**T**********	**R**********	**1690±338**	**117**
G2	**Q**********	**P**********	**774±138**	**-1**
G4	**S**********	**Y**********	**732±105**	**-6**
G6	**W**********	**W**********	**744±156**	**-5**

***Table 5-1 The 8 Adhirons identified and lysis times of Adhirons following addition to pooled, healthy plasma*.**

Due to potential future patent considerations, only the first amino acid is indicated for each loop sequence

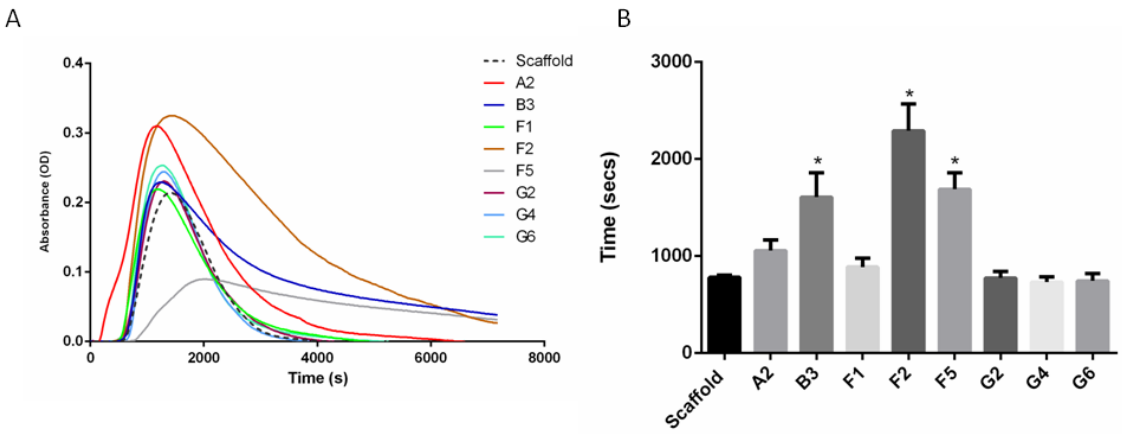


Figure 5-3 Clot lysis parameters of clots formed with Adhirons as measured by turbidimetric assay

*Turbidity and lysis curves of Adhirons in plasma (A) along with the LT (mean±SEM) of Adhirons in plasma (B). Control plasma was pre-incubated with Adhirons followed by the addition of a lysis mix containing tPA and an activation mix containing thrombin and $CaCl_2$. A total of 3 independent experiments were performed in duplicate .*p<0.05*

Of the five Adhirons that prolonged LT in plasma, it was decided to study A2 in more detail given that it had a clear effect on LT without being as extreme as Adhirons B3, F2 and F5. Only Adhiron G4 resulted in a reduction in lysis time greater than 5% and was therefore chosen alongside Adhiron G2 to act as a control Adhiron.

The effects of the presence of Adhirons on plasma clot lysis was further tested using confocal microscopy to i) confirm the turbidimetric findings, ii) visualise clot lysis in real time and iii) investigate whether the Adhirons have similar effects on fully mature clots. Moreover, confocal microscopy was useful to provide preliminary data on Adhiron-induced changes in clot structure.

Serial photographs of the lysis front were observed and lysis time in the presence of scaffold only Adhiron was 463±48 sec. Adhiron G2 had no effect on

fibrinolysis (lysis time 453±46, p=0.67), while A2 showed a trend toward prolongation of LT (600±109 secs p=0.06) in agreement with the turbidimetric analysis. In contrast, Adhiron G4 reduced the lysis time but the effect was much more dramatic than that observed with turbidimetric analysis, reducing LT to 308±101sec ($p<0.05$, Figure 5-4). The effects of Adhirons on LT using turbidimetric assays (during clot formation) and confocal microscopy (of fully mature clots) are shown in Table 5-2.

Adhiron	Difference in LT compared to scaffold from plasma turbidimetric assay (%)	Difference in LT compared to scaffold from real time lysis of plasma confocal clots (%)
G2	**-1**	**-2**
G4	**-6**	**-33**
A2	**35**	**30**

Table 5-2 The effects of Adhirons G2, G4 and A2 on LT in turbidimetric assay and real time lysis of mature plasma clots.

5.2.2.1 The effects of selected Adhirons on clot lysis in a purified system

To understand the effects of Adhirons on PI-induced prolongation of clot lysis time, I used purified proteins (fibrinogen, PI and Adhirons). All samples were supplemented with FXIII to ensure crosslinking of PI into the fibrin network.

In the absence of PI, LT following the addition of an empty scaffold protein to fibrinogen was 606±82 sec. The addition of Adhiron A2 prolonged LT to 993±85 sec ($p<0.05$), whereas Adhirons G2 and G4 had no significant effect at 708±51 and 672±34 sec, respectively ($p>0.1$ for both).

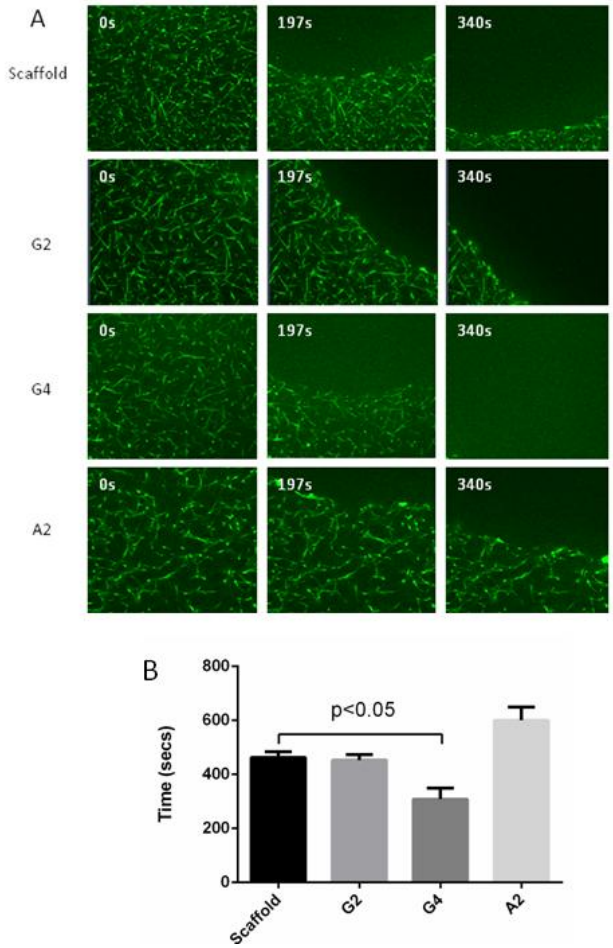


Figure 5-4. The effect of Adhirons G2, G4 and A2 along with scaffold Adhiron on real time lysis of plasma clots

(A) LSCM images of the real time lysis of plasma clots in the presence of G2, G4, A2 and scaffold indicating the increased and decreased LT caused by G4 and A2 respectively, shown graphically (mean LT±SEM) in (B). Clots were prepared by incubating control plasma with Adhiron and 488 Alexa labelled fibrinogen followed by the addition of thrombin and CaCl2. The LT represents the time taken for a lysis mixture containing tPA and plasminogen to completely dissolve the clot within the viewing field. Experiments were performed in duplicate on 3 separate occasions.

In the presence of PI, LT was prolonged to 1440±273 sec, which was further increased to 1880±300 sec ($p<0.05$) following the addition of Adhiron A2, indicating an additive effect of PI and A2 on clot lysis. In contrast, G4 inhibited the effect of PI on LT, which was reduced from 1440±273 to 940±175 sec ($p<0.05$). Finally, G2 had no significant effect on PI-mediated prolongation of clot lysis (1188±303 sec $p=0.16$). The results can be seen in Figure 5-5

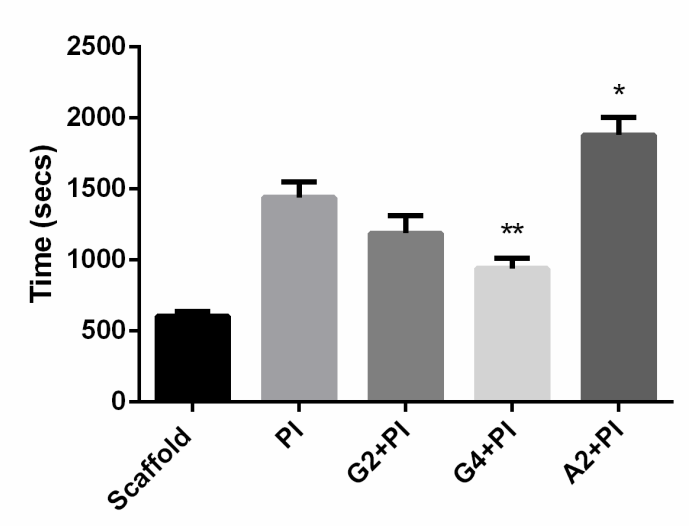


Figure 5-5 The effect of Adhirons on LT in the presence of PI in a purified system

*Adhiron G4 significantly reduces PI prolongation of lysis, with a significant increase observed with A2. No difference was noted in LT with the addition of G2. Purified fibrinogen was pre-incubated with Adhiron followed by addition of either Tris buffered saline or PI. A total of 3 independent experiments were performed in duplicate. *p<0.05,** p<0.005.*

5.2.2.2 The effects of Adhirons on clot structure

Given the effects of Adhirons on fibrinolysis, I further studied the effects of these peptides on fibrin network structure. This was performed using turbidimetric

analysis, scanning electron microscopy and confocal microscopy of clots prepared from plasma samples or purified fibrinogen.

Plasma samples

In a plasma system and in the presence of the scaffold only Adhiron, FT was 0.228±0.007. Higher final turbidity was noted following incubation with Adhiron G2 (0.246±0.013), G4 (0.250±0.005) and A2 (0.322±0.006, $p<0.05$ for all). Given that plasma clot final turbidity represents both fibre thickness and clot density, I further analysed clot ultrastructure using scanning electron microscopy. Control plasma clots, with the scaffold Adhiron added, had an average fibrin fibre diameter of 106.8±22.1 nm. Fibre thickness was not affected by the addition of G2 (109.3.4±23nm; $p=0.39$), whereas G4 resulted in increased fibre diameter (124.6±24.9nm; $p<0.05$). The reverse was observed in the presence of Adhiron A2 with fibre diameter decreasing to 95.3±22.7nm ($p<0.05$). The 3-dimensional structure of plasma clots prepared with the Adhirons was analysed by preparing z-stack images using the LSCM. The addition of Adhirons G2 and G4 both appeared to affect the structure of the clot in the same way which is unusual given the differences observed with the SEM clots. The structures appear less dense with increased porosity. The addition of A2 produces even more changes to the architecture of the fibrin network, again with increased pores compared to control clots, but in the areas where fibrin bundles have formed these appear thicker. These results are summarised in Figure 5-6.

Purified systems

To further understand the direct role of Adhirons on clot structure, I performed purified experiments to eliminate the potential influence of various plasma

proteins. Using the conditions described above for purified experiments, FT with scaffold Adhiron alone was 0.147±0.02 which was similar in the presence of G2. (0.1548±0.04, p=0.89). The numerical difference with G4 failed to reach statistical significance (0.1823±0.04, p=0.26), whereas Adhiron A2 caused a significant increase in FT to 0.2405±0.02 ($p<0.01$).

Scanning electron microscopy showed that fibre diameter with the scaffold Adhiron was 85.9±22.5nm, which did not change in the presence of G2 (80.7±17.1nm p=0.16) or G4 (82.3±18.3nm p=0.37). In contrast, Adhiron A2 produced clots with significantly thinner fibrin fibres at 72.6±17.5 $p<0.001$).

The z-stack images of the purified clots can be seen in Figure 5-7. The clots containing Adhirons G2 and G4 have a structure that is more similar to the control clots and is more in keeping with the EM data. Adhiron A2 again produces a clot with increased pores and fewer fibrin bundles compared to control.

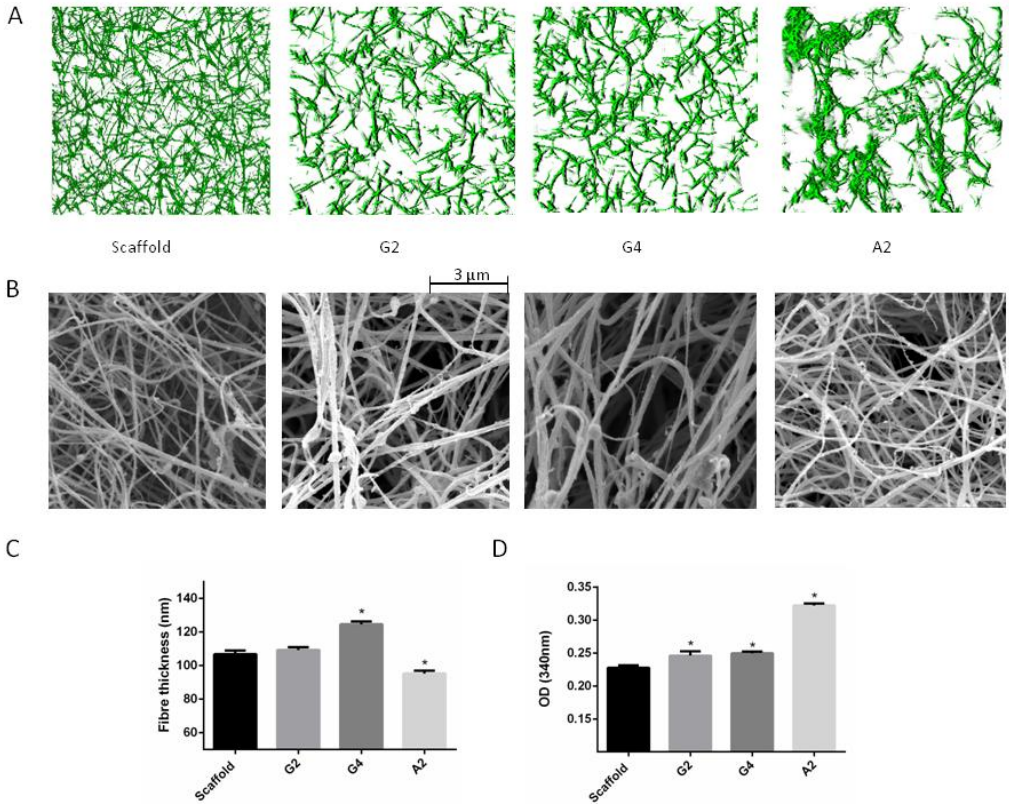


Figure 5-6. LSCM and EM images of plasma clots prepared with Adhirons G2, G4 and A2.

*(A) LSCM z-stack images of plasma clots and (B) EM images of plasma clots all produced following addition of scaffold, G2, G4 and A2 to control plasma. Clots made with G4 had significantly thicker fibrin fibres compared with scaffold Adhiron, whereas A2 produced thinner fibres (C) with a pronounced effect on clot structure. The final turbidity (mean±SEM) of the plasma clots from the turbidimetric assays are shown in (D). 20 fibres were measured from different areas of at least 5 different EM images of each Adhiron. *p<0.05.*

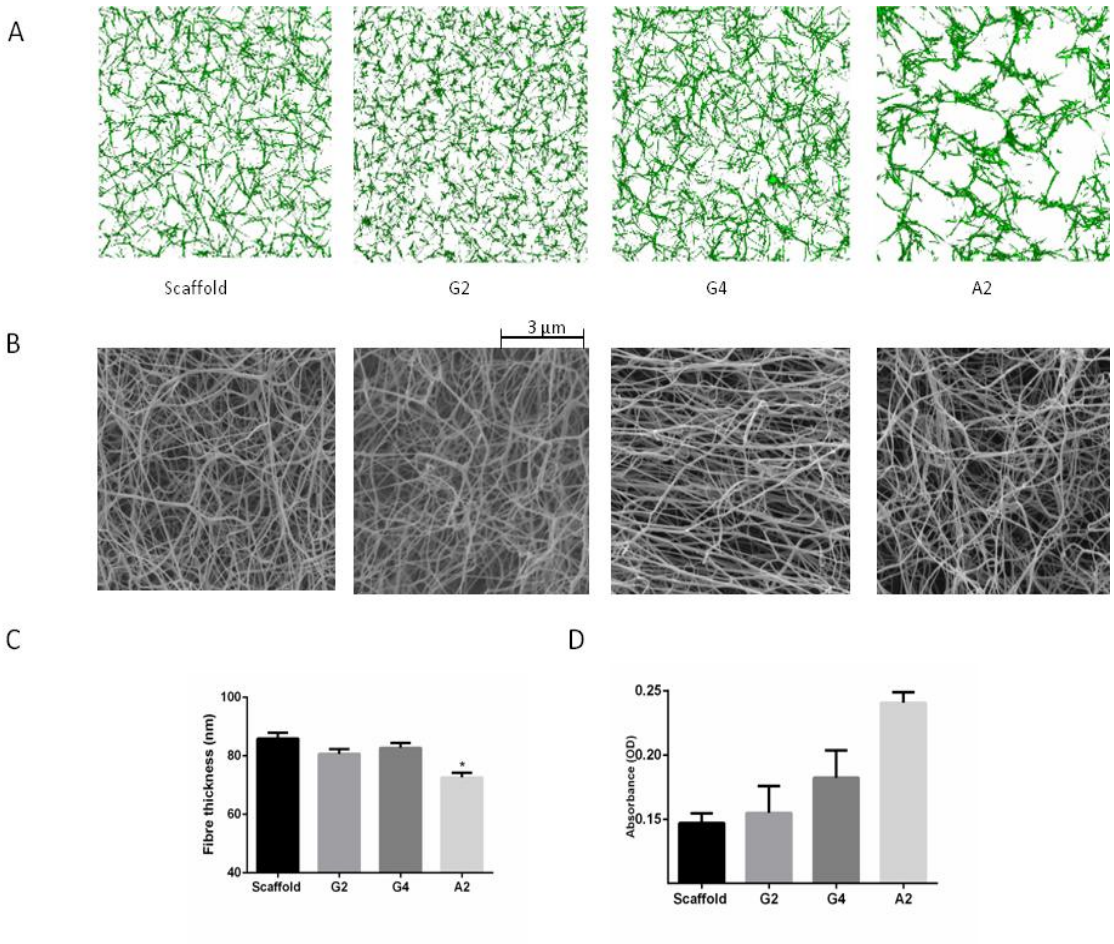


Figure 5-7. LSCM and EM images of clots prepared from purified fibrinogen with Adhirons G2, G4 and A2.

*(A) LSCM, z-stack images of purified clots and (B) EM images of purified clots generated by the addition of scaffold, G2, G4 and A2 to purified fibrinogen. There was no difference in mean (±SEM) fibre diameter between scaffold, G2 or G4 but A2 again produced thinner fibres (C) and altered fibrin network architecture on LSCM images. The final turbidity (mean±SEM) of the purified clots from the turbidimetric assays are shown in (D). 20 fibres were measured from different areas of at least 5 different EM images of each Adhiron and the. *p<0.05.*

5.2.2.3 Incorporation of Adhirons into the clot

Given that Adhirons resulted in a change in clot structure, it was decided to investigate whether Adhirons are directly incorporated into the clot or simply exert their effect through interference with clot formation.

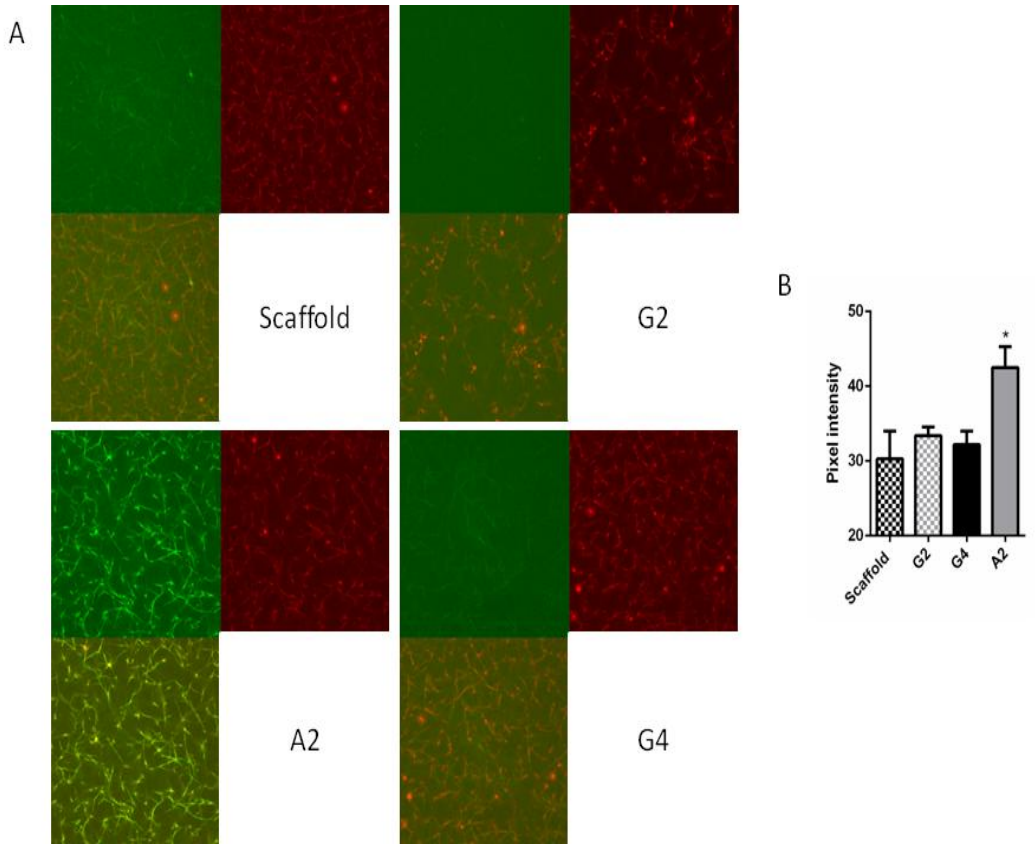


Figure 5-8 LSCM images of dual labelled clots with pixel intensity

*(A) Images of clots viewed by laser scanning confocal microscopy which were prepared using Scaffold Adhiron and Adhirons G2, G4 and A2 (6μM) labeled green and fibrinogen (2.94 μM) labeled red. Top left clockwise, Scaffold, G2, G4, A2. The bottom images represents the overlay of red and green pixels, increased yellow intensity represents increased Adhiron incorporation. (B) Mean±SEM pixel intensities of clots seen in A. *p<0.05.*

By fluorescent labelling of fibrinogen (red) and Adhirons (green), it was possible to determine the degree of incorporation of the Adhirons into the fibrin clot using laser confocal microscopy. Clots prepared using in house labelled scaffold showed no significant incorporation into the fibrin clot, with similar findings mean pixel intensities observed with Adhirons G2 and G4 (pixel intensity 30.3±3.7, 33.4±1.1 and 32.2±1.8, respectively; $p>0.1$ compared with scaffold only). In contrast, Adhiron A2 showed significantly increased incorporation into the fibrin network with an increase in mean pixel intensity to 42.5±2.8 ($p<0.05$).

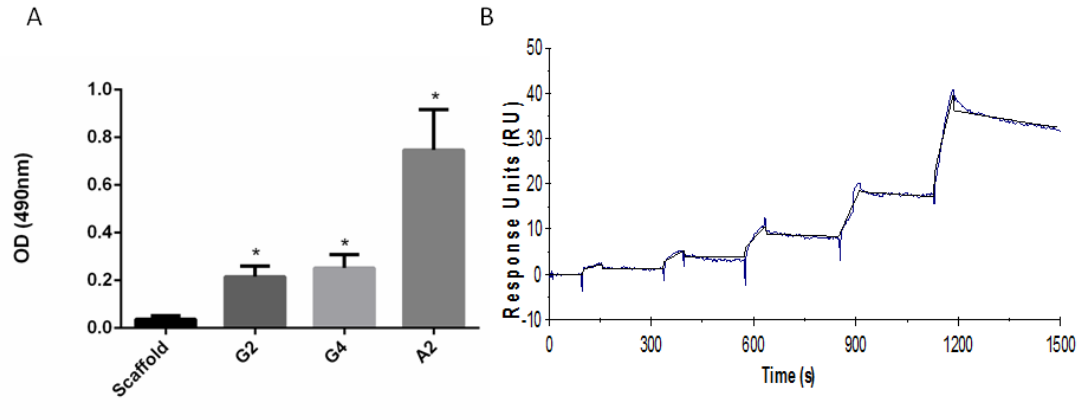
5.2.3 Fibrinogen-Adhiron interaction

5.2.3.1 Enzyme Linked Immunosorbant Assay (ELISA)

ELISA was initially used to confirm the ability of the Adhirons to bind to fibrinogen outside the phage system. All 3 Adhirons showed significant binding to fibrinogen with A2 producing the highest OD at 490nm (Figure 5-9). The OD for scaffold, G2, G4 and A2, were 0.037±0.014, 0.22±0.04 ($p<0.05$), 0.25±0.06 ($p<0.05$) and 0.74±0.17 ($p<0.05$), demonstrating that the purified Adhirons were capable of binding to immobilised fibrinogen.

5.2.3.2 Surface Plasmon Resonance (SPR)

Although ELISA showed binding between fibrinogen and selected Adhirons, the system is not accurate to analyse binding affinities. Therefore, we employed SPR and demonstrated high-affinity binding of Adhirons, G2, G4 and A2 to immobilised fibrinogen, which was concentration dependant. The binding affinities were all in the nanomolar range with K_D of 13.8nM, 1.1nM and 1.85nM for A2, G2 and G4 respectively (Figure 5-9).



C

	ka (M^{-1} s^{-1})	kd (s^{-1})	K_D (nM)	Chi^2 (<2)
A2	2.95×10^{4}	4.07×10^{-4}	13.8	0.615
G2	1.37×10^{6}	1.44×10^{-3}	1.1	1.33
G4	5.85×10^{5}	1.08×10^{-3}	1.85	0.599

Figure 5-9 Binding of Adhirons G2, G4 and A2 to fibrinogen demonstrated by ELISA and SPR

(A) Binding of immobilised Adhirons , G2 G4 and A2 to human fibrinogen using enzyme linked immunosorbant assay (ELISA), captured by HRP conjugated polyconal goat anti-human anti-fibrinogen antibody. (B) Surface plasmon resonance (SPR) using a kinetic titration assay of Adhiron G4 binding to fibrinogen. The blue line represents the experimental data, the black line represents the kinetic titration fitting to a simple 1:1 interaction model. Adhiron A2 and G2 were treated using the same methodology. SPR rate constants and binding affinities (C) of Adhirons A2, G2 and G4

5.2.3.3 Western Blot

In order to establish which fibrinogen chain is the main binding site for the selected Adhirons, a Western Blot was performed. Adhiron G2 showed binding to a band corresponding to the 48 kDa γ chain of fibrinogen. Adhirons A2 and G4 produced similar bands corresponding to the fibrinogen 66kDa α chain

(Figure 5-10). Probing a phage expressing a non-fibrinogen binding Adhiron indicated that the phage itself was not involved in binding.

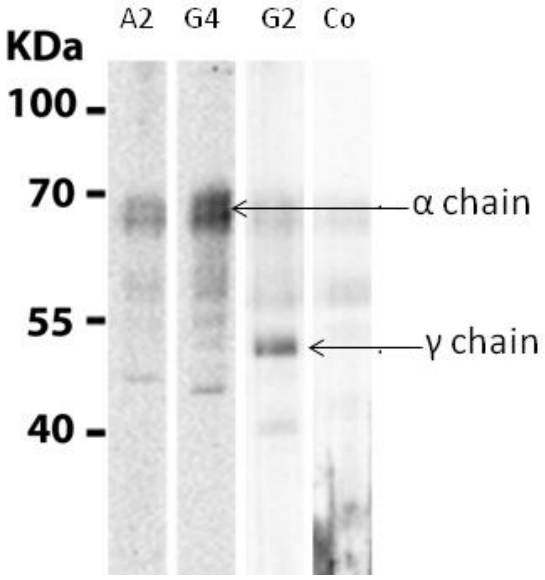


Figure 5-10 Immunoblotting of phage Adhirons to fibrinogen chains

Western blot of phage Adhirons against fibrinogen illustrating Adhirons G4 and A2 binding to the α chain (66kDa) as indicated by the top arrow (lanes 1 and 2) and Adhiron G2 binding to the γ chain (48kDa) as indicated by the bottom arrow (lane 3). Lane 4 non-fibrinogen binding Adhiron.

5.2.3.4 Predicted binding of Adhirons to fibrinogen

Following the western blot results and due to the large size of fibrinogen, it was decided to focus on binding hotspots of loops 1 and 2 of Adhirons G4 and A2 located on the α chain of fibrinogen using the Pepsite2. This identified the central E region of the α chain of fibrinogen to be the most likely area that the Adhirons were binding (Figure 5-11). These results were used to focus the AutoDock docking runs.

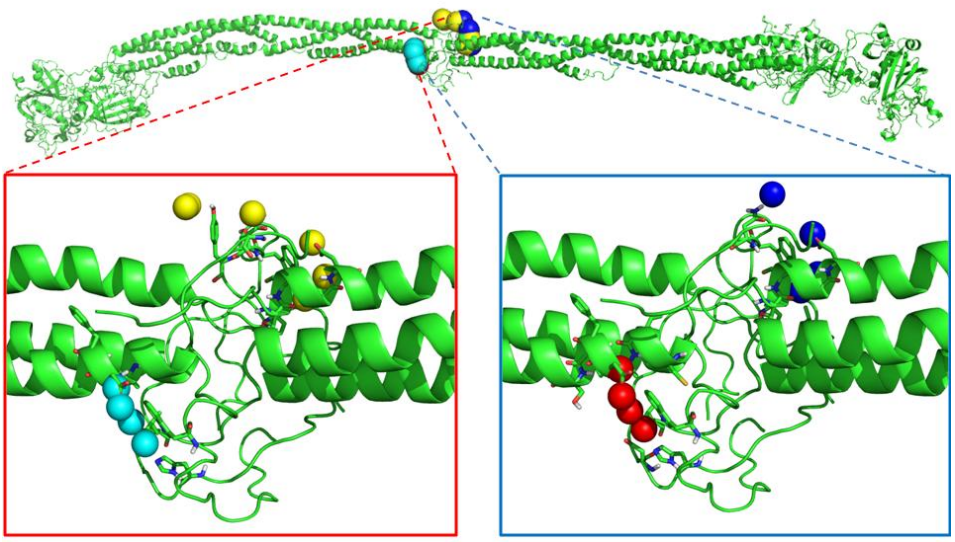


Figure 5-11 Predicted binding sites of Adhirons G4 and A2 to the α chain of fibrinogen

Red dashed lines indicate magnified image of G4 binding sites and blue dashed lines indicate magnified image of A2 binding sites. Yellow spheres are loop1 amino acids on G4, whilst cyan spheres are loop2. Red spheres are loop 1 amino acids on A2 and blue spheres loop 2.

Homology models of Adhiron G4 and A2 were produced using the SWISSMODEL[257] server based on the published Adhiron crystal structure (PDB code 4N6T). The predicted structure of loops 1 and 2 for each Adhiron were then docked to the central E region of the α chain of fibrinogen using the automated docking suite AutoDock[239]. For the docking calculations, the loop backbone was fixed and the amino acid side chains were allowed to be flexible. As illustrated in Figure 5-12, both G4 and A2 were predicted to bind within the central E region of fibrinogen, in close proximity to each other and in the same regions as predicted by Pepsite2.

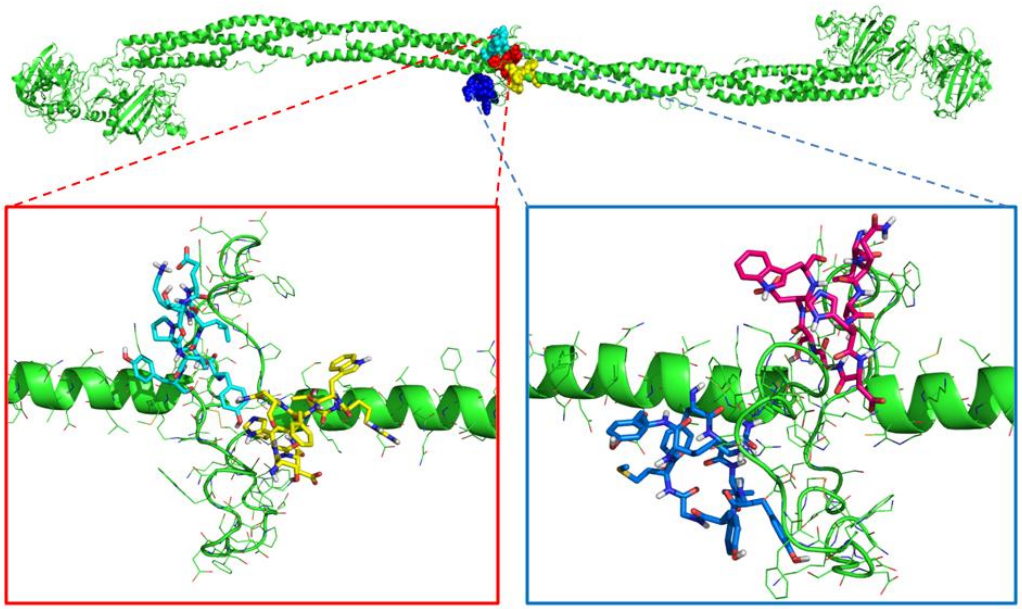


Figure 5-12 Predicted docking of Adhirons G4 and A2 to the α chain of fibrinogen.

Red dashed lines indicate magnified image of G4 binding sites and blue dashed lines indicate magnified image of A2 binding sites. Yellow sticks are loop1 amino acids on G4, whilst cyan sticks are loop2. Red sticks are loop 1 amino acids on A2 and blue sticks loop 2.

5.3 Discussion

This Chapter describes a novel technique to manipulate fibrin clot lysis using small conformational artificial peptides. The scale and quality of the Adhiron phage library permits rapid and reliable screening of a target protein with a large number of potential binding sequences of amino acids. This initial screening identified several Adhirons that bind to fibrinogen and are displaced by PI, and which are able to alter fibrin clot properties and fibrinolytic efficiency.

Of the 8 different Adhirons identified, I chose 3 Adhirons to investigate in more detail. The chosen Adhirons prolonged, reduced or had no effect on clot lysis time. Adhiron G4 was able to significantly enhance lysis of clots made from plasma or purified proteins in the presence of PI. Interestingly, the effect of Adhiron G4 on fibrin clot lysis was far more impressive when studied in fully mature clots (i.e. confocal microscopy) than during clot formation (i.e. turbidimetric assays). This is perhaps not surprising as PI incorporation into the clot during clot formation is limited due to the nature of the technique, whereas lysis conducted on mature clots allows more time for incorporation of PI into the clot allowing the “blocking effects” of G4 to become more obvious.

Western blot analysis confirmed that Adhiron G4 interacts with the α-chain of fibrinogen and molecular modelling predicts that G4-fibrinogen interaction occurs at the central E-domain. Factor XIII induced cross linking of PI to fibrinogen is known to occur at Lys303 within the αC domain of the α-chain. This flexible αC domain, containing Lys303, has not been successfully crystallised to date and so was not available for further interrogation using Pepsite2. Given that the α-chain contains over 600 residues and the C-terminus binds to the β-chain within the central E domain it may be difficult to explain the

PI-inhibitory effects of G4. A possible explanation is that as the αC domain flexes back from the D region towards the E region, the change in conformation brings Lys303 in closer proximity to the E domain than its sequence suggests. Although Adhiron G4 showed high binding affinity to fibrinogen, it failed to show significant incorporation into the clot. This may be related to the methodology used as fluorescent labelling of Adhiron G4 may have affected its ability to be incorporated into the clot. This, however, is unlikely given the successful incorporation of A2 into the clot (detailed below). Despite the failure to detect G4 in the clots, this Adhiron was still able to affect clot structure, resulting in the formation of thicker fibres, confirmed by scanning electron microscopy. There are two explanations for the discrepancy between the absence of G4 incorporation into the clot and an effect on structure. First, it is possible that the sensitivity of confocal microscopy was too low to detect low level of Adhiron incorporation. Second, Adhiron G4 may enhance lateral aggregation during fibrin clot formation, without incorporation into clots, through a mechanism that remains unclear but may involve interference with αC domain tethering to the E region or fibrinopeptide A and B release. This is supported by the molecular modelling results, predicting the binding of the three-dimensional structure of the two loops of G4 to the E region of the α chain. Given that PI is cross linked to the Lys303, it is likely that these effects are independent of PI and lysis is enhanced due to the generation of thicker fibrin fibres, which is known to facilitate plasmin activity.

The main aim of this Chapter was to validate the use of a phage display library to identify Adhirons capable of interfering with PI-fibrinogen interaction, prior to its use with C3. With this in mind it was envisaged that the Adhirons identified

would facilitate clot lysis, however, a number of Adhirons that prolonged clot lysis were also isolated, one of which was further studied in detail. Adhiron A2 acted in a similar manner to PI both in plasma and purified systems and showed high binding affinity to fibrinogen. Confocal microscopy demonstrated significant incorporation of Adhiron A2 into the clot, which was associated with structural changes in the fibrin network. The presence of Adhiron A2 resulted in the formation of clots with thinner fibres and denser structure, contributing to resistance of these clots to lysis[258]. Interestingly, FT of both purified and plasma clots was higher in the presence of Adhiron A2, consistent with the formation of thicker fibrin fibres. However, EM showed that the opposite is the case with Adhiron A2 resulting in the formation of compact clots with thinner fibres. This highlights an important deficiency of the turbidimetric assay and demonstrates that multiple techniques are necessary to properly study fibrin network structure. Molecular modelling predicted that the three-dimensional structures of the two loops in A2 may also bind to the E region within the α chain of fibrinogen, relatively close to the predicted binding sites of G4 loops. This may be difficult to explain given the different effects of both Adhirons on clot formation and lysis. If A2 is binding in this region, one explanation may be that it is having the opposite effect to that of G4 in terms of fibrinopeptide A and B release and αC disengagement, thus reducing lateral aggregation of protofibrils and forming a clot composed of thinner fibrin fibres that is more resistant to lysis. Another possibility is that the presence of A2 within the fibrin structure directly affects the plasmin degradation of fibrin fibres.

Both Adhiron G4 and A2, modulating clot lysis, showed binding to the α-chain of fibrinogen, which is established to be important for interaction with other

proteins. In contrast, Adhiron G2, which had no significant effect on clot structure or lysis, demonstrated binding to the γ-chain of fibrinogen. One criticism that may be directed at this finding is the absence of interaction sites between PI and the γ-chain of fibrinogen, questioning the release of Adhiron G2 by PI. This may be due to the presence of PI interaction sites on the γ-chain of fibrinogen, which have not been described before. However, this is unlikely and the more plausible explanation is related to “spatial displacement” of the Adhiron by PI, which can occur despite different binding areas on the fibrinogen molecule. Further work is required to accurately identify interaction sites between relevant Adhirons and fibrinogen. Given the likely conformational nature of the interactions, linear peptide studies will not be useful and site directed mutagenesis offers a tool to pinpoint binding sites, which will lead to identification of novel therapeutic targets.

The identification of the Adhirons that bind fibrinogen and are released by excess PI certainly validates the use of the phage library to target the fibrin structure in an attempt to modulate clot formation and lysis. Not only was one capable of reducing the anti-fibrinolytic actions of PI, as was the intention at the outset, but an Adhiron that appeared to have an agonistic effect on PI was also discovered. These Adhiron sequences may be used to generate constrained peptides capable of interfering with fibrinogen-PI interaction and ultimately small molecules for use therapeutically to both facilitate clot lysis, as in CVD, and inhibit fibrinolysis in individuals with bleeding disorders or following major trauma. An analogue of another fibrinolytic inhibitor, PAI-1, which has been modified to have an extended half life, has been trialled as a topical therapy to minimise bleeding[40] and this may be a potential use of Adhirons such as A2.

The results are encouraging and provide sufficient evidence to justify the use of the same phage library to screen for binders to fibrinogen that are released by C3 and also binders to C3, released by excess fibrinogen.

Chapter 6

Interference with C3-fibrinogen interactions as a novel methodology to modulate clot lysis

Introduction

The previous chapter was largely aimed at assessing the validity of a phage display library as a method of identifying fibrinogen-specific Adhirons that can modulate clot formation, structure and lysis. The discovery of several Adhirons that interfere with dynamic clot turbidity experiments certainly emphasise the potential use of this technique. Given the lysis prolongation that occurs with C3, and its exaggeration in diabetes samples, it was hoped that Adhirons can be identified that act at fibrinogen-C3 interaction sites and form the basis of future novel, diabetes specific, therapeutic agents for use in CVD.

Agents that inhibit different components of the complement system have been trialled in CVD with limited success. A C5 monoclonal antibody, pexelizumab, has been used following myocardial infarction (MI) and coronary artery bypass grafting with inconsistent reductions in mortality and major cardiac events[216-218]. Inhibition of the C1 esterase enzyme appears to reduce myocardial reperfusion injury but this has not been translated into improved mortality outcomes post MI[219;221]. It is intuitive that the complement and coagulation pathways are so closely linked for example following vascular injury, localising components such as C3 to the site of injury thus preventing external pathogens from entering the blood stream[259;260]. This cross-communication can also have deleterious effects in other circumstances, such as the prolongation of clot lysis mediated by the presence of C3 within the clot. Specific targeting of these effects, rather than systemic, non-specific inhibition of complement proteins, may prove increasingly beneficial.

In the previous Chapter, I have shown that Adhirons can be used to modulate PI-induced prolongation of clot lysis. In this Chapter, I attempt to interfere with

C3-mediated prolongation of fibrinolysis using Adhirons. I try to further refine the technique by implementing a dual approach to investigate i) fibrinogen-binding Adhirons that are released by excess C3 and ii) C3 binding Adhirons that are released by excess fibrinogen. Identifying C3-specific fibrinogen binding Adhirons may help to block C3 binding to fibrinogen, and hence abolish the effect of this protein on clot lysis. On the other hand, isolating fibrinogen-specific C3-binding Adhirons may offer a tool to absorb out C3 and prevent the protein from binding to fibrinogen.

6.1 Methods

6.1.1 Adhiron identification

The Adhiron phage library was screened against biotinylated fibrinogen as discussed in Chapter 2, and after three rounds of panning the bound phage were eluted by addition of C3 (0.5mg/ml) to identify phage binding at the fibrinogen-C3 interaction site. The screening was also performed in reverse, by incubating the phage library with biotinylated C3 and eluting bound phage with excess fibrinogen (1mg/ml) following the first three rounds of panning.

6.1.2 Adhiron production

The selected Adhirons were produced as described in Chapter 5.

6.1.3 Turbidimetric analysis

Turbidimetric analysis was used as a high throughput assay to investigate the role of Adhirons on plasma clot lysis. These were performed as described in Chapter 2 and also in Chapter 5 following the addition of Adhirons to plasma and fibrinogen. To investigate the effects of the Adhirons released from fibrinogen by excess C3 on prolongation of lysis, human C3 (1.34μM) was added to fibrinogen that had been pre-incubated with the Adhiron of interest as described for PI in Chapter 5.

6.1.4 Laser scanning confocal microscopy

This technique was used to investigate lysis of mature clots in real time and to study major differences in the structure of hydrated clots. Fibrin networks were prepared as described in Chapter 2, using the same microscope settings. The addition of Adhirons to plasma and purified clots was performed as described in Chapter 5.

6.1.5 Scanning electron microscopy

To analyse the effects of Adhirons on the ultrastructure of fibrin networks, clots were viewed under an EM after preparation as described in Chapters 4 and 5.

6.1.6 Molecular modelling

Predicted binding sites of the random peptide sequences constrained within the two loops of Adhirons of interest were generated using the website pepsite2®, as described in Chapter 2. Images were viewed and produced using Pymol software.

6.1.7 Statistical analysis

Between group comparisons of normally distributed variables in the in-vitro studies were carried out using independent samples student t-test. Comparisons of non-normally distributed variables were carried out using Mann-Whitney U tests. Data are presented as mean±SEM unless otherwise stated

6.2 Results

6.2.1 Fibrinogen binding Adhirons released by C3

The Adhiron library was screened against immobilised fibrinogen in the same manner as section 6.3.2, but in order to identify Adhirons that may be binding at fibrin-C3 interaction sites, excess C3 was added to elute the bound phage from fibrinogen. In total 16 different colonies were selected to be sequenced (as in section 5.2.1), revealing 8 different sequences that were available for analysis (Table 6-1).

Adhiron	Loop 1	Loop 2
A2	Y********	K********
A5	Y********	********
A6	M********	H********
C1	D********	Y********
C2	V********	K********
C6	K********	K********
D2	Y********	Q********
F5	I********	K********
G2*	G********	H********
G6	Y********	Q********

Table 6-1 Fibrinogen binding Adhirons that were released by excess C3

Due to potential patent considerations only the first amino acid for each loop sequence is indicated

6.2.1.1 Effect on clot formation and lysis

Plasma experiments: In this set of experiments, time to 50% clot lysis was 564±24sec following the addition of the scaffold Adhiron to pooled control

plasma. Two Adhirons had striking effects on clot formation and lysis (Figure 6-1). Adhiron G2* (to differentiate from Adhiron G2 that was released by the addition of PI in Chapter 5 will be presented as G2*) markedly reduced the lag phase and the clot produced was not lysed during the 2 hours, remaining at its maximum absorbance. Adhiron C2 also produced unusual results in that there was no change in the absorbance in this well, suggesting that either no clot was formed or that lateral aggregation was inhibited, thus preventing the exponential rise in absorbance typically observed. Of the remaining 6 Adhirons, their effects on LT were more subtle, with Adhirons A2, A5, A6 and G6 increasing LT to 664±29sec, 858±68sec, 646±25sec and 656±24sec respectively ($p<0.05$ for all). Three Adhirons had little effect on clot lysis (C6 550±31sec, D2 602±15sec, F5 635±24sec, $p>0.1$ for all compared with scaffold only Adhiron, (Figure 6-1).

In purified experiments the effects of this group of Adhirons were less dramatic, indicating possible interactions with plasma proteins. The control clots (fibrinogen and scaffold protein) produced LT of 390±8sec which was not significantly altered by the addition of Adhirons A2, A6, C6, D2 and G6 (LT 380±32sec, 354±24sec, 362±20sec, 358±26sec, 366±35sec respectively). Adhirons A5 and F5 significantly reduced LT to 306±19sec and 306±16sec respectively ($p<0.05$ for both). Both C2 and G2* formed clots in these experiments and these were fully lysed within the allotted time but with significantly increased LT of 805±105sec and 1311±171sec respectively ($p<0.05$ for both).

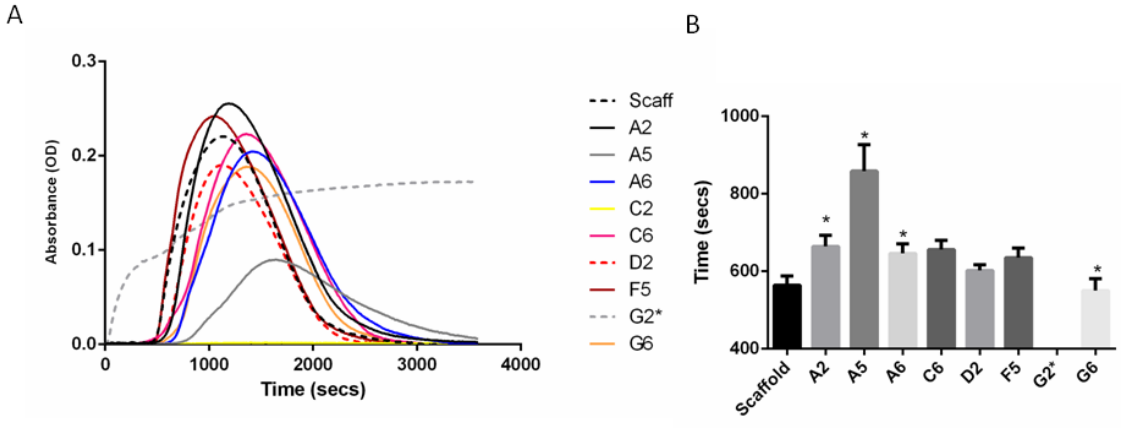


Figure 6-1 Clot lysis parameters of plasma clots formed with fibrinogen binding Adhirons (released by C3) as measured by turbidimetric assay

*Turbidity and lysis curves of fibrinogen binding Adhirons, released by C3, in plasma (A) and the LT (mean±SEM) of these Adhirons in plasma (B). Control plasma was pre-incubated with Adhirons followed by the addition of a lysis mix containing tPA and an activation mix containing thrombin and $CaCl_2$. A total of 3 independent experiments were performed in duplicate .*p<0.05.*

6.2.2 Selection of Adhirons for further analysis

Similarly to the experiments with PI-related Adhirons, I chose 3 Adhirons to study in detail based on i) sequence homology with C3, ii) an effect on clot lysis. Adhiron A6 was chosen as loop 2 shared a sequence homology with a spatially exposed portion of C3, whereas, Adhiron G2* consistently produced a drastic effect on clot formation and prevented fibrinolysis within the allocated time. A third Adhiron, C6, was chosen due to its negligible effect on clot lysis to act as a control alongside the scaffold Adhiron.

6.2.3 Adhiron A6

As mentioned, the sequence of amino acids within loop 2 of Adhiron A6 shared a sequence homology with residues 38-47 of C3 (Figure 6-2). It was tempting to hypothesise therefore that this portion of C3 may be involved in fibrin-C3 interaction and thus A6 warranted further study.

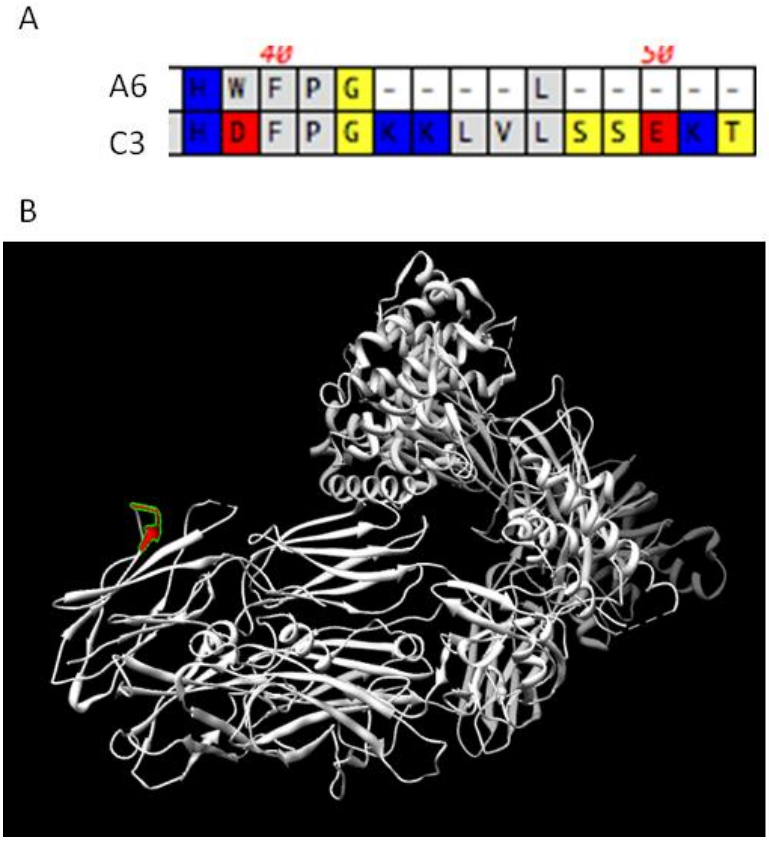


Figure 6-2. Sequence homology between Adhiron A6 and complement C3

Loop 2 of Adhiron A6 that shares a sequence homology with a portion of C3 (A) and the position of this portion of C3 within the crystal structure (B)

6.2.3.1 The effect of A6 on C3 mediated lysis prolongation

Experiments were designed to study the effect of A6 on LT in a purified setting using purified commercial fibrinogen (Calbiochem), in the presence and absence of C3. In the absence of A6, C3 prolonged LT from 644±13secs to 728±25 secs. By pre-incubating A6 with fibrinogen, before the addition of C3 to

the mixture, it was possible to completely abolish the prolongation of LT observed with C3 alone, LT 632±24 secs ($p<0.05$, Figure 6-3).

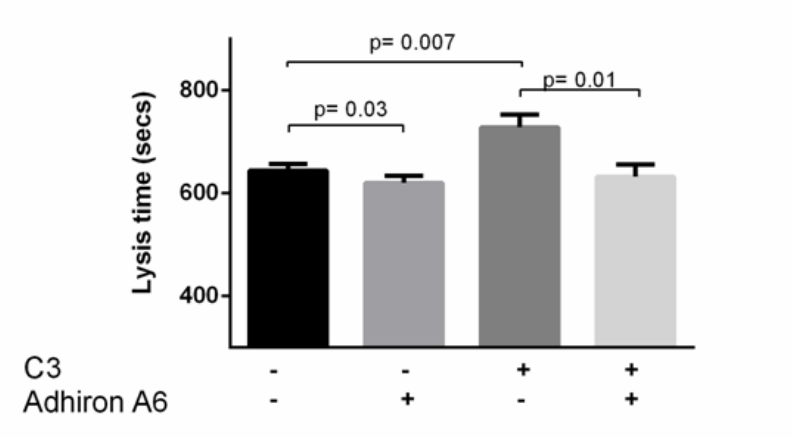


Figure 6-3 Effect of A6 on LT in the presence and absence of C3

Pre-incubation of A6 with fibrinogen prior to adding C3 to the mixture abolishes C3 induced prolongation of lysis. Purified fibrinogen was pre-incubated with A6 or C3 and in a separate experiment followed by addition of either Tris buffered saline or C3 to fibrinogen-A6 mixture .Experiments were performed in duplicate on 3 separate occasions and results expressed as mean±SEM.

6.2.3.2 Effect on clot formation and lysis

As described previously, A6 actually caused a prolongation of LT in plasma which is unexpected given its performance in reducing C3 mediated prolongation of lysis. If A6 is binding to fibrinogen at a relevant site of interaction between fibrinogen and C3 then it may be expected to see a reduction in LT with the addition of A6.

As described in Chapter 3, there is a wide inter-individual variability in the prolongation of lysis following addition of C3, which may relate to the degree of C3 incorporation into the clot and to the presence of glycation. Given that pooled healthy plasma was used in the initial turbidimetric screening assays, I

conducted further experiments using individual plasma samples in the presence and absence of Adhiron A6, (6 non-diabetes, 6 diabetes patients and a pool of both). Of the 14samples, 11 had a significant increase in LT, with 2 only of the diabetes patients producing a reduction in LT (Figure 6-4). Analysing the two groups separately (diabetes and non-diabetes), the non-diabetes group had a significant increase in LT following addition of A6 (529±16secs vs 612±36secs, $p<0.05$) with very little difference observed with the diabetes group (663±49secs vs 685±57secs, $p>0.5$).

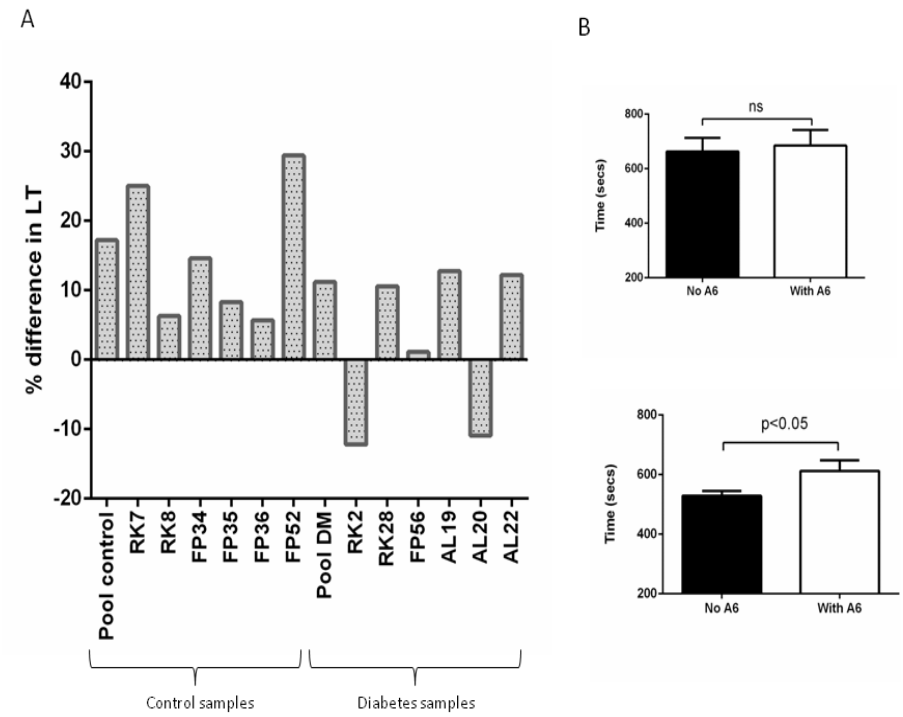


Figure 6-4 Effect of Adhiron A6 on LT in individual samples

The percentage difference in LT (A) following the addition of A6 to individual plasma samples (6 diabetes and 1 pooled sample along with 6 non-diabetes and 1 pooled sample). (B) The mean (±SEM) LT for the diabetes group (top) and non-diabetes group (bottom) with and without A6.

6.2.3.3 Effect of A6 on clot structure

There was no difference in the appearance of plasma clots prepared for LSCM with the addition of scaffold, Adhiron A6 or C6 (Figure 6-5). The mean fibrin fibre thickness was also similar between the 3 groups (106.8±2.2nm, 104.9±2.2nm and 109.2±2.2nm $p>0.05$).

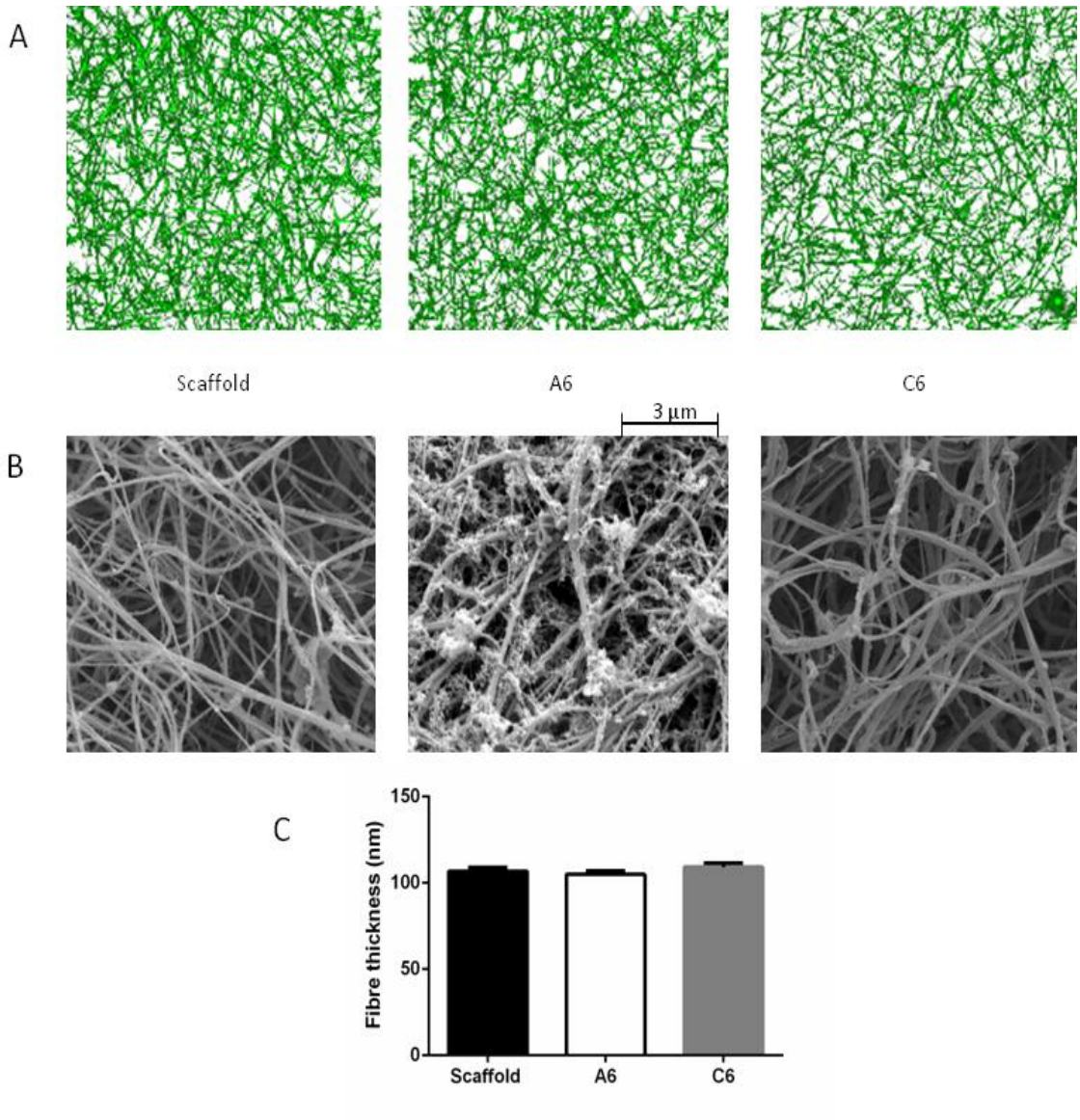


Figure 6-5. LSCM and EM images of plasma clots prepared with Adhirons A6 and C6 with EM fibre thickness.

*(A) LSCM images of plasma clots and (B) EM images of plasma clots, all prepared by the addition of scaffold, A6 and C6 to control plasma. There was no difference in mean (±SEM) fibre diameter between the 3 different clots (C). 20 fibres were measured from different areas of at least 5 different EM images of each Adhiron. *p<0.05.*

In purified experiments, there was again no observed difference between LSCM clots with the addition of scaffold, A6 or C6 (Figure 6-6). Mean fibrin fibre diameter measured from EM clots following the addition of scaffold was 85.9±2nm as mentioned previously and this was also not affected by the addition of A6 or C6, 86.6±1.6nm and 85.3±1.5nm respectively ($p>0.5$ for both). The lack of effect of Adhiron A6 on fibrin network architecture in both plasma and purified experiments suggests that its effect in turbidimetric studies are related to the interaction between the fibrin-A6 complex and surrounding proteins such as C3.

6.2.3.4 Predicted binding of A6 to fibrinogen

Following the results of the microarray screening of C3 and fibrinogen indicating that C3 is likely to bind to portions of the β chain of fibrinogen (as discussed in Chapter 4), the sequences for the two random loops of A6 were individually entered into the Pepsite2 website to predict where A6 might be binding to the β chain of fibrinogen (PDB code 3GHG). The strongest binding prediction for the two loops on the β chain was adjacent to the C-terminal peptide sequences (peptides B and C) identified by the microarray screening (Figure 6-7).

Re-analysing the predicted binding sites of peptides B and C on C3b not only demonstrates that they are predicted to bind in close proximity to the portion of C3b that shares a sequence homology with Adhiron A6 (Figure 6-8), but also to a predicted binding site identified by the initial AutoLigand screening (indicated by the grey spheres in Figure 4-6). This commonality strengthens the evidence that this area of C3b may be a genuine, additional interaction site with fibrinogen, in particular the β chain.

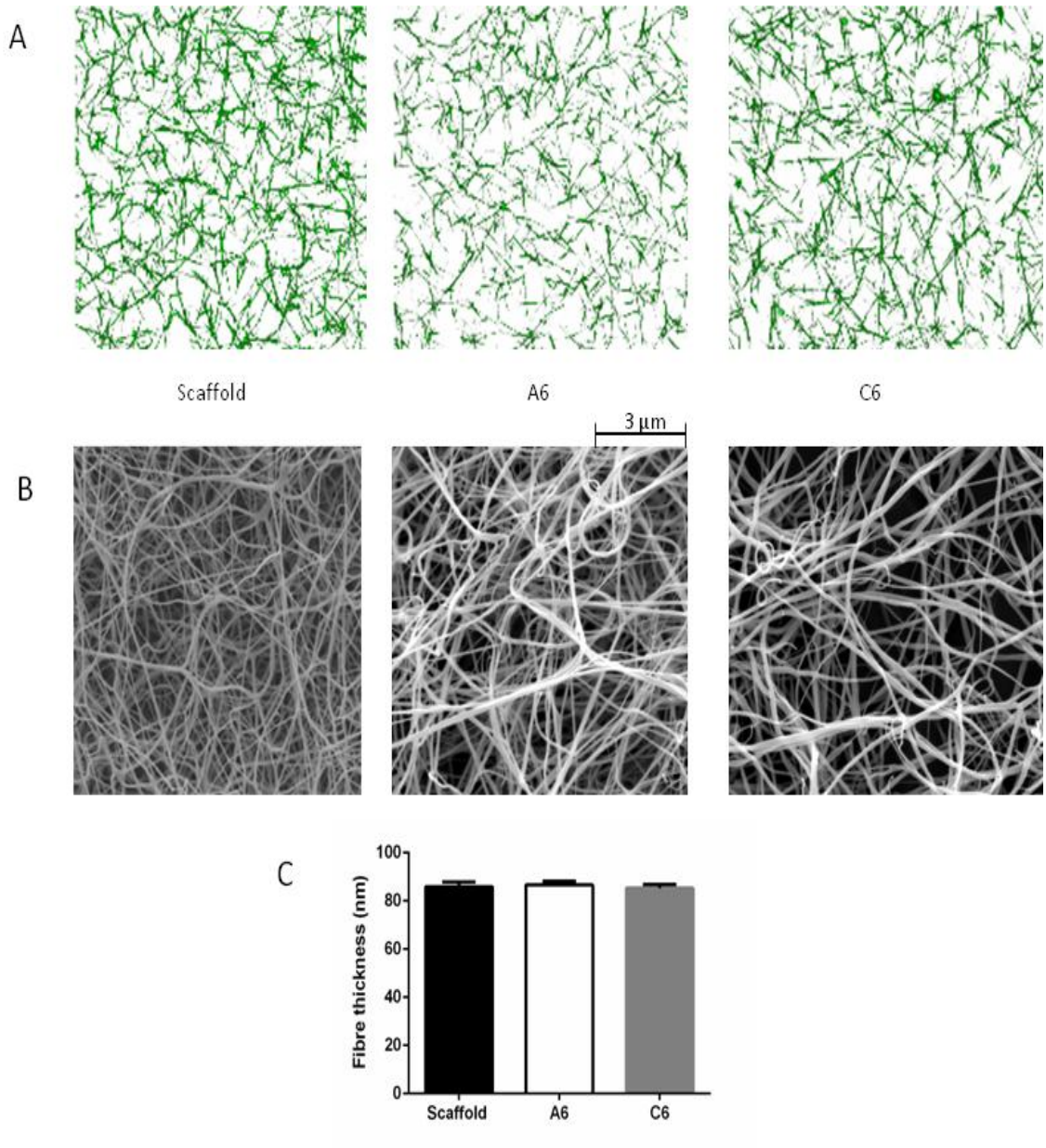


Figure 6-6 LSCM and EM images of purified clots prepared with Adhirons A6 and C6 with EM fibre thickness.

*(A) LSCM images of purified clots and (B) EM images of purified clots prepared by the addition of scaffold, A6 and C6 to purified fibrinogen. There was no difference in mean (±SEM) fibre diameter between the 3 different clots (C). 20 fibres were measured from different areas of at least 5 different EM images of each Adhiron. *p<0.05.*

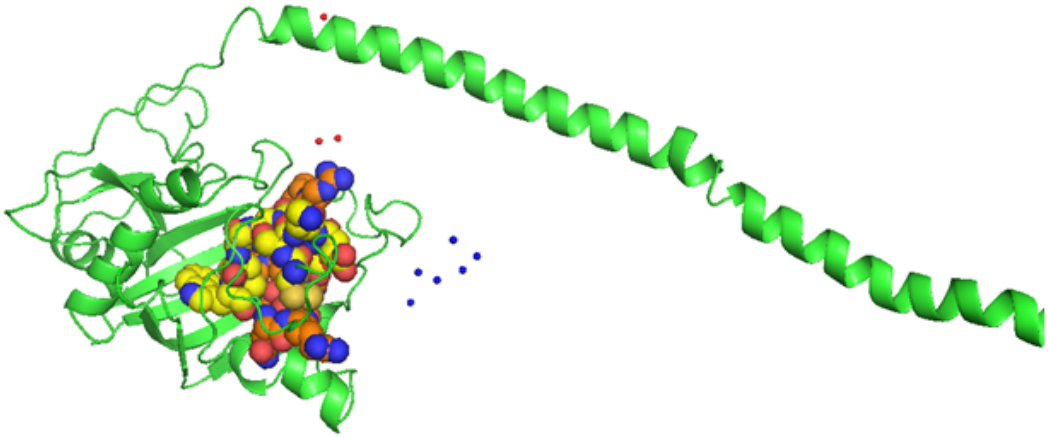


Figure 6-7 Predicted binding of A6 to fibrinogen

The predicted binding areas of A6 (loop 1 red, loop 2 blue) within the β chain of fibrinogen (PDB code 3GHG) as determined by the website pepsite2. The multicoloured spheres within the fibrinogen molecule represent peptides B and C, identified by the microarray screening as potential fibrinogen-C3 interaction sites.

6.2.4 Adhiron G2*

From the initial turbidity and lysis experiments with the Adhirons released by C3 it was evident that Adhiron G2* had a drastic effect on clot formation at the fibrinogen:Adhiron molar ratio of 1:5. The extent of the changes made it an ideal candidate to study in further detail. Its effect on clot formation, lysis and structure were assessed at different concentrations in plasma and purified settings attempting to mirror fibrinogen:Adhiron molar ratios of 1:5, 1:0.5 and 1:0.05 in plasma and 1:2, 1:0.2 and 1:0.02 in purified experiments.

Figure 6-8 Predicted binding of fibrinogen β chain peptides on C3b

The predicted binding sites (from Pepsite) of peptides B (pink spheres) and C (orange spheres) from the microarray screening on C3b are in close proximity to the portion of C3(b) that shares a sequence homology with Adhiron A6 (blue spheres).

6.2.4.1 Effect on clot formation and lysis

The first thing to note from the plasma experiments is the dramatic reduction in lag phase, which suggests that it is interacting at a site involved with fibrinopeptide A release. It is clear that lateral aggregation is permitted given the sudden rise in absorbance, but the lack of a lag phase implies that elongation and protofibril formation is perhaps diminished (Figure 6-9). At the greatest concentration, the clot is not lysed after the allocated time, and the final turbidity or maximal absorbance is reduced compared to the scaffold Adhiron (0.1750±0.02, 0.2598±0.0, $p<0.05$). Dilution of Adhiron G2* corrects the effect

on the lag phase and the clot is broken down to 50% of its final turbidity (LT 1493±51secs, $p<0.05$). Unexpectedly, however, reducing the concentration by 10 fold to 3μM lowers the final turbidity even further to 0.0625±0.005.

At the lowest concentration of 0.3μM, equal to Adhrion:fibrinogen molar ratio of 1:20, the effects of G2* are eventually lost, with all parameters of LT, FT and lag phase similar to the scaffold Adhiron (LT 957±86secs $p=0.3$, FT 0.2335±0.003, $p=0.06$).

In purified experiments, the dose response of G2* was similar to plasma (Figure 6-9). It is difficult to be certain whether the effect on lag phase transfers to the purified setting as the exponential increase in absorbance that occurs with lateral aggregation begins far quicker than in plasma, although there is a suggestion that at the highest concentration (6μM) the lag phase is reduced. The highest concentration is lysed in the purified experiments and the LT normalises with the reduction in concentration (1569±138sesc, 609±77secs, 414±11secs, and scaffold 492±49secs). A similar trend to plasma is noted with regard to FT of the clots. The highest concentration again has a slightly lower FT than the scaffold Adhiron (0.126±0.02 vs 0.1908±0.02 $p=0.13$), and this is reduced further still with a 10 fold reduction in G2* concentration (0.049±0.01, $p<0.05$). The final and lowest dilution of G2* produces a curve that is similar to the scaffold, and the apparent lower FT was not significant (0.1558±0.007, $p=0.4$).

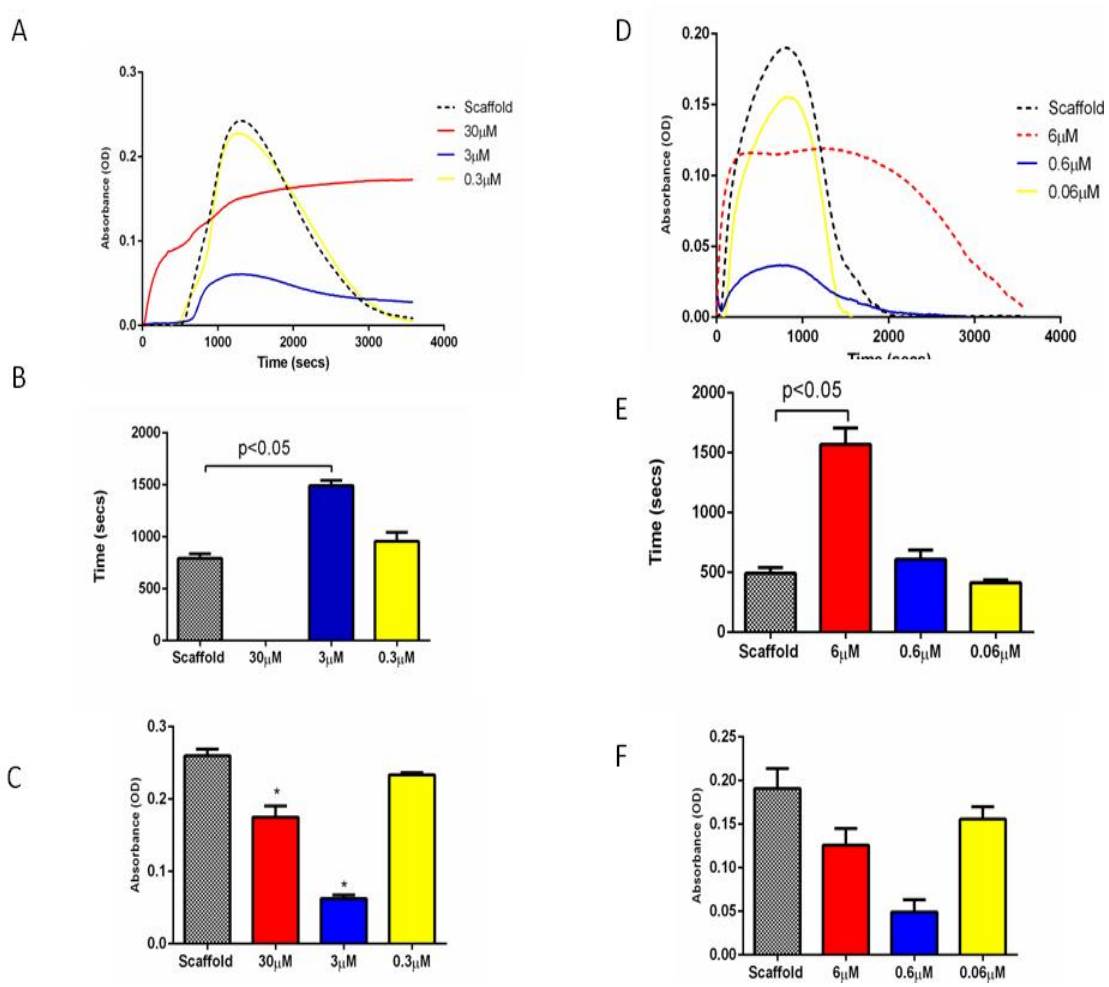


Figure 6-9 Clot lysis parameters of clots formed with varying concentrations of Adhiron G2* as measured by turbidimetric assay

A-C Turbidity and lysis traces, lysis times and final turbidity from G2 at 30μM, 3μM and 0.3μM in plasma and D-F at 6μM, 0.6μM and 0.06μM in purified system. The absence of the red bar in figure B is due to the fact that the clot did not lyse within the allotted time at this concentration of Adhiron G2* (30μM). Clots were prepared by the addition of G2* at varying concentrations to control plasma or purified fibrinogen. A total of 3 independent experiments were performed in duplicate. *p<0.05. Results are presented as mean±SEM.*

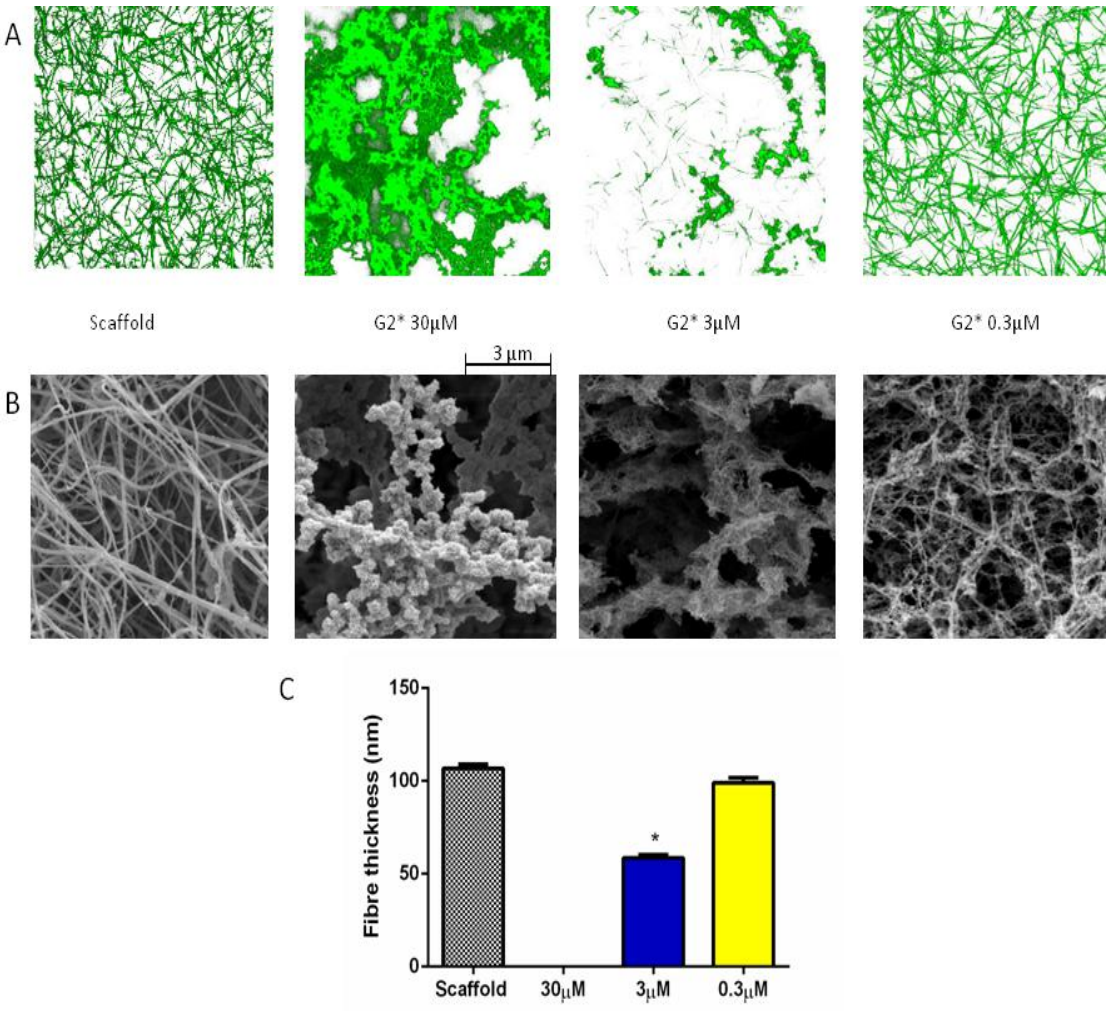


Figure 6-10. LSCM and EM images of plasma clots prepared with varying concentrations of Adhiron G2* with EM fibre thickness and final turbidity.

(A) LSCM images of plasma clots prepared in the absence and presence of G2 at 30µM, 3µM and 0.3µM respectively. (B) EM images of plasma clots in the absence and presence of G2* at the same molar concentrations (C). Mean (±SEM) fibre thickness of EM clots at 3µM and 0.3µM. It was not possible to measure the fibrin fibres at the highest concentration of 30µM hence the empty bar. Twenty fibres were measured from different areas of at least 5 different EM images of each Adhiron. *p<0.05.*

6.2.4.2 Effect on clot structure

In keeping with the dramatic effect that G2* had on clot LT and final turbidity, significant structural changes were noted in the fibrin network following the addition of G2* to plasma and fibrinogen (Figure 6-10). In plasma and at 30µM clot architecture is greatly affected. The confocal and EM images demonstrate that fibrin fibres appear to be clumped together in dense areas. Such is the extent of this clumping it was not possible to measure the individual fibrin fibre diameter from the EM images at this concentration. At the middle concentration (3µM), the fibrin fibres are a little more distinct and thus the diameter can be measured, showing a significant reduction in diameter compared to the scaffold Adhiron (58.56±1.6nm, 106.8±2.2nm $p<0.05$), which fits with the reduced FT observed at this concentration.

The areas of clumping appear less dense on the Z-stack images compared to the highest concentration and would explain the higher FT observed at 30µM. The lowest concentration of G2* produces a clot that is much more recognisable in terms of fibrin fibres and overall structure. The mean fibrin fibre diameter at 0.3µM was 99.09±2.7nm ($p=0.05$) suggesting that even at this relatively low concentration G2* is still able to interfered with fibrin clot formation.

In the purified experiments the same trend was observed in terms of disruption to clot structure (Figure 6-11). At 6µM again the fibrin fibres could not be reliably measured and the clumping of fibrin bundles appeared denser than at 0.6µM. The average fibrin diameter was significantly reduced by 0.6µM (56.65±1.6nm vs 85.86±2nm, $p<0.001$) and although, as with the plasma clots, 0.06µM of G2*

produced a much more normal clot, the fibrin fibres were still significantly thinner than scaffold Adhiron (73.17±1.4nm p<0.001).

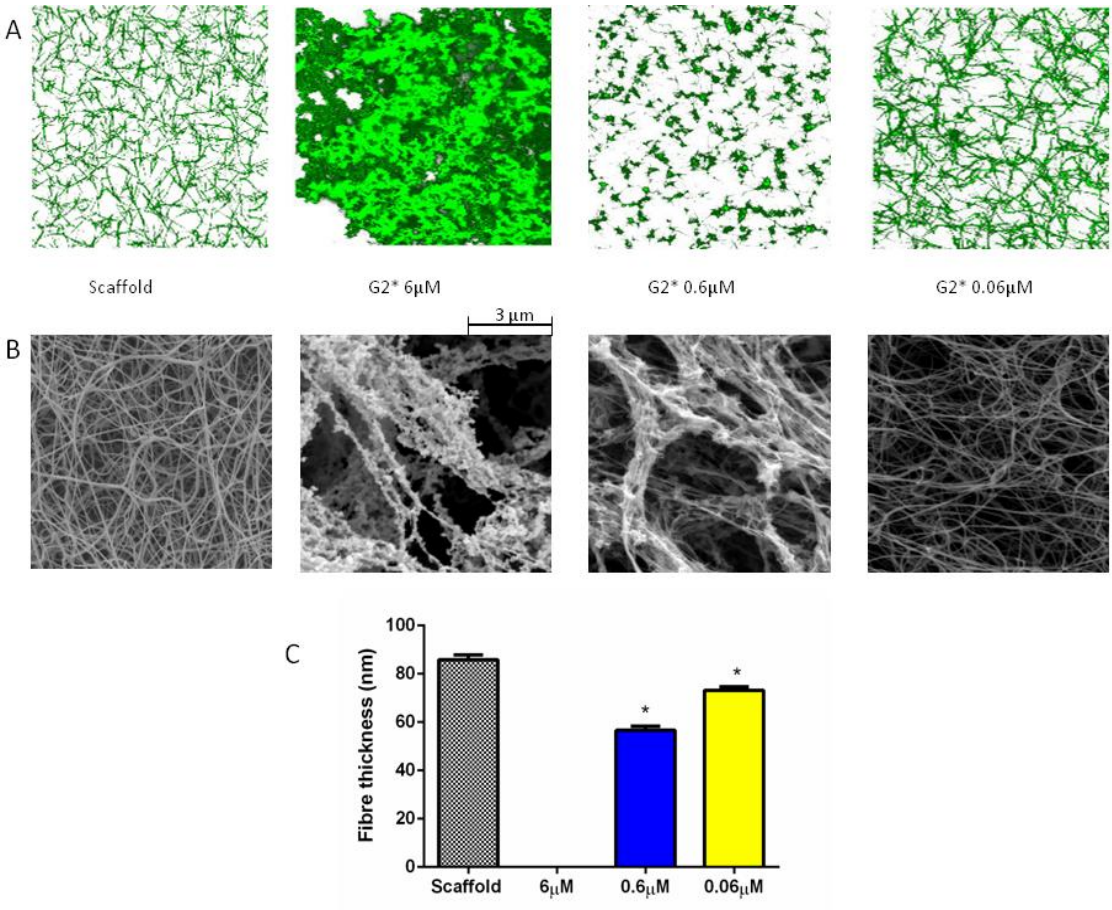


Figure 6-11. LSCM and EM images of purified clots prepared with varying concentrations of Adhiron G2* with EM fibre thickness and final turbidity.

(A) LSCM images of purified clots prepared in the absence and presence of G2 at 6µM, 0.6µM and 0.06µM respectively. (B) EM images of purified clots in the absence and presence of G2 at the same molar concentrations. (C) Mean (±SEM) fibre thickness of EM clots 0.6µM and 0.06µM. It was not possible to measure the fibrin fibres at the highest concentration of 6µM, hence the empty bar in (C).20 fibres were measured from different areas of at least 5 different EM images of each Adhiron. *p<0.05.*

To ensure that the unusual clots observed at the highest concentration were in fact a fibrin clot network, real time lysis of the confocal clots was performed. The clots did indeed lyse but without the presence of a “lysis front” typically observed when lysis is preformed under visualisation with the LSCM (Figure 6-12). Instead the lysis mix appears to attack the clot from all sides, presumably as it flows through what appear to be large pores within the clot. As expected following the turbidimetric experiments, LT is prolonged significantly.

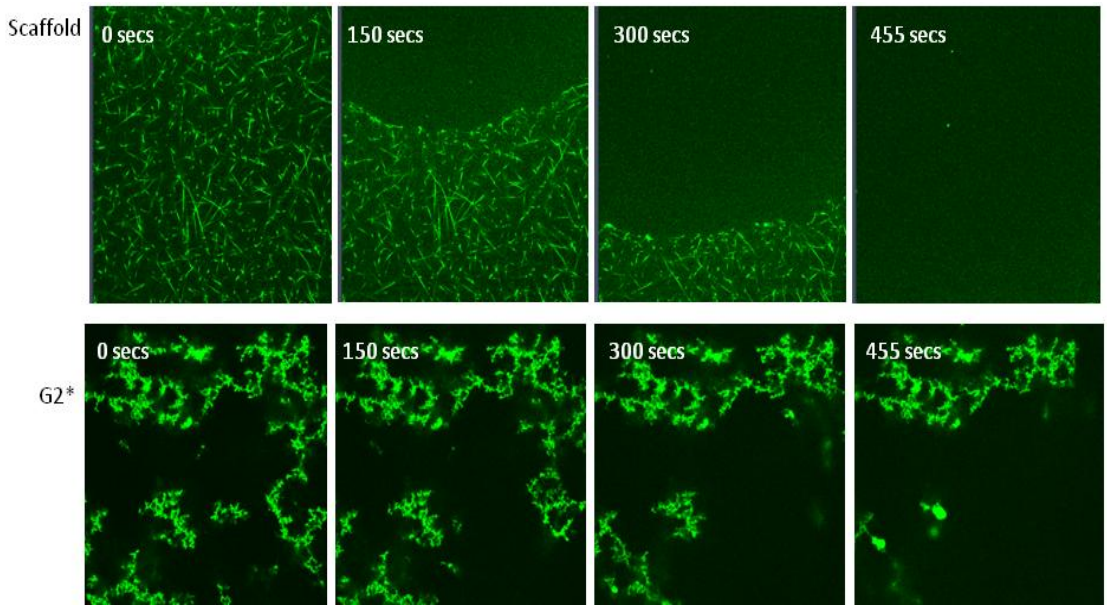


Figure 6-12 Real time lysis of Adhiron G2*

The top image illustrates the movement of a lysis front through a control clot (fibrinogen and scaffold), whereas the bottom image no lysis front is observed following the addition of Adhiron G2 to fibrinogen (images are all in the same orientation) and the LT of the clot is much longer. Clots were prepared by incubating control plasma with Adhiron and 488 Alexa labelled fibrinogen followed by the addition of thrombin and CaCl2. Clot lysis was initiated by the addition of a lysis mixture containing tPA and plasminogen to the edge of the clot.*

6.2.5 C3 binding Adhirons

The Adhiron phage library was screened against immobilised C3. Fibrinogen was added in excess to the display to out compete Adhirons at the C3-fibrinogen interaction site. Following phage ELISA and sequencing of 8 different colonies, 4 C3 binding Adhirons with different sequences were identified and available for analysis (Table 6-2).

Adhiron	Loop 1	Loop 2
G7	N********	L********
A7	V********	N********
C8	T********	N********
B3	S********	T********

Table 6-2 C3 binding Adhirons that were released by excess fibrinogen

Due to potential future patent considerations only the first amino acid from each loop sequence is indicated.

6.2.5.1 Effect on clot lysis

Of the 4 C3 binding Adhirons, only one (B3) demonstrated any effect upon the turbidimetric assays in both plasma and purified settings. The resulting turbidity and lysis curves and lysis times can be seen in Figure 6-13. In a plasma system Adhiron B3 significantly prolonged LT (3419±278secs vs 870±73secs, $p<0.05$). There was no effect on LT from the 3 remaining Adhirons

6.2.6 Selection of Adhirons for further analysis

As with the previous groups of Adhirons, a limited number were chosen for further analysis based on i) sequence homology and ii) effect on clot lysis. None of the 4 C3 binding Adhirons shared sequence homolgy with fibrinogen. Adhiron B3 was the only one to effect clot formation and lysis and was studied along with G7 to act as a control with the scaffold Adhiron.

Confocal microscopy was used to further test the effect of these Adhirons on clot lysis by studying the real time lysis of mature plasma clots in the presence and absence of B3 and G7. The addition of Adhiron B3 to plasma significantly slowed the rate of clot lysis compared to the scaffold alone, whereas G7 had no

effect on LT (Figure 6-14) which was in agreement with the initial turbidimetric assays.

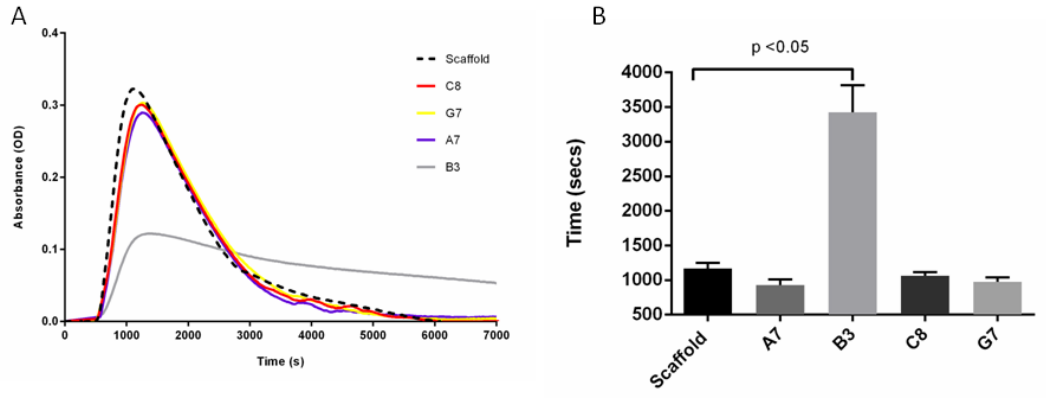


Figure 6-13 Clot lysis parameters of clots formed with C3 binding Adhirons as measured by turbidimetric assay

Turbidity and lysis curves of C3 binding Adhirons in plasma (A) and the LT (mean±SEM) of the Adhirons in plasma (B). Control plasma was pre-incubated with Adhirons followed by the addition of a lysis mix containing tPA and an activation mix containing thrombin and $CaCl_2$. A total of 3 independent experiments were performed in duplicate. *p<0.05

6.2.6.1 Effect on clot structure

Clots prepared for scanning electron microscopy using pooled healthy plasma incubated with scaffold Adhiron produced fibrin fibres with an average thickness of 106.8±2.2nm which was reduced to 64.9±1.5nm by Adhiron B3 (p<0.001). No significant difference in fibre diameter was observed with Adhiron G7 (102.4±2.4nm, p=0.07). Comparisons of Z-stack images of plasma clots prepared with Adhirons G7, B3 and scaffold demonstrated a macroscopic alteration in the structure of the clots, with the B3 clots appearing denser than the other two (Figure 6-15).

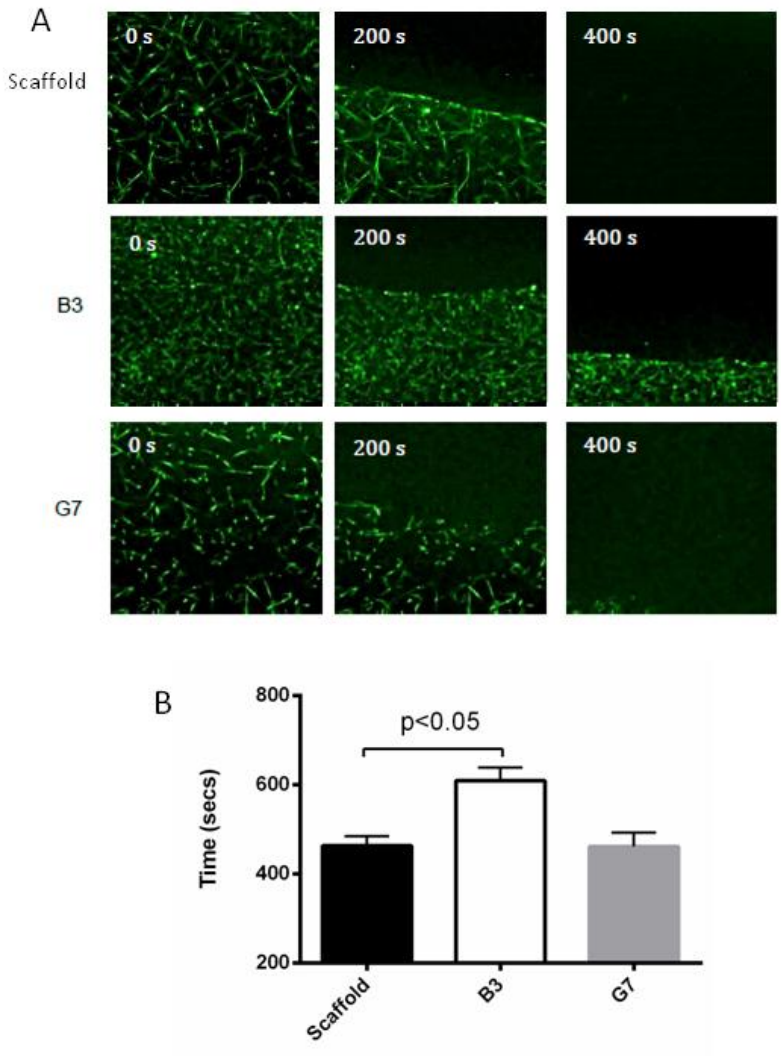


Figure 6-14 Real time lysis of plasma clots in the presence of Adhirons B3 and G7

LSCM images of real time lysis of clots prepared with scaffold, B3 and G7 (A) demonstrating prolonged lysis following incubation with B3 compared to scaffold. The LT (mean±SEM) from the experiments (B) is significantly prolonged with B3. Clots were prepared by incubating control plasma with Adhiron and 488 Alexa labelled fibrinogen followed by the addition of thrombin and CaCl2. The LT represents the time taken for a lysis mixture containing tPA and plasminogen to completely dissolve the clot within the viewing field. Experiments were performed in duplicate on 3 separate occasions.

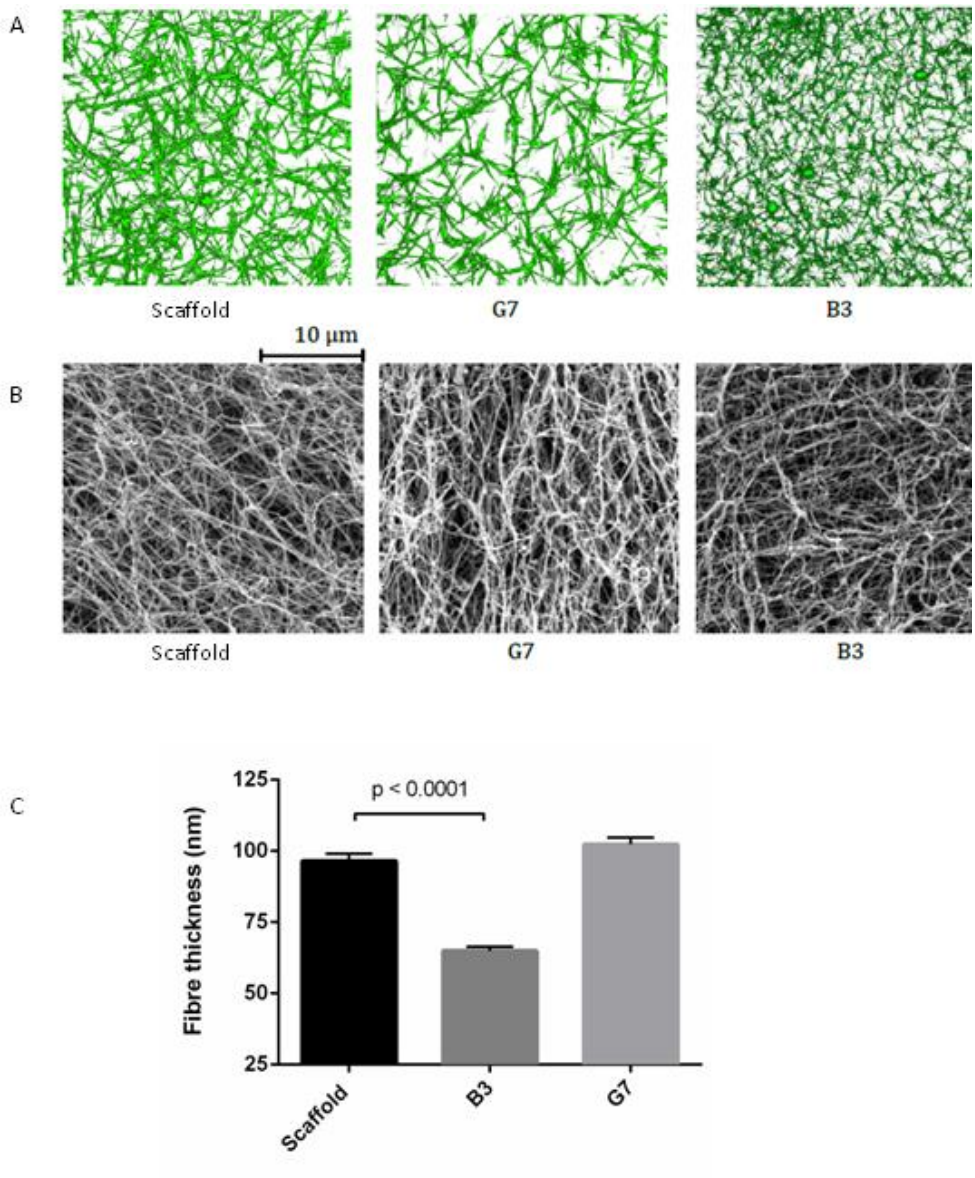


Figure 6-15 LSCM and EM images of plasma clots prepared with Adhirons G7 and B3 with EM fibre thickness.

(A) LSCM images of plasma clots and (B) EM images of plasma clots prepared with scaffold, G7 and B3. Clots made with B3 had significantly thinner fibrin fibres compared with scaffold Adhiron and G7 (C). 20 fibres were measured from different areas of at least 5 different EM images of each Adhiron. Results are presented as mean±SEM).

6.3 Discussion

The use of a phage library to target fibrinogen-C3 interaction sites was able to identify binders to both fibrinogen and C3 that affected clot formation and lysis, to varying degrees. The main findings of this Chapter can be summarised as follows: i) fibrinogen binding Adhirons released by C3 have varying effects on the fibrinolytic process, ii) one Adhiron (A6) shared sequence homology with C3, abolished C3-induced prolongation of clot lysis in a purified system and was predicted to bind fibrinogen in a similar area to C3, iii) despite C3-specific activity of A6, this Adhiron failed to reduce clot lysis in plasma system and appeared to show differential effect in diabetes and non-diabetes subjects, iv) Adhiron G2 demonstrated very significant effect on clot structure and lysis both in plasma and purified system, v) C3 binding Adhirons released by fibrinogen may also affect fibrin clot structure/lysis.

The analysis of fibrinogen binding Adhirons that were released following addition of excess C3 produced dramatic effects within turbidimetric assays, with variability observed between plasma and purified settings. Several Adhirons appeared to be having little effect on clot formation and structure. In plasma turbidimetric assays, three Adhirons, A5, C2 and G2, severely affected both LT and FT, indeed C2 caused no increase at all in absorbance of the reaction mixture, suggesting no clot was formed. These effects were greatly reduced in a purified setting, implying that interaction between the fibrin network, Adhiron and plasma proteins affects both clot structure and fibrinolysis.

Following incubation of fibrinogen with Adhiron A6 prior to the addition of C3 it was possible to abolish C3 induced prolongation of lysis. Clots containing A6

appeared structurally very similar to control with no difference observed in the 3 dimensional structure of the fibrin network from LSCM images and no difference in fibrin fibre thickness from EM images. It is likely that the presence of A6 inhibits C3-fibrinogen interaction to a certain extent, thus reducing incorporation of C3 into the fibrin network and facilitating fibrinolysis. However, the lack of effect of A6 in plasma setting, and indeed the prolongation of lysis in some individuals is puzzling and indicate that A6 mimics the action of C3 in a plasma environment. Moreover, there does appear to be a difference between diabetes and non-diabetes individuals in terms of their response to A6. A significant increase in LT was observed with non-diabetes individuals, with a definite reduced effect in diabetes patients, and enhanced LT in two individuals. It may be that A6 has a diabetes specific effect relating to glycation of fibrinogen and or C3, with the possibility of additional interaction sites between A6 and fibrinogen. Although results were disappointing, molecular modelling provided further evidence of the potential importance of A6. The predicted binding sites of both loops of A6, using Pepsite, were in close proximity within the β-chain of fibrinogen. What was more encouraging was that these predicted binding sites were in the same region of the β chain that included the peptides B and C identified by the microarray screening. Furthermore, these β chain peptides were predicted to bind to C3b in an area adjacent to the portion that shared sequence homology with A6. This commonality of results provides compelling evidence that these are genuine interaction sites between C3b and fibrinogen, in addition to the binding of native C3 to fibrinogen. This may provide further explanation to the lack of effect of A6 in plasma, in particular that of healthy controls. It may be that A6 is able exert some of its effect by inhibiting C3b

incorporation into the clot, and that reduced activation of the complement pathway in healthy individuals translates into lower levels of C3b compared to diabetes. This however would not explain the increase in LT observed in the T2DM control samples and many of the T2DM samples. The most likely explanation for the failure of A6 to facilitate lysis in plasma is related to interaction with other plasma proteins. Further experiments using immunoprecipitation techniques may help to investigate this possibility and this remains an area for future research. Although a promising start, further work is required to identify Adhirons capable of interfering with the interaction between C3(b) and fibrinogen.

Although the main aim of this work was to identify Adhirons capable of reducing C3 medicated prolongation of lysis, similar to the PI results, several Adhirons were identified that dramatically altered clot structure and with it fibrinolysis. Adhiron G2 was studied in more detail and images of the clot structure revealed a very abnormal appearance. The fibrin fibres appear very thin and fragile and at increasing concentration they clump together, thus creating the impression of thicker fibres or denser clot network from the FT values. As the concentration of G2 is reduced there is less clumping and therefore the FT reduced, reflecting the thinner fibres. Even at very low molar concentration (1:0.2 fibrinogen:Adhiron molar ratio), Adhiron G2 significantly alters the clot structure and prolongs LT in both plasma and purified experiments. At the highest concentration studied, there is a clear reduction in lag phase, suggesting that lateral aggregation of the fibrin protofibrils is enhanced. It is possible therefore to hypothesis that G2 is interacting with fibrinogen at the sites of fibrinopeptide A and B release. Although these results are very striking and certainly warrant

further investigation, it is likely that they are C3 independent effects. In terms of fibrinogen-C3 interactions, the results obtained with Adhiron A6 are far more relevant and exciting.

Fewer C3 binding Adhirons were identified and of the four available for analysis, only B3 produced any significant effects. There was a reduction in FT in both plasma and purified settings, suggesting formation of thinner fibrin fibres which was confirmed by measurement of fibres from EM clots. Adhiron B3 also significantly and reproducibly increased lysis time in turbidimetric assays in plasma, suggesting an interference with the process of fibrinolysis. The effects of B3 on clot structure are difficult to explain as in theory, it should represent a portion of fibrinogen binding to C3 and shouldn't therefore have the dramatic effects observed. A possible explanation may be that B3 is able to non-specifically bind to fibrinogen, as well as C3, and is not actually involved in the interaction site between the two. Given the more compact structure induced by Adhiron B3, by whatever means, it is possible that this results in increased incorporation of anti-fibrinolytic agents into the clot during formation, thus prolonging lysis.

Overall, the results presented in this Chapter are encouraging in terms of utilising this Adhiron library to target the interaction between C3 and fibrinogen and modulate clot lysis. Further refinement of the technique, to identify Adhirons with varying binding affinity for example, may lead to the discovery of Adhirons with similar pro-fibrinolytic properties in plasma as well as in purified settings.

Chapter 7

Conclusions and future work

The increasing prevalence of diabetes worldwide shows little sign of abating, with estimations from the International Diabetes Federation that over 500 million will be affected worldwide by 2035, an increase of 55% from the current levels of 382 million in 2013. Diabetes is associated with a number of health complications and is most strongly linked with cardiovascular disease (CVD). In addition to increased risk of myocardial infarction (MI), the prognosis following a vascular event in diabetes remains worse than individuals with normal glucose metabolism[5]. This association is related to clustering of cardiovascular risk factors in diabetes, coupled with a deranged haemostatic system that is driven by inflammation, insulin resistance and hyperglycaemia. A pro-thrombotic environment is generated secondary to a number of abnormalities including increased platelet activation and elevated levels of proteins such as coagulation factors VII and VIII, fibrinogen and plasminogen activator inhibitor 1 (PAI-1)[35, 37]. In addition to increased prothrombotic protein levels, diabetes is associated with functional abnormalities in various proteins in the coagulation pathways, which contribute the thrombotic milieu in diabetes. Moreover, increased incorporation of proteins such as plasmin inhibitor (PI) and complement C3 contribute to a hypofibrinolytic state[6;133], compounding matters further. Interaction between the complement and coagulation pathways is increasingly understood providing a link between the inflammation observed in diabetes and the pro-thrombotic environment.

Current treatment strategies for preventing atherothrombotic events in diabetes are aimed at the cellular phase of coagulation and inhibition of platelet activation. The fibrin network has been successfully targeted in the treatment of acute arterial thrombotic occlusion through the use of fibrinolytic compounds,

although the introduction of primary percutaneous intervention for MI now largely limits this option in some centres to the treatment of acute stroke disease only. Inhibiting coagulation factors to prevent arterial thrombotic events has been trialled using warfarin and more recently factor X inhibitors but concerns persist regarding the bleeding risk associated with broad inhibition of clot formation using these agents. Targeted interference with fibrinolysis is a potential area for the development of new therapeutic agents, such as inhibition of fibrinogen interactions with the anti-fibrinolytic compounds PI and C3. The aims of my work were therefore to i) further study the role of C3 in hypofibrinolysis in relation to diabetes ii) elucidate potential binding sites between fibrinogen and C3 and iii) modulate fibrin clot lysis by targeted interference with fibrinogen-PI and fibrinogen-C3 interactions.

The effects of diabetes on the fibrinolytic properties of C3 and potential mechanisms

The analysis of plasma C3 levels in a cohort of 838 T2DM patients demonstrated a strong correlation with clot lysis time and confirms the contribution of C3 to hypofibrinolysis in T2DM in addition to T1DM as previously described[6]. The lack of association between C3 and PAI-1 levels strengthens the belief that both molecules contribute to the resistance of fibrinolysis in this population via different mechanisms. Fibrinolysis is reduced by PAI-1 due to the inhibition of plasmin generation, whereas increased incorporation of C3 into the fibrin network appears to increase the mechanical resistance to lysis. This notion is supported by the observation that C3 incorporation into the clot correlates with lysis time [214].

Turbidimetric experiments using fibrinogen and C3 purified from T1DM, T2DM and matched controls demonstrated a prolongation in clot lysis time by C3 and this effect appeared to be most pronounced in the T1DM cohort. It may be unfair to compare the T1DM and T2DM groups however given that the concentration of tPA in the T2DM experiments was adjusted to facilitate clot lysis and permit analysis of the samples. It is likely that the C3 mediated prolongation of lysis in these samples was artificially low compared to the T1DM group. There was a wide variability in LT prolongation in both diabetes samples and controls. This observation implies a variation in the degree of C3-fibrinogen interaction and subsequent incorporation of C3 into the clot and may relate to differences in glycation of fibrinogen and C3, and also interaction with other coagulation factors such as FXIII which is responsible for cross-linking C3 to fibrin.

Although *in-vivo* glycation of C3 has been demonstrated previously, the site(s) of glycation has not been described. Mass spectrometry analysis of pooled DM and control samples failed to demonstrate measurable glycation. Individual sample analysis did however reveal multiple Lysine residues with evidence of glycation with a large amount of heterogeneity between samples. Moreover, the T1DM samples contained more glycation sites per sample than the T2DM group with a greater degree of overlap between samples. These findings may explain the increased prolongation in lysis time caused by C3 observed in the T1DM group compared to the T2DM and suggests that glycation of certain, key, Lysine residues is an important factor in the interaction of fibrinogen with C3 and its incorporation into the clot. In addition to post translational modifications caused by hyperglycaemia, elevated glucose concentrations may contribute further by

altering the structure of the C3 molecule, exposing hidden residues and increasing their affinity to other proteins. An example of this mechanism is the glycation of Lysine 839 (K839) which was noted in one T2DM sample. The study by Hair et al (2012) described increased biotinylation of K839 with increased glucose concentration and thus interaction between C3 and fibrinogen at this site may be dependent upon conformational changes within C3 induced by hyperglycaemia.

Future work

The turbidimetric experiments using purified proteins from diabetes and control subjects demonstrated a trend towards increased lysis prolongation by C3 in diabetes compared with controls but fell short of achieving statistical significance. This is likely a matter of sample size as evidenced by the inter-individual variability in lysis time. Repeating the experiments with a larger sample size would be an obvious direction for future work related to this area. Consideration, however, needs to be given to the amount of work this would entail and the additional information derived from it. The purification of C3 from individual samples is very time consuming and protein yield can be unpredictable as occurred in this project. The addition of perhaps 5-10 extra samples would likely reach statistical significance with the T1DM group and possibly the T2DM. Another interesting addition to this work would be to study the effect of C3 glycation on C3 incorporation. This would require analysing more diabetes samples by mass spectrometry and quantifying the amount of C3 incorporated into the clot of the same sample. This might help to identify any glycated Lysine residues that are linked with C3 incorporation.

Assessing C3-fibrinogen interactions

Perhaps the most striking result obtained from this section of work was the discovery that the β chain of fibrinogen was important in binding to C3 rather than the α chain as was previously thought. This finding was from the results of the phage display screening with the cDNA library of the fibrinogen α chain and microarray screening. Both experiments rely on linear interaction, however, It may be worth noting that both C3 and fibrinogen are presented as linear peptides in the phage display system, whereas only the immobilised fibrinogen fragments are presented in a linear fashion with the microarray screening as C3 is added to the plate in solution, thus preserving it conformation. Although three different peptide motifs from the β chain appeared to be involved in binding to C3, two of these (peptides B and C) were separated by a single amino acid and thus it is possible that they represent a single interaction site. Such large scale screening of fibrinogen and C3 was required given the lack of published work regarding possible sites of interaction. The use of molecular modelling techniques allowed these peptides to be tested for their likelihood of binding to C3 and identify potential interaction sites. Peptides B and C were predicted to bind in very close proximity to each other on C3, strengthening the argument that they represent one interaction site. Residues on the surface of C3 that were predicted to bind to these peptides included K839, further implicating the role of hyperglycaemia in binding fibrinogen to C3. Unfortunately 3-dimensional structures of the β chain peptides were predicted to dock to different regions of C3, however these sites did appear to encompass other glycated Lysine residues. The predicted docking pose of peptide B was in the C345C domain an area that includes glycated lysine residue K1505. Peptide C was predicted to

dock to C3 around the MG6 domain containing glycated lysine residue K655. A potentially important finding from this work is the discovery that the active component of C3, C3b may also be able to bind to fibrinogen and be incorporated into the clot structure. Although there is a big difference in the structure of the two molecules, they share a very similar molecular weight and assuming it is the physical presence of C3 that leads to the resistance to lysis then it is feasible that C3b shares this property. The amino acid sequence and 3-dimnesional structure of the β chain peptides were predicted to bind in virtually the same region of C3b, indicating that fibrinogen may be binding to activated C3b as well as native C3. This observation may be of particular relevance to diabetes given that the complement pathway is increasingly activated in diabetes, generating elevated C3b levels. Indeed, preliminary experiments by another member of the team has shown that C3b was able to increase clot lysis. However, only a limited number of experiments were conducted and this will require detailed investigation using samples from different individuals and remains an area for future research.

Future work

To assess whether the β chain peptides identified from the microarray screening are genuine sites of interaction with C3 it would be worth producing them as peptides and assessing their interaction in an ELISA and also in turbidimetric assays. By pre-incubating C3 with the peptides, they should in theory prevent fibrinogen binding to C3. In the case of turbidimetric analysis this should enhance fibrinolysis and in an ELISA a lower OD if an anti-fibrinogen antibody is used as the secondary antibody.

In order to confirm the interaction site of fibrinogen and C3 it may be possible to co-crystallise the D-fragment of fibrinogen (containing the sequences of peptides B and C) with C3. The fibrinogen molecule has a whole is difficult to crystallise due to its size and mobile αC domain and therefore the microarray results allows a more focussed approach.

Modulating clot lysis by interfering with fibrinogen-PI interactions

The use of the Adhiron library to screen for fibrinogen binders capable of disrupting clot formation and lysis is a new and useful technique for the identification of novel therapeutic agents. The method also allows great flexibility in selecting Adhirons of interest as demonstrated by the use of PI in the latter rounds of panning to elute Adhirons binding to fibrinogen at sites of PI interaction. These Adhirons bind to fibrinogen with high affinity, alter the composition of the fibrin network and modulate fibrinolysis in plasma and in purified setting in the presence and absence of PI.

Adhiron G4 enhanced lysis time in plasma and in purified experiments in the presence of PI, suggesting that it is exerting an inhibitory effect on PI-fibrinogen binding. Immunoblotting identified that G4 is binding to the α chain and the use of molecular modelling narrowed the area to the central E region. This is a considerable distance from the known interaction site of PI and fibrinogen (Lys303 in the αC domain) and thus it is difficult to postulate how G4 is directly inhibiting the actions of PI from such a relatively remote area. Difficulties in crystallising this region of the α chain meant it was not possible to interrogate it in terms of Adhiron binding. The clots produced in the presence of G4 contained thicker fibrin fibres than controls and may offer an explanation for its effect on

clot lysis. Fibre diameter is governed by lateral aggregation which relies upon the release of fibrinopeptides (Fp) A and B from the α and β chains respectively. FpA release allows the formation of elongated protofibrils, which are subsequently linked to other protofibrils following FpB release and disengagement of the αC domain from residues 1-66 within the β chain of the central E region. It may be that G4 facilitates the dissociation of FpB and the αC region thus increasing lateral aggregation, producing clots with thicker fibrin fibres that are known to be less resistant to fibrinolysis by plasmin. The lack of change in the lag phase suggests that the rate of FpA dissociation is unaffected.

By using PI to elute fibrinogen bound Adhirons during the latter biopanning rounds, it was envisaged that these Adhirons would facilitate clot lysis by interfering with PI-fibrinogen interaction. In fact the Adhirons identified predominantly inhibited lysis. Of the five that prolonged lysis in plasma, only Adhiron A2 was studied in greater detail, with consistent prolongation in lysis in plasma and purified settings, and an additive effect on lysis prolongation in the presence of PI. Compared to the Adhirons G2 and G4, it showed increased incorporation into the clot with the formation of thinner fibrin fibres. SPR studies showed high affinity binding of Adhiron A2 to fibrinogen, whereas immunoblotting demonstrated an interaction with the α chain of the protein. These techniques were complemented by molecular modelling that predicted the binding site to be within the E region of the α chain, in very close proximity to that of G4. Given the very different effects of these two Adhirons on clot formation and lysis it is interesting that predicted binding sites on fibrinogen are similar. It may be that subtle changes in the binding sites have opposing effects

on fibrinopeptide release and αC disengagement, leading to reduced lateral aggregation and thinner fibres in the presence of A2. One possible explanation for the differing effects is related to the incorporation data. It is possible that the two Adhirons bind on a similar region but the sequence difference allows A2 incorporation into the clot (thus preventing lysis) whereas G4 binds and and prevent PI incorporation without getting cross-linked itself (thus facilitating lysis). Although my aim was to identify Adhirons that facilitate fibrinolysis, the “by product” of discovering anti-fibrinolytic Adhirons may prove to have important implications. These could be developed further for use therapeutically in patients with bleeding tendencies or perhaps as clotting agents following major trauma. There are precedents to such an approach as an analogue of PAI-1 is under development as a topical agent to minimise bleeding.

Future work

In addition to analysing the other Adhirons that prolonged lysis in greater detail there is further work that can be done in an attempt to clarify the binding sites of Adhirons A2 and G4. A modified ELISA could assess the binding of these Adhirons to commercially available D and E fragments of fibrinogen. Adhirons can be variable in their ability to stick to ELISA plates and thus biotinylating the Adhirons prior to incubating them on streptavidin coated plates would eliminate this problem and maintain the conformation of the Adhiron when presented to the fragments. Many commercially available polyclonal anti-fibrinogen antibodies are able to bind to D and E fragments, as well as the whole molecule, and so could be used to monitor the degree of binding.

If Adhirons are to be developed for use therapeutically then they would need to be assessed in animal models. This could be done relatively simply initially by substituting human plasma for murine plasma in turbidimetric assays *in vitro*. More detailed analysis could involve administering the Adhirons intravenously to mice followed by blood sampling and turbimetric analysis *ex-vivo.*

Modulating clot lysis by interfering with fibrinogen-C3 interactions

Using the same Adhiron library to study fibrinogen-C3 interactions identified binders to both fibrinogen and C3 that were capable of disrupting clot formation and lysis. In particular, one Adhiron, A6, that was eluted from fibrinogen by excess C3 shared a sequence homology with a portion of C3, suggesting that this region of C3 might be involved in binding to fibrinogen. Further analysis in purified turbidimetric assays revealed that A6 was able to abolish C3 mediated prolongation of clot lysis without altering the final clot structure and moreover, molecular modelling analysis predicted a binding site that was in close proximity to regions within the β chain of fibrinogen implicated in binding to C3.

These positive results were tempered slightly by a lack of effect in plasma turbidity and lysis experiments. Pooled plasma samples did not reveal a reduction in lysis time and analysis of individual plasma samples demonstrated a reduction in only 2/6 diabetes samples and 0/6 control samples. It may be that A6 is interacting with other plasma proteins, thus reducing its ability to bind to fibrinogen. Given the variation observed in LT between control and diabetes samples, an alternative explanation is that the effects of A6 are diabetes specific; relating either to increased glycation of fibrinogen and or C3, or perhaps changes in the conformation of C3 due to hyperglycaemia that impact

on ability to bind to fibrinogen. Diabetes is associated with enhanced complement activation, which translates to increased conversion of C3 to C3b. The discovery that the fibrinogen β chain peptides (identified form the microarray screening) were predicted to bind adjacent to the area of C3b containing the sequence homology to A6 raises the possibility that C3b is able to bind to fibrinogen in addition to C3. It maybe that A6 is more efficient at blocking the binding of C3b to fibrinogen in plasma, and a change in the C3:C3b ratio in diabetes plasma results in decreased C3b incorporation into the clot compared to healthy controls.

Much like the work regarding interfering with fibrinogen-PI interactions, several Adhirons released from fibrinogen by C3 prolonged clot lysis. Adhiron G2 greatly prolonged lysis time in plasma and purified assays, along with a reduction in final turbidity of the clot and lag phase. These changes in clot parameters persisted even at relatively low fibrinogen:Adhiron molar ratios and correspond with dramatic changes to the clot structure. Electron and confocal microscopy revealed very thin fibrin fibres that appear to clump together at higher concentrations of G2, greatly distorting clot architecture and increasing its resistance to fibrinolysis by plasmin. The mechanisms behind these changes have not been studied but the shortened lag phase, particularly in plasma, coupled with the generation of thin fibrin fibres suggest that protofibril elongation is affected along with lateral aggregation and so the gaze of suspicion should fall on interference with fibrinopeptide A and B release.

Future work

In order to further investigate the possibility that A6 is interacting with other plasma proteins and thus explain its lack of effect in plasma, its behaviour in fibrinogen depleted plasma could be tested. The Adhirons contain a His-tag and thus by coating magnetic IgG beads with an anti-His antibody the beads could be used to draw out A6 from the fibrinogen depleted plasma and with it other proteins that may have bound. The eluted A6-antibody complex could then be run on an SDS-PAGE electrophoresis gel to look for a shift in the observed band. The site of interaction of A6 with fibrinogen could be confirmed in a similar manner to the PI released Adhirons, to i) identify which chain is involved by immunoblotting and ii) determine which region of fibrinogen by incubating fragments D and E to biotinylated A6 captured on streptavidin coated plates. Another modified ELISA could be performed to demonstrate inhibition of fibrinogen-C3 interaction. By coating an ELISA plate with fibrinogen in the presence and absence of A6 followed by incubation with C3 and detection with a polyclonal anti-C3 antibody, a lower OD in the presence of A6 would suggest reduced binding of C3 to fibrinogen. The same method could be used to test for C3b binding to fibrinogen in the presence and absence of A6.

An alternative strategy would be to repeat the initial screening of fibrinogen with the Adhiron library. Only high affinity binders were tested initially and lower affinity Adhirons may also have a role, as reversibility of action is important in clinical applications. Therefore, by altering the stringency during the panning rounds, using different incubation times and washing steps Adhirons with different binding affinities can be identified. It should be noted that only fibrinogen and C3 from healthy controls were used in the initial panning

experiments and therefore using fibrinogen and C3 purified from pooled plasma samples of patients with diabetes would ensure that alternative fibrinogen-C3 interaction sites in diabetes are accounted for.

In summary, my work helped to shed more light on the role of complement C3 in hypofibrinolysis in diabetes and identified some of the interaction sites of this protein on fibrinogen. Moreover, the new methodology that I developed, employing Adhirons, opens the door for identification of novel therapeutic targets that can be used to enhance the fibrinolytic process and reduce thrombosis risk in vascular conditions. Moreover, these Adhirons may be used to impair fibrinolysis and subsequently limit blood loss in bleeding complications following trauma, surgical intervention or over-treatment with anticoagulants.

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Appendix

Leeds Institute of Genetics, Health and Therapeutics

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The Role of Complement C3 in Diabetes

PARTICIPANT INFORMATION SHEET

PART 1

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.

PART 1 tells you the purpose of this study and what will happen to you if you take part.

PART 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.
Thank you for reading this.

What is the purpose of the study?
People with diabetes have an increased risk of developing heart disease, compared to people without diabetes. This can lead to a heart attack which is caused when a blood vessel supplying the heart becomes blocked. This blockage is caused when small cell particles clump together and form a clot. In this study we are interested in looking at some of the processes involved in the formation of blood clots in more detail. In particular, we will study a new protein, called complement C3, which may have an important role in blood clot stability and resistance to breakdown in people with diabetes, leading to increased risk of heart attacks.

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We are asking people with both type 1 and type 2 diabetes, taking different treatments, with and without some of the problems that can be associated with diabetes to take part. We will also ask some people who do not have diabetes to take part as "control" subjects. In total 120 people will be asked to take part. The results from the different groups will then be compared.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time. If you withdraw after you have given a blood sample, unless you object, your data and samples will remain on file and will be included in the final study analysis.

What will happen to me if I take part?

We will ask you to come to the LIGHT Laboratories in the University of Leeds on 1 occasion where you will see Dr King or Penny Rice, the study research nurse. The appointment will be in the morning and will last about 20 minutes. You will need to fast from midnight on the day of your visit (water is allowed) and not take any morning medication you would normally have. If you bring them
with you we will provide breakfast of toast and a drink after the appointment and you can take them at this time.

If you are a participant with diabetes please check your blood sugar when you get up in the morning as per your normal routine. If your reading is low or you are experiencing symptoms consistent with low sugar levels do not fast and treat this low sugar as you would normally. You can still attend the study appointment.

At this study visit you will be asked to sign a consent form. You will have a 60 ml blood sample taken (this is about 4 tablespoons). Some of the sample will be sent for routine laboratory tests and the remainder will be used to analyse some of the proteins that affect blood clotting. We will also ask you some general health questions and check your weight and blood pressure.

What are the possible disadvantages and risks of taking part?

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You will have to attend an appointment in the morning lasting about 20 minutes. We will arrange this at a time to suit you and can reimburse travel expenses. Having a blood sample taken can be uncomfortable and you may develop a bruise over the site. This will disappear after a few days. Some people feel light-headed or faint giving a blood sample and there is also a very slight risk of developing an infection at the site where the sample is taken.

What are the possible benefits of taking part?

There will be no direct benefit to you from taking part in this study but we hope the results will give us more information about the factors affecting the development of heart disease. This may help with future treatments for cardiovascular disease in people with diabetes.

What happens when the research study stops?

Any other medical care you are receiving will continue as before.

What if there is a problem?

If you have a concern about any aspect of this study, you should contact one of the researchers who will do their best to answer your question. Our contact numbers are at the end of this information sheet.

If you are harmed by taking part in this study there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

If you wish to complain formally you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of this research will be kept strictly confidential. Details are in Part 2 of this information sheet.

Contact Details

If you require any further information please contact us on the telephone numbers below.

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Thank you for reading this information sheet and for considering participating in this research.

Dr. Rhodri King Sr. Penny Rice – Research Nurse

TEL: 0113 3437719 0113 3437702 or 07762 824470

This completes Part 1 of the information sheet. If this information has interested you and you are considering participation please continue to read Part 2 before making a decision

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PART 2

What happens if I don't want to carry on with the study?

If you change your mind about participating you can withdraw from the study. A decision to withdraw, or a decision not to take part, will not affect the standard of care you receive or your relationship with any medical staff involved in your care. If you withdraw after you have given a blood sample, unless you object, your data and samples will remain on file and will be included in the final study analysis. If you object to this your sample and all study data will be destroyed and you will not be included in the analysis.

Will my part in this study be kept confidential?

If you consent to take part in this study, the records obtained while you are in this study as well as related health records will remain strictly confidential at all times. Study information will be held securely on paper and electronically in the LIGHT Building at the University of Leeds under the provisions of the 1998 Data Protection Act. Your name will not be passed to anyone else outside the research team or the sponsor, who is not involved in the trial. You will be allocated a trial number, which will be used as a code to identify you on all samples. This number will also be used on all study forms and databases. We will keep a separate record of your name and a contact detail for the duration of the study which will be destroyed when the study is completed.

Your records will be available to people authorised to work on the trial but may also need to be made available to people authorised by the Research Sponsor, which is the organisation responsible for ensuring that the study is carried out correctly. A copy of your consent form may be sent to the Research Sponsor during the course of the study. By signing the consent form you agree to this access for the current study and any further research that may be conducted in relation to it, even if you withdraw from the current study.

The information collected about you may also be shown to authorised people from the UK Regulatory Authority and Independent Ethics Committee; this is to ensure that the study is carried out to the highest possible scientific standards. All will have a duty of confidentiality to you as a research participant.

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In line with Good Clinical Practice guidelines, at the end of the study, your data will be archived securely for a minimum of 15 years. Arrangements for confidential destruction will then be made.

Informing your General Practitioner (GP)

Your GP will not be told you are taking part in this study. However if we find any abnormalities in your routine blood tests we will inform you, and with your permission, your GP, and any other doctors who may be treating you. We will also document in your hospital notes that you are taking part in a study.

What will happen to any samples I give?

All samples will be labelled with a study number which will be unique to you. Only Sister Rice will be able to identify you from this number. The blood samples will be analysed in the LIGHT Laboratories in the University of Leeds.

If you agree we will store some of your blood sample in the freezer in the LIGHT Laboratories for use in future studies. However, if you prefer not to do this, any remaining blood sample will be destroyed once this study concludes. Your sample will remain anonymous to other researchers at all times even after this study has ended.

Will any Genetic testing be done?

General genetic testing is not a part of this study.

What will happen to the results of this study?

The results of the study will be available after it finishes and we will be able to send you a summary if you are interested. Results will usually be published in medical journals or be presented at scientific conferences. The data will be anonymous and none of the people who take part in the trial will be identified in any report or publication.

Who is organising and funding this research?

This research is organised by the Leeds Teaching Hospitals Trust and the University of Leeds and funded by the Sir Jules Thorn charity.

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Who has reviewed the study?

The scientific content of the study has been through national reviewing processes. The study was given favourable ethical opinion for conduct in the NHS by Leeds East Research Ethics Committee.

Contact for further information

You are encouraged to ask any questions you wish, before, during or after taking part. If you have any questions about the study or the procedures involved, or would like to read the research on which this study is based please speak to your study nurse or doctor. Our contact details are at the end of Part 1 of this information sheet. If you require any further information or have any concerns while taking part in the study please contact one of us.

Alternatively if you or your relatives have any questions about this study you may wish to contact your GP or specialist member of staff in the diabetes centre to discuss it further.

The hospital also has a Patient Advisory Liaison Service (PALS) who may be able to offer independent advice.

If you decide you would like to take part you will be asked to read and sign the consent form. You will be given a copy of this information sheet and the consent form to keep. A copy of the consent form will be filed in your patient notes, one will be filed with the study records and one may be sent to the Research Sponsor.

You can have more time to think this over if you are at all unsure.

Thank you for taking the time to read this information sheet and to consider this study

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CONSENT FORM

Complement C3 and inflammation in diabetes

Subject Identification Number:

Please initial box

1. I confirm that I have read and understand the information sheet dated …… (version .....) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that sections of my medical notes may be looked at by authorised individuals from the Sponsor for the study, the UK Regulatory Authority of the Independent ethics Committee in order to check that the study is being carried out correctly. I give permission, provided that strict confidentiality is maintained, for these bodies to have access to my medical records for the above study and any further research that may be conducted in relation to it. I also give permission for a copy of my consent form to be sent to the Sponsor for the study. regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree that my blood sample is a gift and may be retained for use in future research. I agree to transfer any property rights to the samples I provide to the University of Leeds on the understanding that it will only be used for research. I understand that even if I withdraw from this study the data and samples collected from me may be used in analysing the trial results, unless I specifically withdraw consent for this. I understand that my identity will remain anonymous. ☐

5. I consent to the storage including electronic, of personal information for the purposes of this study. I understand that any information that could identify me will be kept strictly confidential and that no personal information will be included in the study report or other publication. ☐

6. I agree to take part in this study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature