Identification and characterisation of novel plasma clot components

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Intellectual Property and Publication statement

I confirm that the work submitted is my own, except where work which has formed part of jointly authored publications has been included. The contribution of me and the other authors to this work has been explicitly indicated below. I confirm that appropriate credit has been given within the thesis where reference has been made to the work of others.

I confirm that information and data in chapters 1, 4 and 5 form part of work from jointly authored publications as outlined below.

Original Publications

Role of proteomic technologies in understanding risk of arterial thrombosis. VR Polkinghorne, KF Standeven, V Schroeder, AM Carter (2009) Expert Reviews of Proteomics **6** (5) 539-550

- I produced the figures, helped write the manuscript (Abstract, Introduction, Proteomic analysis of plasma, Conclusions, Key Issues)
- The remaining authors wrote the remainder of the manuscript

Complement C3 is a novel plasma clot component with anti-fibrinolytic properties. JM Howes, VR Richardson, KA Smith, V Schroeder, R Somani, A Shore, K Hess, R Ajjan, RJ Pease, JN Keen, KF Standeven, AM Carter (2012) Diabetes and Vascular Disease Research **9** (3) 216-225

- I performed the experiments, analysed the data and wrote the relevant sections of the methods, results and discussion for the purified turbidimetic data for purified C3 and purified factor H and the percentage incorporation of factor H into plasma clots
- The other authors performed the experiments, analysed the data and wrote the relevant sections for the remainder of the manuscript.

Elevated properdin and enhanced complement activation in first-degree relatives of South Asian subjects with type 2 diabetes. R Somani, VR Richardson, KF Standeven, PJ Grant, AM Carter (2012) Diabetes Care **35** 894-899

- I performed the factor H ELISA on the subjects plasma and analysed the data in relation to the differences in South Asian relatives and controls
- The other authors performed the experiments and analysed the data for the remainder of the manuscript.

Substrates of Factor XIII-A: Roles in thrombosis and wound healing. VR Richardson, P Cordell, KF Standeven, AM Carter (2013) Clinical Science **124** 123-137

- Wrote the manuscript except for the section on cross-linking in cartilage and bone, which was written by P Cordell. I produced the table and figures.

Complement C3 is a substrate for activated factor XIII that is cross-linked to fibrin during clot formation. VR Richardson, V Schroeder, PJ Grant, KF Standeven, AM Carter (2012) British Journal of Haematology **DOI: 10.1111/bjh.12096**

- I performed the experiments, analysed the data, produced the figures (Figure 1, Panels A, B and D, Figure 2, Panels A-J) and wrote the manuscript.
- V Schroeder performed the experiment and analysed the data for figure 1 panel C.

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Abstract

Plasma clot structure/function is a major determinant in cardiovascular disease risk and severity. Plasma proteins are incorporated into plasma clots via binding and factor XIII-dependent cross-linking, with complement C3 and factor H previously identified as plasma clot components using proteomics. The aim of this current project was to validate the role of C3 and factor H in fibrin structure and function and to establish a proteomics method for the identification of novel factor XIII substrates. C3 did not affect fibrin structure; however C3 induced a concentration-dependent prolongation of fibrinolysis. C3 was cross-linked to fibrin within purified and plasma clots and bound to plasma clot components. C3 was a substrate for plasmin, with cleavage occurring in the presence and absence of fibrin. C3 also influenced angiostatin production and t-PA and plasminogen interactions within fibrin clots to prevent plasminogen cleavage and plasmin generation. All of these interactions were found to influence fibrinolysis. Whereas factor H was confirmed to be a plasma clot component, was associated with inflammation and fibrin structure and function but was not associated with complement activation in individuals at risk of cardiovascular disease. Further in vitro analyses found that factor H did not affect fibrin structure or fibrinolysis. Factor H was not cross-linked to fibrin in purified and plasma clots, but did form homodimers in the presence and absence of fibrin and factor H was a substrate for thrombin and plasmin, with cleavage occurring within fibrin clots. The proteomic techniques were established for the identification of factor XIII substrates however no novel proteins were identified using these methods, suggesting the sensitivity of the technique may be insufficient to detect novel proteins. This study has added to the growing body of evidence which suggests complement and coagulation pathways interact for the purposes of preventing blood loss and pathogen invasion.

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Abbreviations

α2-AP	α2-antiplasmin
ADP	Adenosine diphosphate
aHUS	atypical haemolytic uraemic syndrome
BMI	Body mass index
BP	5-(biotinamido)pentylamine
BSA	Bovine serum albumin
C3aR	C3a Receptor
C3(H_2O)	Hydrolysed C3
C5aR	C5a Receptor
CAD	Coronary artery disease
COAT platelet	Collagen and thrombin activated platelet
CR	Complement receptor
CRP	C reactive protein
CV	Column volume
CVD	Cardiovascular disease
DAF	Decay acceleration factor
DCDR	Division of Cardiovascular and Diabetes Research
DEAE	Diethylaminoethanol
DTT	Dithiothreitol
εACA	ε-aminocaproic acid
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme linked immunosorbant assay
FP	Fibrinopeptide
FXIII	Factor XIII
FXIII-A	Factor XIII A subunit
FXIII-B	Factor XIII B subunit
FXIIIa	Activated factor XIII
FXIII-A2B2	Plasma factor XIII
gC1qR	C1q receptor
HOMA	Homeostasis model assessment
HRP	Horseradish peroxidase
ICAM-1	Intracellular adhesion molecule-1
IFG	Impaired fasting glucose
IL	Interleukin
KCl	Potassium chloride
LC-MS/MS	Liquid chromatography tandem MS
ldlr	Low density lipoprotein receptor
LOX-1	Oxidized low density lipoprotein receptor-1
MALDI-MS/MS	Matrix assisted laser desorption/ionisation tandem MS
MASP	MBL-associated proteins
MBL	Mannose-binding lectin
MES	2-(N-morpholino)ethanesulfonic acid
MI	Myocardial infarction
MMP	Matrix metalloproteinase

MS	Mass spectrometry
NaCl	Sodium chloride
NO	Nitric oxide
OPD	o-Penylenediamine
Ox-LDL	Oxidised low density lipoprotein
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PBS-T	PBS-Tween
PEG	Polyethylene glycol
PMSF	Phenylmethanesulfonylfluoride
PVD	Peripheral vascular disease
PVDF	Polyvinylidene fluoride
SCR	Short consensus repeats
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
T2DM	Type 2 diabetes mellitus
TAFI	Thrombin activatable fibrinolysis inhibitor
TBS	Tris buffered saline
TBS-T	TBS-Tween
TF	Tissue factor
TG2	Tissue transglutaminase
TNFα	Tumor necrosis factor α
t-PA	Tissue-type plasminogen activator

TRAP	Thrombin receptor activating peptide
u-PA	Urokinase plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1

Chapter 1 Introduction

1.1. Cardiovascular disease

Cardiovascular disease (CVD), an illness of the heart and circulation encompasses a number of syndromes including ischaemic heart disease, coronary artery disease (CAD), myocardial infarction (MI), cerebrovascular disease (stroke), hypertensive heart disease, inflammatory heart disease, rheumatic heart disease and peripheral vascular disease (PVD).

A recent World Health Organisation report, indicated CVD was one of the leading causes of mortality across the world, with 17 million deaths during 2008, with low to middle income countries suffering particularly badly (Anon 2011). A 2010 British Heart Foundation report found that mortality from CVD has fallen by 44% over the last ten years in the UK; however, deaths from CVD still remained high at around 191,000 each year with 50,000 premature deaths (before the age of 75 years). In England alone, 101,000 individuals have an MI and 125,000 have a stroke every year, placing enormous pressure on health service resources. These include 266 million prescriptions for anti-platelet, cholesterol-lowering and anti-hypertensive drugs, 80,000 angioplasties and 453,000 in-patient episodes, costing the UK economy £30 billion every year, with this level likely to increase with the ever growing older population (Scarborough et al. 2010b). In developed countries, Black and South Asian ethnic groups are at particular risk of CVD with 15% and 27% of deaths resulting from CAD in men respectively in the UK (Scarborough et al. 2010a).

CVD was originally considered a disease of developed countries; however, the prevalence of CVD is rapidly increasing in developing countries, with deaths as a result of CVD estimated to reach 9 million by 2020. Changes to a Westernized diet are one of the major causes for this increase (Reddy 2004).

1.1.1. Risk factors for Cardiovascular Disease

CVD is a multi-factorial disease, with numerous behavioural, medical, genetic and inflammatory factors influencing disease pathogenesis and outcome. Behavioural risk factors for CVD include smoking, poor diet, physical inactivity and alcohol consumption (Scarborough et al. 2010b). Smoking was a strong risk factor for PVD and CAD in a prospective study (Price et al. 1999); additionally smoking is associated with an increase in intima thickening and atherosclerotic plaque development (Wallenfeldt et al. 2001). Smoking also increases the risk for thrombosis, by promoting platelet aggregation, fibrin accumulation and increasing clot strength (Barua et al. 2010). Poor diet is associated with an increased CVD risk. A western diet of high fat, highly processed foods was associated with un-favourable biomarkers of CVD such as reduced high density lipoprotein, increased plasma cholesterol, insulin resistance and C-reactive protein (CRP) (Fung et al. 2001), whereas an increase in fruit and vegetable intake was found to have a protective effect for CVD (Liu et al. 2000).

Poor diet and physical inactivity can lead to the development of several other CVD risk factors, including increased blood pressure, increased blood cholesterol, obesity and type 2 diabetes mellitus (T2DM). Obesity is a major risk factor for both T2DM and CVD (Wilson et al. 2002), whereas weight loss of >5% was associated with a reduced CVD risk (Wing et al. 2011). Deposits of adipose tissue in the abdominal region has been shown to be a stronger risk factor for MI measured by computed tomography compared with total adipose tissue measured by body mass index (BMI) (Nicklas et al. 2004). Obesity is also associated with other traditional risk factors for CVD including increased blood pressure, cholesterol and triglycerides (Wilson et al. 2002). The age of T2DM onset determines the increased CVD risk; individuals who develop T2DM before 60 years of age have the highest CVD risk (Wannamethee et al. 2011). Insulin resistance in T2DM individuals is also associated with a pro-coagulant (increase in plasminogen activator inhibitor 1 (PAI-1) and fibrinogen) and a pro-atherogenic (increase triglycerides and cholesterol) state, suggesting insulin resistance contributes to CVD risk (Haffner et al. 1999).

The genetic component of CVD risk was highlighted by twin studies. In a large prospective study in Sweden, the risk of coronary heart disease was increased 8.1% in male monozygotic and 3.8% in male dizygotic twins if one twin died before

the age of 55 years, whereas in female monozygotic twins the risk was 15.0% and in dizygotic twins was 2.6% if one twin died before the age of 65 years, which suggests that the genetic component of CVD risk is particularly significant if one twin dies young (Marenberg et al. 1994). In the Stockholm Heart Epidemiology Program, family history of coronary heart disease was a risk factor for MI, after accounting for conventional CVD risk factors. In women who had a family history of coronary heart disease and who were current smokers the increased risk for MI was synergistic (Leander et al. 2001). Recent genome wide association studies suggest that the genetic component of CAD, T2DM and blood pressure are likely to be due to small effects of many different genetic variants involved in disease pathogenesis (Burton et al. 2007;Ehret et al. 2011).

1.2. Pathogenesis of Cardiovascular Disease

1.2.1. Atherosclerosis

Atherosclerosis is the term used to describe hardening of the artery wall, which occurs due to the build up of fats, cholesterol and inflammatory cells in the formation of a plaque in the subendothelium. Atherosclerosis occurs early in life with increased intima thickening observed in children as young as 7 years that are overweight or with type 1 diabetes (Jarvisalo et al. 2004;Woo et al. 2004).

The endothelium is regulated by vasodilators (nitric oxide [NO], prostacyclin, bradykinin) vasoconstrictors (endothelin, angiotensin II) and by cellular adhesion molecules, which prevent cell adhesion and suppression of coagulation. During the initial stages of atherosclerosis, endothelial dysfunction, caused by a variety of CVD risk factors, leads to alterations in the balance between vasoconstriction and vasodilation and in endothelial cell expression of cellular adhesion molecules (Davignon and Ganz 2004). The main candidate for endothelial dysfunction is low density lipoprotein (LDL), which when mildly oxidised, forms oxidised LDL (Ox-LDL). Ox-LDL increases the permeability of the endothelium (Essler et al 1999) allowing LDL to be transported across the endothelium through porous pathways between adjacent endothelial cells into the subendothelium (Rutledge et al 1990). Once inside the subendothelium LDL is converted to Ox-LDL by reactive oxygen species released from the endothelium in response to increased glucose and free fatty acids (Inoguchi et al 2000). Ox-LDL binds and activates the up-regulated endothelial receptor, oxidized low density lipoprotein receptor-1 (LOX-1). LOX-1 promotes endothelial abnormalities such as superoxide generation, nuclear factor κB activation, increases adhesion molecule numbers, endothelial cell apoptosis (Chen et al. 2002) and matrix metalloproteinase (MMP) 1 and 3 expression (Li et al. 2003). One of the main effects of endothelial dysfunction is impaired endothelial relaxation, as a result of dysfunction within the NO synthase pathway, which under normal circumstances produces NO, however during endothelial dysfunction produces superoxide instead of NO due to the uncoupling of endothelial NO synthase by tetrahydrobiopterin, thus preventing bioavailability of NO for vasodilation (Kawashima and Yokoyama 2004)..

Dysfunctional endothelial cells also up-regulate a number of adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), P-selectin and E-selectin by Interleukin (IL) 1 and tumor necrosis factor α (TNFα) (Eppihimer et al. 1996;Pober et al. 1986). The selectins are responsible for the initial rolling and tethering of monocytes and T cells to the endothelium through interactions with sialylated carbohydrates, P-selectin glycoprotein-1 and E-selectin ligand-1, whilst firm adhesion to the endothelium is mediated by VCAM-1 and ICAM-1 interactions with α4β1 and leukocyte specific β2 integrin respectively (Blankenberg et al. 2003). Once adhered to endothelial cells, the monocytes migrate to the arterial intima, promoted by the cytokine monocyte chemoattractant protein-1 where they differentiate into macrophages with the aid of macrophage colony-stimulating factor (Linton and Fazio 2003) which is produced/expressed by endothelial cells following a decrease in NO (Peng et al. 1995). The scavenger receptor CD36 is up-regulated on the macrophage surface and internalises Ox-LDL, resulting in the formation of foam cells and ultimately fatty streaks (Huh et al. 1996;Podrez et al. 2002). Foam cells produce a number of inflammatory mediators (IL1β, IL6, TNFα) which promote expression of monocyte chemotactic factor by endothelial cells, which in turn recruit additional monocytes, thus contributing to the growth of the atherosclerotic plaque and to intima thickening (Strieter et al. 1989). As the plaque increases in complexity, vascular smooth muscle cells are recruited and secrete collagen due to the stimulation from cytokines (transforming growth factor-β, IL-1, platelet derived growth factor), which forms a major component of the fibrous cap (Amento et al. 1991). The thickness of the fibrous cap is critical to the overall stability of the plaque, with thin fibrous caps particularly prone to rupture due to the stresses applied to the cap within narrowed arteries (Li et al. 2006). The fibrous cap is weakened by the secretion of MMP's, which break down collagen (Shah et al. 1995), thus increasing susceptibility to plaque rupture.

Plaque rupture exposes collagen, collagen-bound von Willebrand factor and tissue factor (TF) to flowing blood, allowing binding of platelets via GPIb-IX-V, GPVI and α2β1 receptors (discussed in more detail in section 1.3.1) and activation of the extrinsic pathway of the coagulation cascade (Figure 1) (as discussed in more detail in section 1.3.2). Activation of the coagulation cascade leads to the generation

of the serine protease thrombin, which cleaves and activates fibrinogen and factor XIII (FXIII) to form a stable cross-linked fibrin clot (discussed in more detail in section 1.3.3). In some cases the thrombus is incorporated into the atherosclerotic plaque (Bini et al. 1989), furthering its development by promoting smooth muscle cell migration into the fibrin gel (Kodama et al. 2002), in other cases thrombus formation continues until complete vessel occlusion occurs leading to acute MI.

1.3. The coagulation cascade

1.3.1. Platelet plug formation

Following vascular wall damage, platelets adhere to the damaged tissue via interactions between platelet receptors glycoprotein VI and α2β1 which binds directly to collagen (Kehrel et al. 1998) or via glycoprotein Ib which binds to the multimeric protein von Willebrand factor (Kroll et al. 1991), forming a bridge between the collagen and the platelet. FXIII also binds platelets via αIIbβ3 and αVβ3 under flow, leading to platelet adhesion and spreading, additionally when activated FXIII (FXIIIa) is bound to collagen platelet adhesion is synergistic (Magwenzi et al. 2011). Thrombin also activates platelets via protease activating receptors 1 and 4 leading to rapid platelet aggregation and secretion of granular contents (Kahn et al. 1999). Activation of platelets by thrombin, results in several morphological changes leading to the formation of filapodia, this is due to structural changes which occur in the cytoskeleton (Jennings et al. 1981). Whereas conformational changes in the glycoprotein receptor IIb-IIIa exposes binding sites for fibrinogen, von Willebrand factor and fibronectin (Sims et al. 1991), which results in a network of proteins which bridges the platelet aggregates. In a small subset of collagen and thrombin activated platelets (COAT platelets) this network of proteins is the result of FXIII-dependent cross-linking of serotonin to α-granule proteins which bind to an unknown serotonin binding site on platelet bound fibrinogen and thrombospondin (Dale et al. 2002;Szasz and Dale 2002).

1.3.2. Coagulation cascade activation and inhibition

The coagulation cascade is a series of proteolytic cleavages split into two pathways (Figure 1) the first is the extrinsic pathway which is activated following damage to the vascular wall. The extrinsic pathway is initiated when TF present on the membranes of cells around the vascular bed is exposed to flowing blood. TF forms a complex with factor VII in the presence of Ca^{2+} to produce the active serine protease TF/VIIa. The TF/VIIa complex cleaves factor X (Silverberg et al. 1977) and factor IX (Osterud and Rapaport 1977) to form the active serine proteases factor Xa and IXa.

The second pathway is the intrinsic pathway which is initiated when factor XII, high molecular weight kininogen and prekallikrein come into contact with charged surfaces in a process known as the contact activation pathway. In plasma high molecular weight kininogen and prekallikrein are present as a complex (Mandle et al. 1976), which once bound to the negatively charged surface along with factor XII results in the cleavage and activation of factor XII to form factor XIIa. Factor XIIa cleaves prekallikrein into kallikrein, which in turn cleaves more factor XII (Mandle and Kaplan 1976) and digests high molecular weight kininogen to form bradykinin (Thompson et al. 1978). Factor XIIa cleaves and activates factor XI (Brunnee et al. 1993), which in turn cleaves and activates factor IX (Sun and Gailani 1996). Following the activation of factor IX the intrinsic pathway converges with the extrinsic pathway at the stage of factor X activation.

Factor Xa forms a complex with the activated cofactor Va bound to phosphatidylserine on the surface of platelets to form the prothrombinase complex (Krishnaswamy 1990). Factor V can be activated by factor Xa directly or by the first amounts of thrombin generated. The prothrombinase complex cleaves prothrombin at Arg271 and Arg320 to form thrombin (Orcutt and Krishnaswamy 2004). Thrombin forms several feedback mechanisms to further activate the coagulation cascade, firstly thrombin amplifies coagulation by cleaving and activating factor V (Suzuki et al. 1982). Thrombin activates factor VIII and liberates the bound von Willebrand factor (Saenko et al. 1996), allowing binding of factor VIIIa to the platelet surface and the formation of the tenase complex with activated factor IX. Thrombin also cleaves factor XI in the presence of negatively charged surfaces to form factor XIa (Naito and Fujikawa 1991). Thrombin cleaves fibrinopeptides (FP) A and B from

fibrinogen to initiate fibrin polymer formation (as described in section 1.3.3) and cleaves and activates FXIII to initate cross-linking of the fibrin polymer (as described in section 1.3.4). Thrombin also activates platelets via protease activating receptors 1 and 4 (as described in section 1.3.1).

The coagulation cascade is regulated at several stages, firstly TF pathway inhibitor regulates TF/VIIa complex formation and inhibits factor Xa via kunitz domains (Bajaj et al. 2001). Secondly antithrombin III a serine protease inhibitor is responsible for the inhibition of components of the intrinsic and extrinsic pathway including factor IX (Mauray et al. 1998), X (Choay et al. 1983), and thrombin (Maaroufi et al. 1997). As shown by these studies antithrombin III is a poor serpin when free in solution, however in the presence of heparins antithrombin III inhibitory activity is increased. Finally coagulation is regulated by protein C. Protein C is a vitamin K dependent protein which is activated by thrombin and thrombomodulin present on endothelial cells (Esmon and Owen 1981). Activated protein C in the presence of its cofactor free protein S proteolytically inactivates factor V or activated factor Va and factor VIIIa (Dahlback 1991), however factor VIIIa bound to von Willebrand factor is protected from activated protein C cleavage. In plasma protein S can be found bound to complement C4 binding protein (Dahlback 1991), however it is only the free form of protein S which can act as a cofactor for protein C.

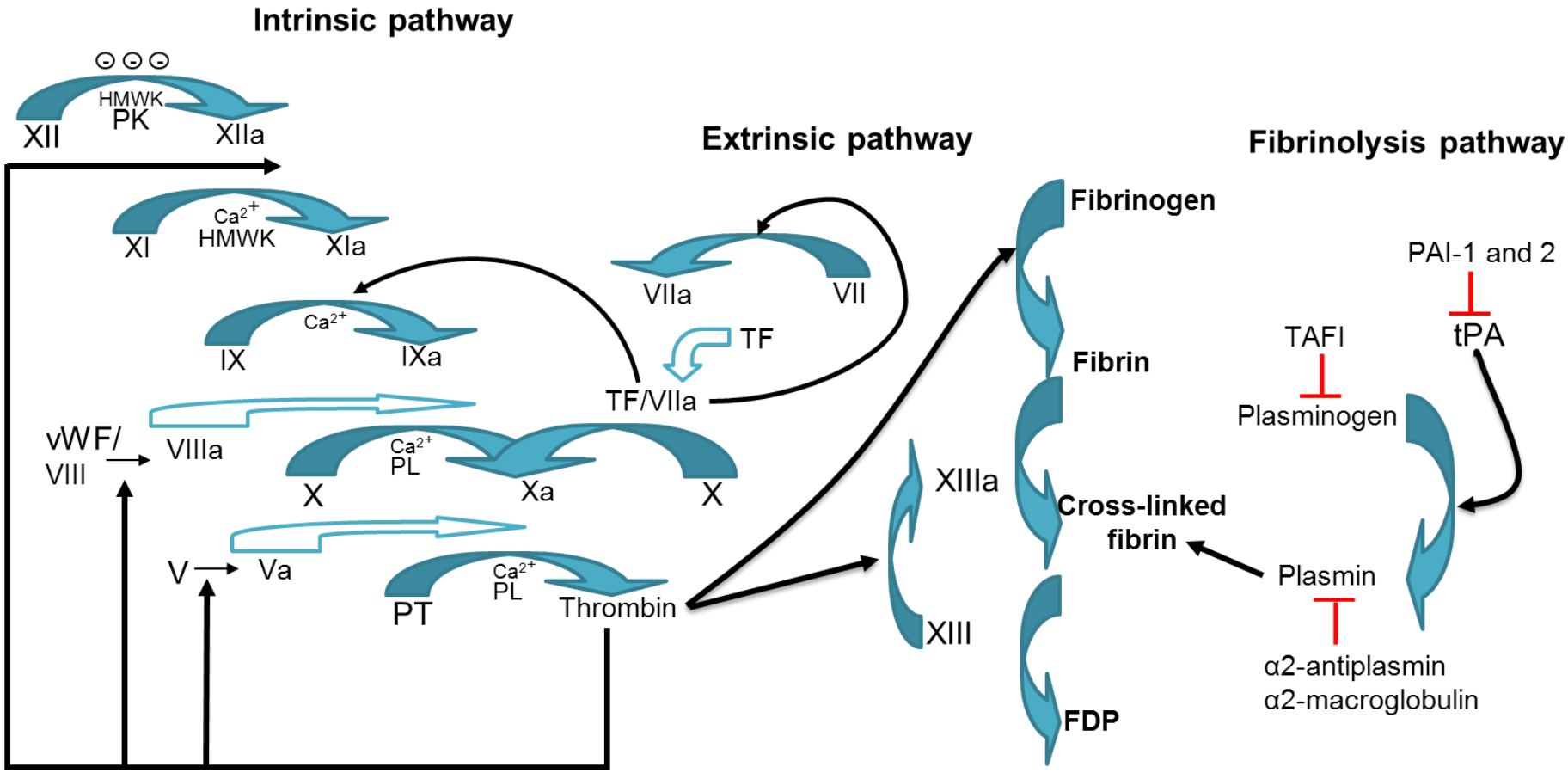

Intrinsic pathway
Extrinsic pathway
Fibrinolysis pathway
HMWK
PK
XII
XIIa
XI
Ca2+
HMWK
XIa
IX
Ca2+
IXa
VIIa
VII
TF
TF/VIIa
vWF/
VIII
VIIIa
X
Ca2+
PL
Xa
X
V
Va
PT
Ca2+
PL
Thrombin
XIIIa
XIII
Fibrinogen
Fibrin
Cross-linked
fibrin
FDP
PAI-1 and 2
tPA
TAFI
Plasminogen
Plasmin
α2-antiplasmin
α2-macroglobulin

Figure 1: The coagulation cascade and fibrinolytic pathway.

In the extrinsic pathway, exposed tissue factor (TF) forms a complex with factor VII, which cleaves and activates factor X and factor IX. In the intrinsic pathway, contact between high molecular weight kininogen (HMWK), prekallikrein (PK) and factor XII with a negatively charged surface results in the activation of factor XII (XIIa), which in turn cleaves and activates factor XI. Activated factor XI (XIa) then cleaves and activates factor IX. The intrinsic and extrinsic pathways converge at the stage of factor X activation. Activated factor X (Xa) forms a complex with activated V (Va) to form the prothrombinase complex which cleaves and activated prothrombin to generate thrombin. Thrombin cleaves and activates fibrinogen and FXIII to form a stable cross-linked fibrin clot. Thrombin also activates factor V, factor XI and cleaves factor VIII bound to von Willebrand factor (vWF) to aid the generation of more thrombin. During fibrin formation the fibrinolytic pathway is activated by tissue plasminogen activator (t-PA) binding to fibrin and activating plasminogen to form plasmin. Plasmin cleaves fibrin to form fibrin degradation products (FDP). Fibrinolysis inhibitors target t-PA (plasminogen activator inhibitor (PAI) 1 and 2), plasminogen (thrombin activatable fibrinolysis inhibitor (TAFI)) and plasmin (α2-antiplasmin and α2-macroglobulin).

1.3.3. Fibrin formation

Fibrin is formed by thrombin-mediated cleavage of fibrinogen in the final stages of activation of the coagulation cascade (Figure 1) to prevent blood loss and promote wound healing. Fibrinogen is a six-chain ($A\alpha_2$, $B\beta_2$, γ_2) polypeptide arranged into two outer D domains joined to the central E domain via a coiled coil region and an αC domain which begins at residue 111 in the D domain and is tethered to the E domain (Mosesson et al. 1998). Fibrinogen is present in plasma at ~ 2.5 mg/ml. During coagulation the serine protease thrombin sequentially cleaves the short N-terminal fibrinopeptides (FP)A and FPB from fibrinogen Aα and Bβ chains, respectively, to initiate polymerisation and the formation of half-staggered double stranded fibrin protofibrils. Protofibrils undergo lateral aggregation when they reach 600-800 nm in length to form fibrin fibres with the aid of αC intermolecular interactions (Weisel 2005). Fibrin fibres branch through binding of fibrin monomers to divergent fibrin monomers to form trimolecular and tetramolecular branch points, which ultimately lead to formation of a complex fibrin network (Figure 2) (Mosesson et al. 1993;Weisel et al. 1993). Fibrin fibres are strengthened by FXIII mediated cross-linking (described in section 1.3.5).

1.3.4. FXIII activation and mechanism of cross-link formation

FXIII is a member of the transglutaminase family, which catalyses the formation of covalent ε-(γ-glutamyl)lysine cross-links between plasma, extracellular matrix and intracellular substrates (Table 1). Plasma FXIII (FXIII-A2B2) has a heterotetrameric structure, composed of pairs of A subunits (83 kDa) and B subunits (80 kDa) (Komaromi et al. 2011;Schwartz et al. 1973) and is present in plasma at a concentration of ~ 22μg/ml. The structure of cellular FXIII is an A subunit homodimer (Henriksson et al. 1985;Schwartz et al. 1973). The FXIII-A subunit contains the active site of the enzyme and is synthesised by the following cell types: monocyte/macrophage, megakaryocyte/platelet, chondrocyte, osteoblast/osteocyte and hepatocytes (Kappelmayer et al. 1995;Komaromi et al. 2011;Nurminskaya and Kaartinen 2006). The FXIII-B subunit acts as a carrier protein in plasma for the hydrophobic A subunit and is synthesised and secreted by liver hepatocytes (Nagy et al. 1986).

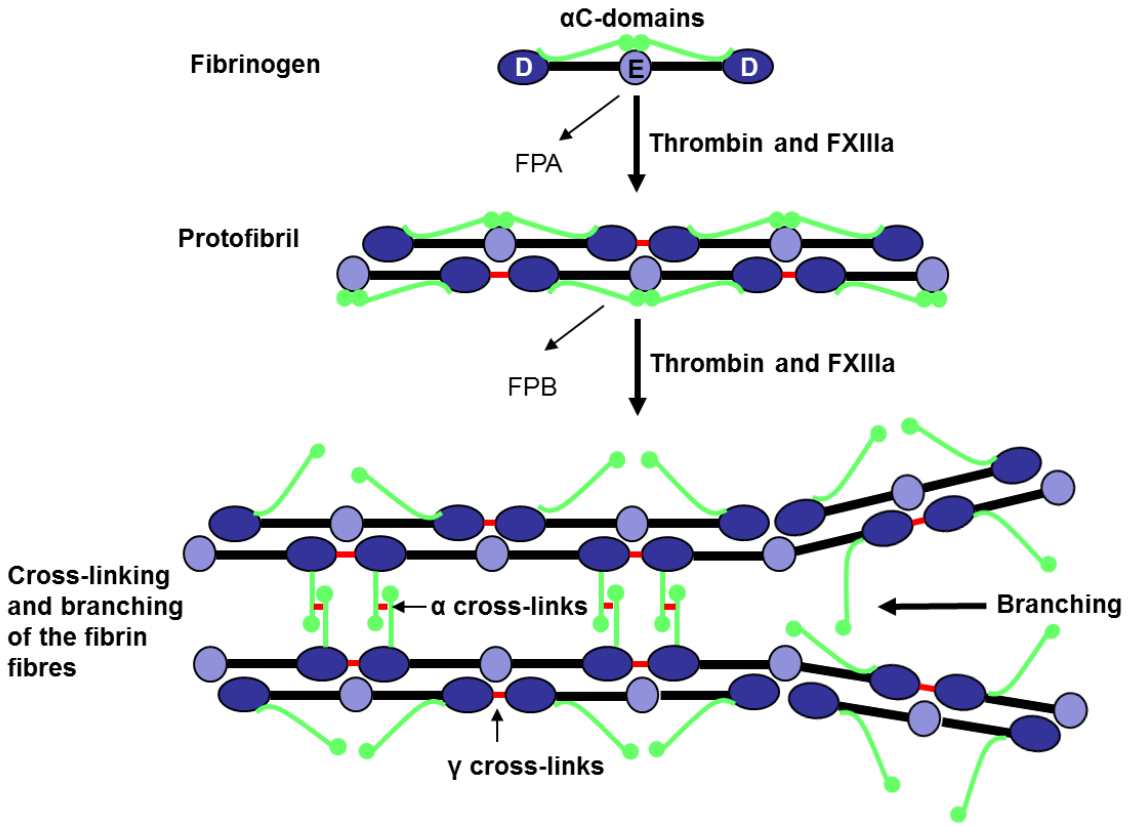


Figure 2: The formation of fibrin fibres

Thrombin cleaves fibrinopeptide A (FPA) from fibrinogen to initiate protofibril formation and activates FXIII to form cross-links between the fibrin γ-chains (red lines between D domains). Following thrombin cleavage of FPB the αC domains (green) are released to allow the formation of α-chain cross-link formation between protofibrils (red lines between αC domains). Fibrin branching occurs when fibrin monomers bind to divergent fibrin monomers.

During FXIII activation, FXIII-A2B2 binds to fibrin via the B subunit thus promoting the correct orientation for cleavage of the peptide bond between Arg37 and Gly38 of FXIII-A by thrombin, leading to the release of the 37 amino acid activation peptide (Hornyak and Shafer 1992;Takagi and Doolittle 1974). In the presence of calcium and fibrin thrombin-cleaved FXIII undergoes several conformational changes; initially FXIII-B dissociates from FXIII-A and then

reorientation of FXIII-A leads to exposure of the active site, forming FXIIIa (Lorand et al. 1974;Moaddel et al. 2000). FXIII-A activation is downregulated following γ-dimer formation, suggesting fibrin plays an activatory and regulatory role in FXIII-A activation (Lewis et al. 1985). Cellular FXIII in comparison is activated by an increase in cellular Ca^{2+} above 2 mM (Kristiansen and Andersen 2011). Following dissociation of the FXIII-B subunit the FXIII-A subunit catalytic triad (Cys314, His373, Asp 396) is exposed to allow substrate cross-linking (Lewis et al. 1985). FXIIIa uses a double displacement mechanism to form covalent ε-(γ-glutamyl)lysine cross-links between the γ-carboxy-amine group of a glutamine (amine acceptor) and the ε-amino group of a lysine (amine donor) residue. In stage 1, Cys314 within FXIII-A attacks the glutamine γ-carboxyamide group of an amine acceptor protein, displacing a molecule of ammonia to form a thioester intermediate. In stage 2, the reactive thioester intermediate is attacked by the lysine ε-amino group of the amine donor protein, displacing FXIII-A resulting in the formation of an isopeptide bond between the two substrate proteins and the release of FXIIIa (Loewy et al. 1961;Lorand et al. 1966). Alternatively in the absence of a lysine donor protein, water reacts with the thioester intermediate converting glutamine into glutamic acid. Based on this reaction FXIII substrates have previously been identified using molecular probes such as putrescine, dansylcadaverine and 5-(biotinamido)pentylamine (BP), which are cross-linked by FXIII to glutamine residues, whereas labelled synthetic peptides based on α2-antiplasmin are cross-linked into lysine residues of FXIII substrates. In these studies the glutamine and lysine residues within the FXIII substrate(s) were characterised by mass spectrometry or Edman sequencing analysis as outlined in Figure 3 and the identified substrates can be found in Table 1.

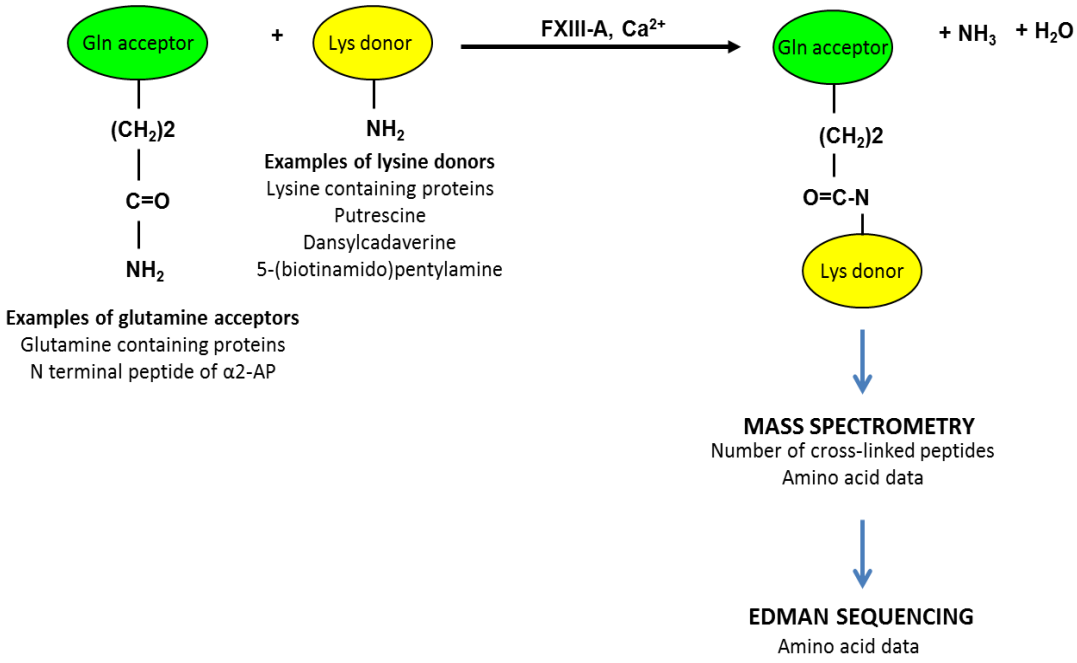


Figure 3: Mechanism for the identification of FXIII substrates

FXIII-A forms a thioester intermediate between a glutamine acceptor (α2-antiplasmin (α2-AP)) and a lysine donor, displacing a molecule of ammonia, water and with the release of FXIII-A. FXIII substrates and their reactive glutamine and lysine residues are identified by mass spectrometry and edman sequencing (Richardson et al. 2013).

1.3.5. Cross-linking of fibrin by FXIIIa

During fibrin formation the fibrin clot is stabilised by FXIIIa-induced cross-linking of the fibrin α and γ-chains as shown in Figure 2. FXIIIa rapidly forms cross-links between γ-chains of two fibrin molecules to form γ-dimers (Siebenlist et al. 2001;Standeven et al. 2007). Cross-link formation occurs between Lys406 on one γ-chain and either Gln399 or Gln398 on the neighbouring γ-chain (Gorman and Folk 1980). The fibrin α-chain is cross-linked by FXIIIa to form α-polymers, albeit at a much slower rate compared to γ-γ cross-links (Siebenlist et al. 2001). Cross-link formation occurs between Gln223 and either Lys508 or Lys539; between Gln237 and either Lys418, Lys508, Lys539 or Lys556; between Gln366 and Lys539; and between Gln563 and either Lys539 or Lys601 (Wang 2011). A number of additional

lysine donor sites in the α-chain have been identified by their ability to incorporate an N terminal peptide of α2-antiplasmin, including Lys580, Lys448, Lys606, Lys427, Lys429, Lys208, Lys224 and Lys219 (Matsuka et al. 1996;Sobel and Gawinowicz 1996). The multiple lysine donor sites identified in the α-chain provide the opportunity for the formation of a complex fibrin polymer whilst retaining the possibility of cross-linking numerous other plasma proteins such as α2-antiplasmin, thrombin activatable fibrinolysis inhibitor (TAFI), PAI-2, fibronectin and von Willebrand factor to fibrin (Table 1).

Cross-linking increases the stiffness and reduces the stretch of the fibrin network (Liu et al. 2010). Analyses of the elastic modulus of individual fibrin fibres, using optical tweezers, showed that cross-linked fibrin fibres were stiffer compared to non-cross-linked fibrin fibres (Collet et al. 2005), specifically fibrin α-chain cross-linking increased clot stability and rigidity by promoting protofibril aggregation and the formation of thicker fibres (Shen and Lorand 1983;Shen et al. 1974;Standeven et al. 2003). Using site directed mutagenesis of Lys406, Gln399 and Gln398 within the fibrinogen γ-chain, it was demonstrated that α-polymers produced an increase in clot stiffness of 2.5 fold, whereas γ-α-heteropolymers produced an increase in clot stiffness of 1.5 fold, compared to a 3.5 fold increase in clot stiffness in the presence of un-mutated fibrin (Standeven et al. 2007). This suggests that all forms of cross-links contribute to fibrin stiffness; however the formation of α-polymers contributes to the greatest extent.

Table 1: Substrates of activated FXIII

FXIII substrates including their reactive glutamine (Gln) and lysine (Lys) residues (Richardson et al. 2013)

Protein	Cross-link sites	Cross-link substrates	References
Fibrin α chain	Gln221, Gln237, Gln328 Gln366, Lys556, Lys539, Lys508, Lys580, Lys583, Lys418, Lys448, Lys601, Lys606, Lys427, Lys446, Lys429, Lys208, Lys224 Lys219	Fibrin α and Fibrin γ chains	(Cottrell et al. 1979;Matsuka et al. 1996;Siebenlist et al. 2001;Sobel et al. 1996;Wang 2011)
Fibrin γ chains	Gln399, Gln398, Lys406	Fibrin α and Fibrin γ chains	(Gorman et al. 1980;Siebenlist et al. 2001;Standeven et al. 2007)
Factor V	---	Factor V	(Francis et al. 1986;Huh et al. 1988)
Thrombospondin-1	---	Thrombospondin-1 and Fibrin α chain	(Bale et al. 1985;Bale and Mosher 1986;Lynch et al. 1987)
α2-antiplasmin	Gln2, Gln21, Gln419, Gln447	Fibrin α chain Lys303	(Lee et al. 2000;Lee et al. 2001)
Thrombin activatable fibrinolysis inhibitor (TAFI)	Gln2, Gln5, Gln292	TAFI and Fibrin α chain Lys77, Lys79, Lys212	(Mutch et al. 2003;Mutch et al. 2007;Valnickova and Enghild 1998)
Vitronectin	Gln93, Gln73, Gln84, Gln86	Vitronectin	(Sane et al. 1988;Skorstengaard et al. 1990)
α2-macroglobulin	Gln670, Gln669	Unknown	(Mosher 1976;Sottrup-Jensen et al. 1983)

Fibronectin	Gln3, Gln4, Gln16	Fibrin α chain and Collagen	(Corbett et al. 1997;Matsuka et al. 1997;McDonagh et al. 1981;Piercy-Kotb et al. 2012;Procyk and Blomback 1988)
Collagen	---	Fibronectin and von Willebrand factor	(Mosher et al. 1980;Mosher 1984;Mosher and Schad 1979;Piercy-Kotb et al. 2012)
Von Willebrand Factor	Gln313, Gln560	Fibrin α chain, Collagen and Laminin	(Bockenstedt et al. 1986;Takagi et al. 1995;Usui et al. 1993)
Plasminogen	Lys298, Gln322	Plasminogen and Fibronectin	(Bendixen et al. 1993;Bendixen et al. 1995)
Plasminogen activator inhibitor 2 (PAI-2)	Gln83, Gln84, Gln86	Fibrin α chain Lys148, Lys176, Lys230, Lys413	(Jensen et al. 1994;Ritchie et al. 1999;Ritchie et al. 2000;Ritchie et al. 2001)
Platelet Actin	Gln41	Fibrin α chain	(Cohen et al. 1980)
Platelet Myosin	---	Platelet myosin	(Cohen et al. 1979)
Vinculin	---	Unknown	(Serrano and Devine 2002)
Filamin	---	Unknown	(Serrano et al. 2002)
Angiotensin type 1 receptor	Gln315	Angiotensin type 1 receptor	(AbdAlla et al. 2004)

1.3.6. Fibrinolysis

Fibrin clot formation is a temporary response to wound healing and, as such, mechanisms involved in the breakdown of fibrin are important in preventing thrombosis. Fibrin is cleaved by plasmin, a serine protease produced upon cleavage of the inactive precursor plasminogen by tissue-type plasminogen activator (t-PA) or urokinase plasminogen activator (uPA) (Figure 1). Fibrin forms a surface for enhanced plasminogen cleavage as t-PA and plasminogen both bind to the αC domains (Tsurupa and Medved 2001) and D regions (Bosma et al. 1988;Schielen et al. 1991) of fibrin to enable production of the enzyme/substrate complex and the generation of plasmin. Fibrin is degraded by plasmin within the coiled coil located between regions D and E to form the following fibrin degradation products D-D:E1, D-D dimer, E3 and remnants of the αC domain (Figure 4). Degradation of fibrin also generates C terminal lysine and arginine residues which act as high affinity binding sites for plasminogen, thus promoting plasmin generation and acceleration of fibrin cleavage (Suenson et al. 1984;Tran-Thang et al. 1986). Resistance of the fibrin clot to plasmin-mediated fibrinolysis was found to be dependent on the formation of γ-dimers or highly complex α-multimers which conferred increased resistance to fibrinolysis (Siebenlist and Mosesson 1994).

To prevent uncontrolled fibrin breakdown fibrinolysis is controlled at numerous levels. Firstly t-PA is inhibited by the plasminogen activator inhibitors (PAI)-1 and 2. PAI-1 and 2 form complexes with free PA, preventing plasmin generation and therefore fibrinolysis and proteolysis (Ritchie et al. 1999). PAI-2 and not PAI-1 is cross-linked to the fibrin α chain by FXIIIa (Ritchie et al. 2000;Ritchie et al. 2001). Cross-linked PAI-2 was also functionally active to inhibit uPA and t-PA (Ritchie et al. 1999;Ritchie et al. 2001). Secondly, plasmin is directly inhibited by the serine protease inhibitor α2-antiplasmin (α2-AP) which forms a covalent bond with the active site serine of plasmin, thus inhibiting plasmin-induced breakdown of fibrin (Longstaff and Gaffney 1991). FXIIIa-dependent cross-linking of Gln 2 of α2-AP to fibrin Lys 303 during clot formation is important in providing localised protection from plasmin-mediated lysis (Fraser et al. 2011). Alternatively TAFI cleaves the C terminal lysine and arginine residues of plasmin digested fibrin thus preventing plasminogen binding, activation and therefore plasmin generation. Physiological concentrations (70 – 250 nM) of activated TAFI also inhibit plasmin directly, to

further inhibit fibrinolysis (Wang et al. 1998). The activation peptide of TAFI is cross-linked to the fibrin α chain by FXIIIa (Mutch et al. 2003), with cross-linking of TAFI prolonging fibrinolysis in plasma and whole blood thrombi (Mutch et al. 2007). Inhibition of fibrinolysis is dependent on FXIII-dependent cross-linking of fibrinolysis inhibitors to fibrin and supports the incorporation of numerous plasma proteins during clot formation.

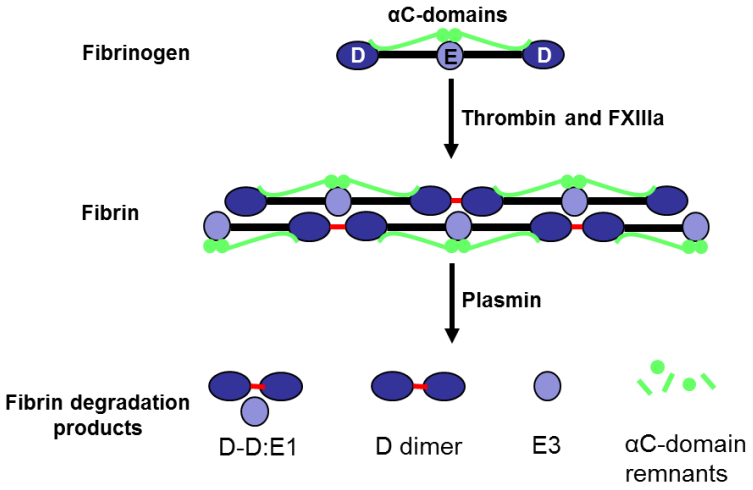


Figure 4: Schematic representation of fibrin degradation products

Plasmin cleaves cross-linked fibrin in the coiled coil region between domains D and E resulting in fibrin degradation products D-D:E1, D-D dimer, E3 and αC-domain remnants.

1.4. The influence of fibrin structure in CVD

Thrombosis is the formation of fibrin clots in the blood vessel resulting in impaired blood flow and ultimately leading to MI and stroke. In prospective studies, fibrinogen and PAI-1 were associated with an increased risk of MI, CAD and stroke (Danesh et al. 2005;Thogersen et al. 1998), additionally fibrinogen was positively associated with established risk factors for CVD including age and smoking (Danesh et al. 2005). Fibrin clots from MI patients have altered fibrin architecture with stiffer

fibrin clots containing fibres which are shorter and increased in number, with smaller pores and are susceptible to slower lysis times and reduced permeability (Collet et al. 2006;Fatah et al. 1996). Individuals with a number of metabolic risk factors for T2DM are at increased risk of CVD and as such also contain altered fibrin clot structure with reduced clot density and increased clot lysis times, with an increase in the number of metabolic risk factors associated with an increase in clot density and fibrinolysis times (Carter et al. 2007). Marchi-Cappelletti (2010) further showed that fibrin clot permeation was reduced and fibrin compaction was increased in individuals with T2DM (Marchi-Cappelletti and Suarez-Nieto 2010). The change in fibrin structure as a result of T2DM was previously found to be the result of fibrinogen concentration rather than fibrinogen glycation (Pieters et al. 2006). Increased fibrinolysis times are also associated with venous thrombosis (Lisman et al. 2005). Changes in fibrin architecture pre-date CVD symptoms, as relatives of CAD patients produce ex vivo plasma clots with reduced permeability and shorter fibrin fibres (Mills et al. 2002). These studies suggest that fibrin structure is a risk factor for future CVD development. Furthermore, fibrin clots formed from platelet poor plasma form slower and contain fibrin fibres 2-4 times larger than fibrin clots formed from purified fibrinogen (Carr 1988), suggesting other plasma proteins contribute to fibrin structure and therefore CVD development.

1.5. The influence of inflammation in CVD

Inflammation occurs as a response to tissue damage or infection. In CVD inflammation occurs at all stages of development from early atheroma formation through to plaque rupture and thrombosis. During early atheroma formation inflammation occurs via monocyte infiltration of the endothelial surface to form macrophages and foam cells. Foam cells release inflammatory cytokines IL1β, IL6 and TNFα, which recruite additional immune cells (T cells and mast cells) and vascular smooth muscle cells to the endothelium (Barillari et al. 2001) and promote up-regulation of vascular adhesion molecules. T cells and mast cells secrete a number of cytokines (IFNγ, IL2 and TNFα) which promote the continued immune cell infiltration (Strieter et al. 1989) and collagen secretion from vascular smooth muscle cells (Amento et al. 1991). Inflammation also promotes a prothrombotic

environment, for example IL6 stimulates production of fibrinogen and C-reactive protein (CRP) in hepatocytes (Castell et al. 1990), whereas mast cells, macrophages and vascular smooth muscle cells synthesise and secrete tissue factor, t-PA, uPA and PAI-1 (Mach et al. 1997;Wojta et al. 1993;Wojta et al. 2003).

In CVD localised cytokine production may lead to systemic inflammation due to the synthesis of inflammatory proteins by the liver during the acute phase response. In recent years systemic inflammatory proteins have been measured as a marker of CVD risk. Of these proteins CRP has been measured in a number of prospective studies, with CRP a predictor of a future CVD event (MI and/or stroke), in men and women with high baseline levels (Ridker 1998;Ridker et al. 1998). CRP is also a predictor of future CVD mortality (Kaptoge et al. 2010;Strandberg and Tilvis 2000). CRP concentrations of <1, 1-3, >3 mg/L are associated with a low, medium and high risk of developing CVD (Ridker et al. 2003). Furthermore, systemic CRP levels are associated with atherosclerotic plaque severity with 3.2 mg/L, 2.9 mg/L and 2.5 mg/L associated with ruptured, eroded and stable atherosclerotic plaques respectively (Burke et al. 2002). In a prospective study, Hashimoto et al (2001) found that high systemic CRP levels were associated with increased plaque number and plaque severity after a 1 year follow-up, even after accounting for conventional CVD risk factors (Hashimoto et al. 2001). Using immunohistochemistry, CRP has been positively stained within atherosclerotic plaques, with increased systemic CRP associated with increased staining of CRP within atherosclerotic plaques (Burke et al. 2002). Within atherosclerotic plaques CRP co-localises with macrophages (Burke et al. 2002;Eisenhardt et al. 2009) and the terminal complement complex, C5b-9 (Torzewski et al. 1998a).

CRP also activates another inflammatory response in the form of the complement cascade. CRP binds to microbial surfaces via phosphocholine and recruits C1q of the classical pathway of complement to initiate complement activation, however CRP also regulates complement activation by binding complement inhibitors C4 binding protein and factor H, thus halting complement activation at the point of C3 (Mihlan et al. 2011). For this reason the role of complement in CVD is a rapidly emerging area of research in the context of CVD risk and pathogenesis.

1.6. Complement

1.6.1. Complement Cascade

The complement system forms a critical component of the innate immune response and consists of plasma and membrane associated proteins, which form three pathways of activation, the classical, lectin and alternative pathways (Figure 5). The classical pathway is initiated when antibodies or CRP binds to foreign surfaces initiating binding of C1q of the C1 complex (C1q, C1r and C1s) which promotes conformational changes in C1r and C1s required for their activation (Golan et al. 1982;Mihlan et al. 2011). C1s cleaves both C2 and C4 to form the classical pathway C3 convertase (C4bC2a) (Nagasawa and Stroud 1977). The lectin pathway is activated by mannose on the surface of pathogens interacting with mannose binding lectin (MBL) (Neth et al. 2000), which binds the MBL associated proteins (MASPs) 1, 2 and 3 initiating a conformational change in MASP2 which cleaves C4 (Moller-Kristensen et al. 2007;Thiel et al. 1997). C4b generated as a result of this cleavage binds to the pathogen surface along with C2 which is then cleaved by MASP1 and 2 to form the C3 convertase (C4bC2a) (Moller-Kristensen et al. 2007). The alternative pathway unlike the classical and lectin pathways is constitutively activated at low levels (tick over) by the hydrolysis of C3 to form C3(H_2O) (Pangburn et al. 1981). C3(H_2O) recruits factor B and factor D to form a soluble C3 convertase (C3(H_2O)Bb) which cleaves C3 to form C3a and C3b deposited on the pathogen surface (Pangburn et al. 1981). The alternative pathway is also activated by C3b generated as a result of the classical and lectin pathways. On the pathogen surface, C3b recruits factor B forming C3bB in an open conformation. Factor D binds to the factor B portion of this complex and cleaves the factor B to form C3bBb with the release of Ba (Forneris et al. 2010). The C3 convertase is stabilised by properdin (Hourcade 2006), preventing its cleavage by factor I in the presence of factor H. The C3 convertase produced via the three pathways cleaves C3 to form C3a and C3b. C3b combines with the C3 convertase to form the C5 convertase (C4bC2aC3b or C3bBbC3b), which is responsible for cleaving C5 into C5a and C5b (Pangburn and Rawal 2002). C5b binds to the pathogen surface and recruits C6, C7, C8 and numerous C9 molecules to form the membrane attack complex C5b-9 (Kolb and Mullereberhard 1975), which is responsible for forming pores in the cell membranes of pathogens resulting in cell lysis (Bhakdi and Tranumjensen 1986). Following

complement activation a number of potent anaphylatoxins are produced including C3a and C5a. Anaphylatoxins are responsible for chemotaxis of phagocytes and degranulation of granulocytes. These effects are mediated by the binding of C3a and C5a to the analphylatoxin receptors C3a receptor (C3aR) and C5a receptor (C5aR) expressed on the surfaces of inflammatory cells (Werfel et al. 2000;Zwirner et al. 1999).

To prevent uncontrolled complement activation causing tissue damage, complement is regulated by soluble and membrane associated inhibitors as outlined in Table 2. Complement is tightly regulated at three points including initiation, C3 convertase formation and membrane attack complex formation. To prevent complement initiation, C1 inhibitor binds and inactivates the serine proteases (C1r, C1s, MASP1 and MASP2) responsible for initiating the classical (Chesne et al. 1982) and lectin (Presanis et al. 2004) pathways of complement. To prevent continuation of the complement cascade at the point of C3 convertase formation several proteins and processes are involved; firstly cofactor inactivation/cleavage of C3b and C4b by factor I with cofactors including membrane cofactor protein which binds C3b and C4b on host cells (Liszewski et al. 2000), C4 binding protein which binds C4b (Fujita et al. 1978) and factor H which binds C3b (Wu et al. 2009b). Secondly dissociation of the already formed C3 convertase (C4b2a and C3bBb) by decay accelerating factor (DAF) resulting in the release of C2a and Bb (Fujita et al. 1987); C4 binding protein resulting in the release of C2a (Gigli et al. 1979) and factor H resulting in the release of Bb (Wu et al. 2009b). Finally to prevent the formation of the membrane attack complex, protectin prevents C9 polymerisation by preventing C9 insertion into the lipid bilayers of host cells (Meri et al. 1990).

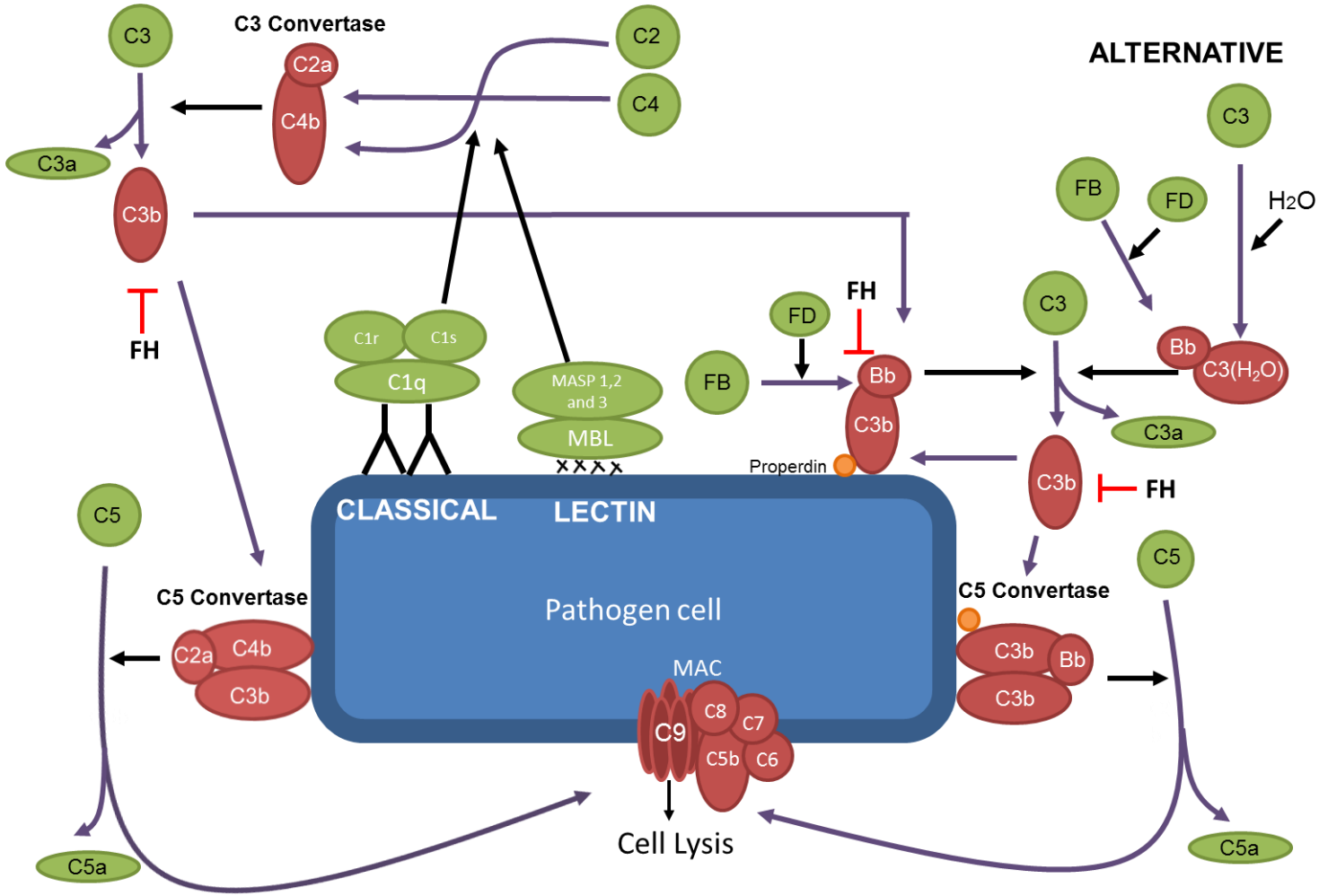

C3
C3 Convertase
C2a
C4b
C2
C4
C3a
C3b
FH
C1r
C1s
C1q
MASP 1,2 and 3
MBL
FD
FB
FH
Bb
C3b
Properdin
ALTERNATIVE
C3
FB
FD
H2O
Bb
C3(H2O)
C3a
C3b
FH
C5
C5 Convertase
C2a
C4b
C3b
CLASSICAL
LECTIN
Pathogen cell
MAC
C9
C8
C7
C5b
C6
C5 Convertase
C3b
Bb
C3b
C5
C5a
Cell Lysis
C5a

Figure 5: The complement cascade

The classical pathway is activated when the C1 complex (C1q, C1r, C1s) bind to antibodies and/or CRP bound to foreign surfaces. The C1s component of the C1 complex cleaves C2 and C4 to form the C3 convertase (C4bC2a). The lectin pathway is activated by the binding of mannose binding lectin (MBL) to mannose on the pathogen surface, which recruites MBL-associated serine proteases (MASP) 1, 2, 3. MASP2 cleaves C2 and C4 to also form the C3 convertase. The C3 convertase cleaves C3 to form C3a and C3b, with C3b recruited to form the C5 convertase (C4bC2aC3b) or recruited to the alternative pathway activation. The alternative pathway is activated by the hydrolysis of C3, with hydrolysed C3 recruiting factor B which is cleaved by factor D to form soluble and membrane bound C3 convertase (C3bBb). Membrane bound C3 convertase is stabilised by properdin. Factor H (FH) inhibits fluid and surface bound C3b by acting as a cofactor for factor I cleavage of C3b. Factor H also aids the dissociation of Bb from C3b, thus inhibiting C3 convertase formation. The C3 convertase cleaves C3 into C3a and C3b, with C3b recuited to the C5 convertase (C3bBbC3b). The C5 convertase from all three pathways cleaves C5 to form C5a and C5b. C5b binds to the pathogen surface and recuits C6, C7, C8 and numerous C9 molecules to form the membrane attack complex (MAC).

Table 2: Regulators of the complement system

Name	Soluble/membrane	Role
C1 Inhibitor	Soluble	Binds and inactivates C1r and C1s of the classical pathway and MASP1 and 2 of the lectin pathway
C4 Binding Protein	Soluble	Cofactor for factor I cleavage of C4b, Accelerates the classical and lectin pathway C3 convertase (C4b2a) decay with the release of C2a
Factor H	Soluble	Cofactor for factor I cleavage of C3b, Accelerates the alternative pathway C3 convertase (C3bBb) decay with the release of Bb
Membrane Cofactor Protein (CD46)	Membrane	Cofactor for factor I cleavage of C3b and C4b
Decay Accelerating Factor (CD55)	Membrane	Prevents C3bBb assembly, aids dissociation of C3bBb and C4bC2a with the release of C2a and Bb
Protectin (CD59)	Membrane	Prevents formation of the membrane attack complex by preventing C9 polymerisation

1.6.2. Complement C3

Complement C3 is a two chain (α chain 110 KDa; β chain 75 KDa) polypeptide connected by a disulphide bond, which is present in plasma at a concentration of ~1.2 mg/ml. Complement C3 is cleaved by all three pathways of complement in the generation of C3a and C3b which function in many roles within immunity as highlighted in Figure 6. C3a is an anaphylotoxin which recruits phagocytic cells (neutrophils and monocytes) via chemotaxis to the injury site (Sarma and Ward 2011), causes degranulation of eosinophils (Daffern et al. 1995) and the proliferation and differentiation of T cells (Strainic et al. 2008). These effects are mediated by the binding of C3a to C3aR expressed on the surfaces of these inflammatory cells (Werfel et al. 2000;Zwirner et al. 1999).

C3b binds and is covalently attached to cell surfaces and recruits factor B forming C3bB in an open conformation which is cleaved by factor D to produce the alternative pathway C3 convertase (C3bBb) with the release of Ba (Forneris et al. 2010) to initiate the alternative pathway. C3b is also recruited to the C3 convertase to form the C5 convertase allowing completion of the complement cascade and in the generation of the membrane attack complex (C5b-9) as described in section 1.6.1. Alternatively the C3 convertase amplifies the complement response by cleaving C3 molecules to generate more C3a and C3b. C3b is also an opsonin and binds to nucleophilic groups and carbohydrates on cell surfaces via its thioester domain forming an intramolecular acyl-imidizole bond (Law and Dodds 1997;Sahu et al. 1994). The thioester domain is exposed as a result of C3 cleavage to form C3b (Janssen et al. 2005). The C3b α chain is cleaved by factor I in the presence of the cofactor factor H between residues 1281/1282 and 1298/1299 to form iC3b with the release of C3f (Sahu and Lambris 2001), this process is sufficient to prevent the formation of the alternative pathway C3 convertase (Sahu et al. 1998). C3b and iC3b are opsonins and deposit on bacteria such as *S. aureus*, with conversion of C3b to iC3b occurring by factor I in the absence of factor H (Cunnion et al. 2004). Complement receptor (CR) 1 on eosinophils (Hatano et al. 2009) and neutrophils (Gros et al. 2008) and C3Ig receptors on macrophages bind foreign particles opsonised with C3b and iC3b, whereas CR3 and CR4 on leukocytes bind foreign particles opsonised with iC3b to initiate phagocytosis of pathogens (Gros et al. 2008).

The iC3b α chain is further cleaved by factor I at a third site between residues 932/933 to form C3c and C3dg (Sahu et al. 2001). The activation products iC3b and C3d/C3dg immobilised to a cell surface or in solution bind to CR2 (Sarrias et al. 2001) mainly expressed on B cells. Antigen particles coated in the opsonin C3dg stimulate B cell proliferation and differentiation 1000-fold, due to the combined binding of C3dg to CR2 and the antigen to the B cell antigen receptor (Morgan et al. 2005), thus enhancing the adaptive immune response.

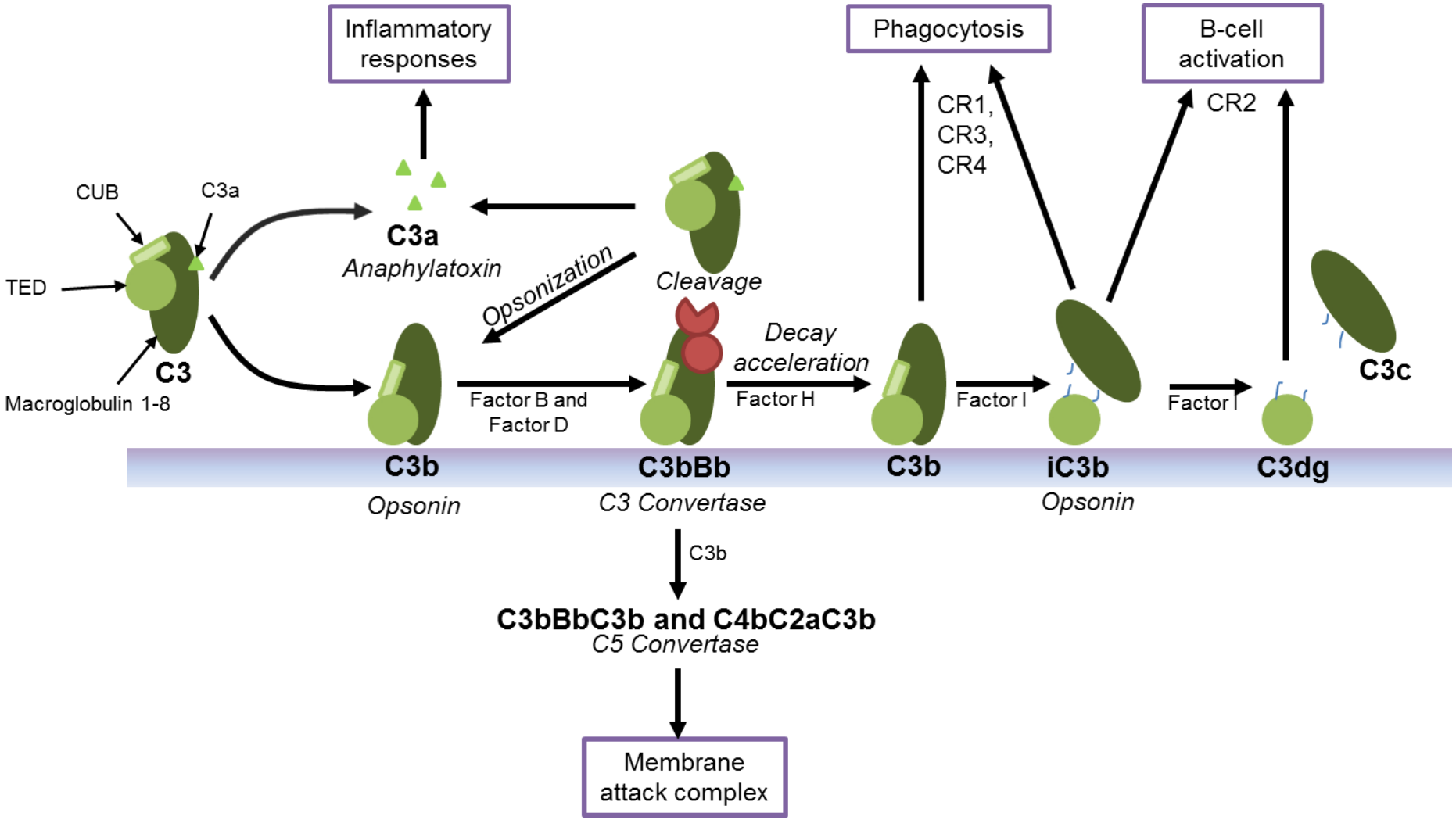

Inflammatory responses
Phagocytosis
B-cell activation
CR1, CR3, CR4
CR2
CUB
C3a
TED
C3
Macroglobulin 1-8
C3a
Anaphylatoxin
Opsonization
Cleavage
Factor B and Factor D
Decay acceleration
Factor H
Factor I
Factor I
C3c
C3b
Opsonin
C3bBb
C3 Convertase
C3b
iC3b
Opsonin
C3dg
C3b
C3bBbC3b and C4bC2aC3b
C5 Convertase
Membrane attack complex

Figure 6: Schematic representation of the role C3 fragments play in immunity.

C3 is made up of the following domains; macroglobulin domains 1-8, thioester containing domain (TED), complement C1r/C1s Uegf,Bmpl (CUB) and C3a domain. C3 is cleaved by the C3 convertase to form the anaphylotoxin C3a and the opsonin C3b. In the presence of factor B, factor D and C3b the alternative pathway C3 convertase is formed, cleaving more C3 into C3a and C3b. C3b combines with the C3 convertase to form the C5 convertase with completion of the complement cascade in the formation of the membrane attack complex. The alternative pathway C3 convertase is broken down in a process known as decay acceleration to form C3b. C3b is further cleaved by factor I in the presence of factor H to initially form the opsonin iC3b and finally C3dg and C3c. C3b and iC3b promote phagocytosis by binding to complement receptors (CR) 1, 3 and 4, whereas iC3b and C3dg promote B cell activation by binding to CR2. Figure adapted from (Gros et al. 2008).

1.6.3. Factor H

Factor H is a single polypeptide chain of 155 KDa, made up of 20 short consensus repeats (SCR) and is present in plasma at 110-615 µg/ml. Factor H is important for cofactor activity and decay acceleration activity. In decay acceleration the SCR 1 and 2 of factor H bind macroglobulin domains 2, 6 and 7 of C3b, thus dislodging Bb and therefore resulting in decay of the alternative pathway C3 convertase (Wu et al. 2009b). Alternatively during cofactor activity the SCR 1-4 bind the CUB domain of C3b; this interaction is essential in allowing the generation of an interface for factor I binding and subsequent cleavage of C3b to iC3b (Wu et al. 2009b). Factor H regulates both C3b in the fluid phase and membrane bound C3b, whilst inactivation of C3b in the fluid phase is rapid, inactivation of membrane bound C3b is dependent on the composition of the cell surface. Cell surfaces containing sialic acids, glycosaminoglycans or sulphated polysaccharides (heparins) increase the affinity of factor H for C3b as simultaneous recognition of both the cell surface and C3b is required for effective inactivation of C3b (Rodriguez de et al. 2004). This process is highlighted by the large amount of C3b and iC3b opsonisation on surfaces in the absence of sialic acids, glycosaminoglycans or sulphated polysaccharides (Rodriguez de et al. 2004). Alternatively polyanion binding sites in the SCR 19-20 are responsible for protecting host cells from alternative pathway complement activation (Morgan et al. 2011). This was highlighted using sheep red blood cells whereby cell lysis occurred in the presence of recombinant factor H lacking SCR 19-20 (Pangburn 2002). SCR 7 and SCR 12-14 also contribute to host cell defence, albeit to a lesser extent compared to SCR 19-20 (Pangburn 2002). Host cell protection by factor H is achieved by binding of SCR 19-20, 7 and 12-14 to the polyanions on host surfaces, thus preventing C3b deposition (Pangburn 2002). In the absence of factor H, the alternative pathway is uncontrolled, with consumption of C3 and components of the membrane attack complex.

1.7. Clinical studies of complement activation and CVD

As CRP is a strong predictor of CVD risk and CRP is known to activate the complement system, activated components of the complement system including soluble C5b-9, C5a and the central effector in complement activation, C3 have been investigated for an association with CVD risk. In a prospective study, patients with pre-existing atherosclerosis, followed for 22 months, had an increased CVD risk in the presence of increased C5a, with a 37% increased risk in the highest quartile (Speidl et al. 2005). In stroke patients, levels of soluble C5b-9 increased until day 7 and remained high until day 12 following a stroke, with soluble C5b-9 levels correlating with the infarction size (Pedersen et al. 2004). In a case control study soluble C5b-9 was increased in stroke patients compared to controls with atherosclerosis, with an increase in soluble C5b-9 correlating with stroke severity (Szeplaki et al. 2009). In a case-control study soluble C5b-9, C4d, C3 activation products (iC3b, C3a and C3d) and Bb were all found to be increased in acute MI patients compared with controls (Yasuda et al. 1990). Martel et al (2012) further found that levels of C5a and soluble C5b-9 increased 37% and 96% respectively at 24 hours following MI compared with controls (Martel et al. 2012), suggesting complement activation following MI may lead to tissue damage and further CVD risk. Siezenga et al (2009) further showed that soluble C5b-9 and C3 were significantly higher in South Asian diabetics and non-diabetics compared with healthy Caucasian controls. When the diabetic South Asian group was compared with the non-diabetic South Asian group the levels of soluble C5b-9 and C3 remained significantly higher (Siezenga et al. 2009). These studies suggest that complement activation products are a marker of current inflammation and disease severity in CVD patients.

1.7.1. Associations between complement C3, CVD and T2DM

Complement C3 is the central effector in complement activation and as such many studies have focused specifically on the association between C3 and CVD and T2DM development. In prospective studies, individuals in the highest C3 tertile had a 5.8-fold increased risk of an MI (Muscari et al. 1995). Onat et al. (2005) found that C3 levels above 1.6 g/L gave a 1.9-fold increase in coronary heart disease prevalence

(Onat et al. 2005). Whereas analysis of C3 levels following acute ischaemic stroke showed that levels increased to a peak at 3-5 days, after which levels steadily decreased, however C3 levels remained significantly higher than healthy controls (Tamam et al. 2005). Measuring the C3/C4 ratios in acute coronary syndrome patients over a year showed that it was a strong predictor of future cardiovascular events compared to measuring C3 or C4 alone, with half the individuals in the highest C3/C4 ratio likely to have a cardiovascular event during the follow up period (Palikhe et al. 2007).

In case-control studies levels of C3 were significantly higher in South Asian stroke patients and relatives with a family history of stroke compared to healthy white controls (Somani et al. 2006), suggesting that C3 may be a marker of CVD development and that this development may pre-date disease presentation. In patients with coronary artery disease, including those with undetectable, minimal or extensive stenosis on angiogram, C3 levels were significantly higher than healthy controls (Ajjan et al. 2005). C3 levels were also increased in both male and female patients with coronary heart disease and C3 was associated with previous MI and stroke (Muscari et al. 2000;Onat et al. 2005). C3 was also found to be elevated in patients with MI compared to healthy controls even after adjusting for conventional CVD risk factors (Carter et al. 2009), suggesting C3 is also a marker of current inflammation in CVD patients. Within these patient groups, individuals with T2DM or impaired fasting glucose (IFG) had significantly higher C3 levels compared with patients without T2DM or IFG (Ajjan et al. 2005;Carter et al. 2009) and C3 was also found to predict future development of T2DM (Engstrom et al. 2005). In relatives with a family history of diabetes, levels of C3 were significantly higher than healthy controls, with a significant association of C3 with prolonged fibrinolysis times observed in these individuals (Schroeder et al. 2010), suggesting C3 is pro-thrombotic and may contribute to increased CVD risk. C3 also significantly correlated with conventional cardiovascular risk factors (cholesterol, triglycerides, fibrinogen, CRP, BMI and waist to hip ratio, fasting glucose, HbA1c, insulin and homeostatic model assessment (HOMA)) in a number of these studies (Ajjan et al. 2005;Carter et al. 2009;Onat et al. 2005;Onat et al. 2010).

Obesity is a known risk factor for the development of diabetes and CVD. In non-diabetic healthy individuals, C3 predicts the risk of future weight gain, with

higher levels of C3 associated with higher BMI and weight (Engstrom et al. 2005). In obese patients undergoing a six week low calorie diet, weight loss coincided with a decrease in C3 (Hernandez-Mijares et al. 2012). Weight loss and increased physical activity was responsible for a reduction in C3 levels, with little influence of drugs towards diabetes and hypertension on C3 levels (Muscari et al. 2005). These results are understandable as adipocytes contribute to the C3 plasma pool, by producing 10% of the amount produced by the liver (Gabrielsson et al. 2003).

1.7.2. Associations between factor H, CVD and T2DM

Factor H is a regulator of complement, and as such alterations in factor H may therefore play a role in inflammatory aspects of T2DM and CVD. People with T2DM have increased factor H and factor B levels, with factor H being negatively associated with insulin sensitivity. Improvements in insulin sensitivity as a result of treatments with rosiglitazone or weight loss were also shown to decrease factor H levels in these individuals (Moreno-Navarrete et al. 2010). The up-regulation of factor H in this study as a result of insulin sensitivity may be important in combating the up-regulation observed in C3 as a result of T2DM as discussed in section 1.7.1

A number of studies have looked at the role of the factor H polymorphism Y402H in determining risk of CVD. The factor H Y402H polymorphism involves the substitution of a tyrosine residue for a histidine in the SCR 7. This mutation was found to affect binding of factor H to CRP, but not heparin (Laine et al. 2007). In two case control studies looking at Caucasian males and relatives with a family history of MI, the Y402H polymorphism was not associated with any ischemic event (Stark et al. 2007;Zee et al. 2006). In a similar study looking at both male and female Caucasians, the same observation was noted in men; however, in females the HH genotype was inversely associated with coronary heart disease when onset was before 65 years (Pai et al. 2007). Caucasians in a prospective study had a greater incidence of the HH genotype compared to African Americans, and this genotype was a significant predictor of ischemic stroke, with a significant increase in hypertension and carotid artery thickening in these individuals (Volcik et al. 2008). In a prospective study looking at an elderly population (>85 years) the YH and HH genotypes had a 1.23 and 1.51 fold increase in cardiovascular mortality compared to

the YY genotype. This study also found that individuals with the HH genotype had increased CRP and IL-6 levels during the follow-up period of the study (Mooijaart et al. 2007). The Rotterdam study showed that individuals with the HH genotype and no history of CHD had an increased risk of MI (Kardys et al. 2006). As highlighted by these few studies, the role of factor H in CVD and T2DM is limited with currently contradictory findings. As discussed in section 1.7 complement activation is an important contributor to CVD risk, since complement activation is regulated by complement inhibitors it suggests the down-regulation of inhibitors such as factor H as a risk factor for CVD.

1.8. In vitro studies of complement activation and CVD

1.8.1. The influence of complement in atherosclerosis

The role of complement in CVD is a rapidly developing area of research with evidence suggesting a role for complement in both atherosclerosis and thrombosis as highlighted in the following sections. Complement components have been observed in atherosclerotic plaques at all stages of development. During the initial stages of atherosclerotic plaque development, the classical pathway of complement is activated as a result of binding between C1q and gC1qR receptor on vascular endothelial cells. In response to increased sheer stress, endothelial cell expression of gC1qR is upregulated leading to deposition of activated complement components C3b and C5b-9, suggesting endothelial cells can support classical pathway activation and full complement activation (Yin et al. 2007). In a study by Yasojima et al. (2001) atherosclerotic plaque tissue was found to contain mRNA and protein relating to classical pathway components from C1r, C1s to C8. Interestingly this study did not show up regulation of mRNA or protein relating to complement inhibitors C1-inhibitor, DAF, C4 binding protein or CD59, suggesting ongoing synthesis of complement components within the plaque, with uncontrolled complement activation (Yasojima et al. 2001). Oksjoki et al. (2003) further found that in the superficial layer of early atherosclerotic plaques factor H bound to proteoglycans, thus limiting complement activation to C3 convertase stage, whereas deeper in the intima factor H

was absent enabling full activation of the complement cascade and the generation of C5b-9 (Oksjoki et al. 2003).

Complement activation within atherosclerotic plaques has been observed by a number of studies using immunohistochemistry. In early atherosclerotic plaques C5b-9 was found to co-localise with CRP (Torzewski et al. 1998a) and enzymatically modified low density lipoproteins (Torzewski et al. 1998b), due to binding between C1q and enzymatically modified low density lipoprotein resulting in the activation of the C1 complex (Biro et al. 2007). Ruptured and non-ruptured atherosclerotic plaques removed from the coronary arteries of individuals who died of acute MI were examined for complement components and inflammatory cells. Immunostaining identified C5b-9 present in the intima of both ruptured and non-ruptured plaques with iC3b present at the rupture site only (Laine et al. 2002). These observations support the suggestion that deep in the intima complement inhibitors are absent allowing uncontrolled complement activation, whereas at the rupture site C3 is cleaved by plasma factor I in the presence of plasma factor H to form inactivated iC3b.

Complement activation also results in the production of anaphylatoxins C3a and C5a, which are responsible for inflammatory cell recruitment. In ruptured plaques high concentrations of T cells, macrophages and mast cells were observed, suggesting that anaphylotoxins may contribute to plaque instability (Laine et al. 2002). Histological staining and mRNA analysis of atherosclerotic plaques shows that expression of the anaphylotoxin receptors C3aR and C5aR were increased 5 fold, with expression occurring on macrophages, T cells, endothelial cells and smooth muscle cells (Oksjoki et al. 2007). Speidl et al. (2011) further showed that C5a production within the lipid rich regions of the atherosclerotic plaques were increased, with co-localisation of C5a with C5aR on macrophages. In vitro binding between C5a and C5aR on macrophages was found to promote the expression and production of the MMP-1 and 9, with an anti-C5aR antibody preventing the expression and production of MMP-1 and 9 (Speidl et al. 2011). Taken together the evidence fom these studies suggest that the complement system is active within atherosclerotic plaques; that complement promotes atherosclerotic plaque development and may aid in atherosclerotic plaque rupture.

1.8.2. The influence of complement in thrombosis

There is increasing evidence to suggest that complement plays a role in thrombosis through interactions with platelets and coagulation proteins. Complement activation is initiated following platelet activation. In a subset of platelets, rapid (within 30 seconds) C5b-9 formation was found following platelet activation by adenosine diphosphate (ADP), thrombin receptor activating peptide (TRAP) or collagen independent of P-selectin expression on the platelet surface (P-selectin binds C3b). Formation of C5b-9 in this subset of platelets was thrombin dependent as hirudin blocked C5b-9 formation, and these platelets were found to specifically interact only with collagen fibres, with upregulation of C3aR and C5aR as a result of this interaction (Martel et al. 2011). It is currently unknown why only subsets of platelets form C5b-9 upon activation; however this subset may form an important role along with the previously identified pro-coagulant subset of COAT activated platelets (Dale et al. 2002). Platelets treated with C5b-9 also promote FVa and FXa binding to the platelet surface which in turn promotes the prothrombin converting activity of the platelet. C5b-9 was also found to stimulate α granule release of FV (Wiedmer et al. 1986).

Platelet activation results in activation of the alternative and classical pathway of complement on the platelet surface. Platelets activated by sheer stress, ADP or TRAP, contained complement C3 deposition, with deposition occurring as a result of P-selectin-mediated binding of C3b. Once bound C3b was able to recruit factor B to form the alternative pathway C3 convertase, with the increased generation of C3a and deposition of C5b-9 on the platelet surface (Del, I et al. 2005). Hamad et al. (2010) also found that C3(H_2O) deposition on TRAP-activated platelets was independent of complement activation; however, unlike in the previous study, C3 was cleaved by factor I in the presence of factor H to generate iC3b, which in turn bound soluble CR1 (Hamad et al. 2010). This might suggest that platelets are a target for phagocytosis as a result of C3 opsonisation (Flaumenhaft 2006). Factor H binds to resting and activated platelets, with activation by thrombin and ADP increasing factor H deposition by 22% and 89% respectively (Vaziri-Sani et al. 2005). Recombinant constructs of the factor H SCR incubated with heparin confirmed that binding to platelets took place within the heparin binding region in SCR 19-20 via GPIIb/IIIa and thrombospondin-1 receptors present on the platelet surface (Vaziri-

Sani et al. 2005). On normal platelets, 47% of plasma factor H is present on their surface; however, this level is reduced to 18% in patients with atypical haemolytic uraemic syndrome (aHUS), which is caused by a mutation in SCR 20. As a result of this mutation, surface levels of complement components, C3 and C9 and the platelet receptor P-selectin are all increased, with increased platelet microparticle release (Stahl et al. 2008). As platelet microparticles are known to be pro-thrombotic (Keuren et al. 2006), this study in aHUS patients suggests a protective role of factor H in thrombosis. Similar to platelets, complement activation and regulation also occurs on platelet microparticles, with expression of gC1qR, IgG, C1-inhibitor, CD55 and CD59 (Yin and Peerschke 2007). Platelet microparticles incubated in plasma undergo full complement activation with iC3b and C5b-9 deposition occurring within 30-60 minutes (Yin et al. 2007).

Alternatively, platelets activated by TRAP or sheer stress, can also activate the classical pathway of complement, with deposition of C1q and C4b on the platelet surface.Deposition coincided with increased gC1qR expression, with deposition inhibited by antibodies against gC1qR. Classical pathway component deposition also resulted in complement activation with the generation of soluble C3a (Peerschke et al. 2006). The C3 activation products C3a and C3 desArg promote platelet aggregation and serotonin release, and these effects are synergistic in the presence of ADP (Polley and Nachman 1983). These studies suggest that complement activation on the surface of platelets results in increased inflammation by the production of complement dependent inflammatory mediators and promotes thrombosis via platelet aggregation at the site of vascular damage. Platelets also influence the complement response. Platelet casein kinase is responsible for phosphorylating the C3 α chain (Ekdahl and Nilsson 1995;Ekdahl and Nilsson 1999) and phosphorylation of C3b prevents its cleavage by factor I in the presence of factor H (Ekdahl et al. 1995) as a result of decreased binding of factor H to phosphorylated C3b (Ekdahl and Nilsson 2001). Phosphorylated C3b produced by the C3 convertase, binds more readily to CR1 on cell surfaces (Ekdahl et al. 1999;Ekdahl et al. 2001). This suggests that platelet phosphorylation is responsible for promoting C3 opsonisation instead of complement inactivation.

Recent investigations into the lectin pathway have highlighted the role of complement in thrombus formation. Recombinant MASP1 directly cleaves the

FXIII-A chain to release the activation peptide and FPB from the fibrinogen β chain, with a catalytic efficiency 650-fold slower than thrombin; however, amino terminal sequencing confirmed that cleavage occurs at the same sites as thrombin. MASP1 cleavage of FXIII and fibrinogen resulted in γ dimer and a small amount of α polymer formation; however, the majority of the α chain was degraded, suggesting MASP1 cleaves at numerous sites within fibrinogen (Krarup et al. 2008). A recent study by Hess et al. (2012) confirmed cleavage and activation of FXIII by MASP1. MASP1 was also found to cleave TAFI, prothrombin, and FPA from the fibrinogen α chain; however, FPA cleavage required the presence of prothrombin for its actions, suggesting MASP1 cleaves prothombin first to form thrombin, which then cleaves FPA. Analysis of fibrin fibres by scanning electron microscopy in the presence of MASP1 showed that the fibrin network contains large pores and coarse fibres compared to thrombin (Hess et al. 2012), suggesting MASP1 is protective for CVD in this result, however cleavage of prothrombin by MASP1 may promote a pro-thrombotic state for CVD. Comparable to MASP1, MASP2 is also capable of cleaving prothrombin, with thrombin generation by MASP2 enhanced in the presence of FXa (Krarup et al. 2007). MASP2, in complex with MBL, generated thrombin from prothrombin in the presence of Ca^{2+}, with thrombin generation resulting in polymerisation of fibrin; this process also occurred when the MBL-MASP2 complex was bound to *S.aureus*, suggesting fibrin deposition occurs on bacterial surfaces following MASP2 activation (Krarup et al. 2007). In enzyme-linked immunosorbant assays (ELISA)-based binding assays, MBL has also been shown to bind the α and β chains of fibrin, with fibrin binding promoting MBL binding to *S.aureus* (Endo et al. 2010), suggesting fibrin plays a role in promoting complement activation via the lectin pathway. The anticoagulant antithrombin III inhibits MASP1 and MASP2 and therefore C4 activation in the presence of heparin (Presanis et al. 2003). These studies suggest that when the lectin pathway is activated it promotes thrombosis by activating/cleaving prothrombin, fibrinogen and FXIII, however in the presence of an anticoagulant (antithrombin III), lectin pathway activation and thrombosis are inhibited.

Although extensive work has been performed on the lectin pathway, several interactions between components of the classical and alternative pathways with components of coagulation have also been observed. The initiating component of the

classical pathway, C1q, has been shown to bind the FPA and B on fibrinogen via two high affinity sites on C1q (collagen-like and globular domains) (Entwistle and Furcht 1988), suggesting localisation of complement activation to sites of injury. Similarly, Lu et al. (1999) found that the endothelial cell C1q receptor (gC1qR) also bound to fibrinogen via the D domain. This binding interaction was found to prevent fibrin polymerisation with an increase in clotting time and a decrease in fibrin monomer formation (Lu et al. 1999). Alternatively, in zymosan treated plasma (treatment activates the alternative pathway of complement) clots, fibrin fibres were found to be thinner with increased clot strength and reduced clot permeability, additionally following clot formation complement activation continued with the generation of C3a (Shats-Tseytlina et al. 1994). This study suggests a component of the alternative pathway is involved in altering fibrin clot structure, although none were specified in this study. This study also suggests that fibrin clots can support complement activation. Factor B, a component of the alternative pathway, was also found to cleave and activate plasminogen similar to the actions of uPA; the resulting plasmin was functionally active demonstrated by effective cleavage of radioactively labelled fibrin (Sundsmo and Wood 1981) suggesting factor B is pro-fibrinolytic. The anaphylatoxin C5a generated by all three complement pathways, promotes PAI-1 expression and reduces uPA expression and production in macrophages as a result of signalling through the C5aR. Incubations with C3a had no effect, suggesting a C5a specific effect on PAI-1 expression (Kastl et al. 2006). Additionally C5a promotes increased expression and production of PAI-1 in mast cells, PAI-1 production in mast cells was capable of inhibiting t-PA produced by the resting mast cells (Wojta et al. 2002). P-selectin expressed on endothelial cells, binds C3 and activates the alternative pathway of complement resulting in increased C3a generation (Morigi et al. 2011). C3a once bound to C3aR results in thrombomodulin shedding, thrombus formation and platelet aggregation (Morigi et al. 2011) and therefore microvascular thrombosis. These studies highlight the pro-thrombotic actions of the anaphylatoxins C3a and C5a. To prevent uncontrolled anaphylatoxin production the fibrinolysis inhibitor TAFI cleaves and inactivates C3a and C5a resulting in C3desArg and C5desArg, a product with no ability to activate neutrophils (Myles et al. 2003), with inactivation occurring within 5 minutes when incubated in serum or plasma (Campbell et al. 2001). These studies further support the important interactions between complement and coagulation in controlling thrombosis and inflammation.

1.9. In vivo models of complement activation and CVD

1.9.1. The influence of complement on atherosclerosis in in vivo mouse models

The importance of complement in atherosclerosis progression is highlighted in complement deficient animals. During the early stages of atherosclerosis development, in ApoE mice Shields et al. (2011) found that C3 and C4 produced by perivascular adipose tissue bound to collagen and elastin within the adventitia, thus promoting vascular stiffness and future atherosclerotic plaque development. (Shields et al. 2011). Whereas in ApoE $^{-/-}$ CD59 $^{-/-}$ double knockout mice formation of C5b-9 led to damage of the endothelium as assessed by increased staining for von Willebrand factor and increased endothelial cell membrane permeability. Cobra factor venom activation of the alternative pathway confirmed C5b-9 produced by the alternative pathway was responsible for endothelial dysfunction (Wu et al. 2009a). Wu et al (2009a) further showed that macrophages, T cells and apoptotic cells accumulate at the site of atherosclerosis, with C5b-9 promoting the formation of foam cells with increased cholesteryl ester and Ox-LDL accumulation inside the foam cell. In a study by Sakuma et al (2010) DAF $^{-/-}$ mice were monitored following induced endothelial injury, with increased thickening of the neointima and increased recruitment of leukocytes observed compared to wildtype mice. Combined knockout of DAF with either C3aR or C5aR produced the opposite effects on the neointima and leukocytes, suggesting the development of atherosclerosis is dependent on complement activation and in particular anaphylatoxin signalling (Sakuma et al. 2010). DAF $^{-/-}$ and low density lipoprotein receptor (ldlr) $^{-/-}$ double knockout mice fed either a low fat or high fat diet had increased lipid deposition and increased plaque area compared to ldlr $^{-/-}$ littermates. Immunostaining of the plaques found extensive complement activation with increased C3d and C5b-9 deposition in DAF $^{-/-}$ ldlr $^{-/-}$ mice only (Leung et al. 2009). These studies highlight the athero-protective role of complement inhibitors in atherosclerosis development.

Several studies have also investigated in vivo knockout models of components of the alternative pathway of complement. Malik et al. (2010) showed that factor B $^{-/-}$ and ldlr $^{-/-}$ double knockout mice had smaller plaques with decreased complement activation compared to ldlr $^{-/-}$ littermates, suggesting a role for factor B in atherosclerosis. This study further showed that chronic infection as a result of

bacterial lipopolysaccharide administration increased atherosclerotic plaque size in ldlr $^{-/-}$ mice but not factor B $^{-/-}$ ldlr $^{-/-}$ mice, which suggests that the alternative pathway of complement contributes to atherosclerosis development especially during chronic infection (Malik et al. 2010). However in a factor B $^{-/-}$, ldlr $^{-/-}$, ApoE $^{-/-}$ triple knockout mice atherosclerotic plaque size and serum triglyceride levels did not differ from ldlr $^{-/-}$ and ApoE $^{-/-}$ littermates (Persson et al. 2004), this may suggest that ApoE$^{-/-}$ is less dependent on complement. Alternatively C3 $^{-/-}$ and ldlr $^{-/-}$ double knockout mice have increased lipid and macrophage deposition within the atherosclerotic plaque with decreased collagen and smooth muscle cell deposition (Buono et al. 2002), suggesting C3 is important in the formation of the fibrous cap and the continual development of the atherosclerotic plaque. Persson et al (2004) found that in C3 $^{-/-}$, ldlr $^{-/-}$, ApoE $^{-/-}$ triple knockout mice atherosclerotic plaque size was larger with increased serum triglyceride levels compared to ldlr $^{-/-}$, ApoE $^{-/-}$ littermates, this finding is consistent with an increase in lipid and macrophage deposition observed by Buono et al. (2002). These studies in both human and mouse atherosclerotic plaques have highlighted the role complement and in particular complement activation plays in both the development and instability of atherosclerotic plaques.

1.9.2. Influence of complement on thrombosis in in vivo mouse models

The in vitro data determining the role of the lectin pathway in thrombosis suggests that MBL and MASPs are pro-thrombotic, which is confirmed in the following studies using MBL and MASP1/3 knockout mice. In MBL and MASP1/3 knockout mice tail tip bleeding times were prolonged compared to wildtype mice, with clotting times decreased after the addition of recombinant MBL into MBL knockout mice. Analysis of bleeding times in C3 knockout mice were the same as those of wildtype mice, thus confirming activation of the lectin pathway is important in thrombus formation (Takahashi et al. 2011). In MBL and MASP1/3 knockout mice no occlusion of blood flow or thrombi formation were observed after $FeCl_3$ induced endothelial injury compared to wildtype mice and C2/Factor B knockout mice which showed occlusion of blood flow and thrombi formation by 10 minutes and 20 minutes respectively, with occlusion of blood flow restored to the MBL knockout mice following the addition of recombinant MBL. Immunostaining of MBL within

wildtype and MBL knockout thrombi after restoring MBL confirmed a strong presence of MBL within the thrombi. Additionally, wildtype whole blood was able to promote platelet aggregation due to the presence of MBL and MASPs, however using MBL knockout whole blood no difference in platelet aggregation was observed. Using a thrombin substrate cleavage assay, MBL or MASP1/3 knockout plasma was unable to cleave thrombin however after the addition of recombinant MASP1 to MASP1/3 knockout plasma thrombin generation was restored to that of a wildtype mouse (La Bonte et al. 2012). These studies show that the lectin pathway was important in thrombi formation, as a result of thrombin generation. Although downstream complement activation products were found to be less important in the inital thrombi formation, no conclusive evidence has been shown to suggest that downstream complement components do not play a role in fibrin structure and function *in vivo*.

1.10. Proteomics

More recent evidence for the influence of complement in CVD has been derived from proteomic studies. Proteomics is the study of proteins expressed at a given period in time by cells or tissues (the proteome). Proteomics can be used in protein identification, quantification, protein modification and interactions. Mass spectrometry (MS) uses the mass-to-charge ratio of proteins to determine its identity and any potential modifications, making it a widely used tool in proteomics. To allow the effective identification of proteins by MS, proteomes or subproteomes are separated either by two-dimensional gel electrophoresis or by one or more chromatography steps (Polkinghorne et al. 2009). Two-dimensional gel electrophoresis has been used extensively in the past in the identification of large, complex mixtures of proteins according to their isoelectric point and molecular mass, as it can separate up to 2000 proteins simultaneously and detect protein as low as 1 ng per spot. Two-dimensional gel electrophoresis is particularly useful in monitoring protein expression and changes in molecular weight associated with post-transcriptional modifications (Gorg et al. 2005). Liquid chromatography separates proteins/peptides based on their size, affinity, ion exchange or reversed-phase, with the efficiency of these methods based on the packing material (particle size, pore size and surface area). Reversed-phase high performance liquid chromatography has been used extensively in proteomics due to its ability to flow peptides directly into the mass spectrometer (Neverova and Van Eyk 2005). Separated proteins are proteolytically digested by enzymes (e.g trypsin), resulting in peptides that are ionised by either electrospray ionisation or matrix–assisted laser desorption ionisation (MALDI) within the mass spectrometer. The mass-to-charge ratio is determined for each peptide and is compared to known peptide masses in an extensive protein database. Information regarding the peptide sequence can be generated from further fragmentation of the peptide by collision induced dissociation and analysed by tandem MS/MS (Aebersold and Mann 2003;Arrell et al. 2001).

1.10.1. Use of proteomic technologies in CVD research

Within the last 10 years the use of proteomics in cardiovascular research has expanded rapidly, with numerous studies looking to identify biomarkers of disease and therefore future disease progression. The largest of these studies is the HUPO plasma proteome project, which looked to extending the knowledge of the plasma proteome. The HUPO project identified 3020 proteins in plasma and by comparing the proteins analysed and the current literature they assigned proteins related to CVD. Of these, 345 proteins with known cardiovascular functions were identified and assigned to the following protein groups: inflammatory and cardiovascular markers, vascular and coagulation, signalling, growth and differentiation, transcription factors, channels and receptors, heart failure and remodelling (Berhane et al. 2005). By comparing plasma from patients with CAD with healthy age and sex matched controls, 95 proteins were differentially expressed, the majority (75 proteins) of which were up-regulated in the CAD patients, including proteins from the following protein groups: complement (C1, C3, C4, C5a and factor B), coagulation (fibrinogen γ chain, prothrombin) and fibrinolysis (α2-antiplasmin) (Donahue et al. 2006). Alternatively, MI patients have a distinctive peptide pattern with regards to C3f and FpA, which were found to be amino terminally cleaved by endopeptidases (Marshall et al. 2003). Proteins associated with the known cardiovascular risk factor high density lipoprotein, analysed from CAD patients and healthy controls, saw 48 proteins involved in cholesterol/lipoprotein metabolism, inflammation, peptidase inhibitors and heparin binding proteins (Vaisar et al. 2007). These few studies have highlighted the importance of inflammation (in particular complement) and coagulation proteins in CVD.

Proteomics has also been used to characterise the proteins which make up atherosclerotic plaques. Several studies have compared stable atherosclerotic plaques to unstable atherosclerotic plaques. In a study by Lepedda et al (2009) unstable atherosclerotic plaques were found to contain increased concentrations of fibrinogen fragment D, ferritin, and superoxide dismutase 2 compared to stable atherosclerotic plaques (Lepedda et al. 2009). Donners et al (2005) further showed that 71 spots (relating to angiotensin I, lactoferin, lipocalin-1 and proline rich lacrimal protein) were unique for stable atherosclerotic plaques with 29 spots (relating to vinexin β and α1-antitrypsin) unique for atherosclerotic plaques containing thrombi (Donners

et al. 2005). A recent study comparing systemic plasma to plasma from an atherosclerotic plaque rupture site from MI patients, found that the latter contained 9 proteins which were differentially expressed including an increase in complement factor H related protein 2 and a decrease in complement factor B, C5, C7, carboxypeptidase N, plasminogen, protein S, pigment epithelial derived factor and matrix metalloprotease 9 (Distelmaier et al. 2012). This study has highlighted the local complement activation and potential regulation (complement factor H related protein 2) that occurs at coronary atherosclerotic plaque rupture sites. Additionally the results from these studies confirm the pro-coagulant environment associated with atherosclerotic plaques.

1.10.2. The use of proteomic technologies in thrombosis

Fibrinogen is a major determinant of clot density and susceptibility to fibrinolysis; however, numerous other plasma proteins are incorporated into plasma clots by binding or FXIII-dependent cross-linking, suggesting other plasma proteins may play a role in thrombosis. This is highlighted in plasma clots, where fibrin formation is slower and fibre diameter is larger than in fibrin clots produced from purified fibrin (Carr 1988). For this reason, several proteomic studies have looked at identifying the components of plasma clots. Comparisons between systemic plasma and plasma derived from thrombi after acute MI found that C3c α chain fragment 1, C3a, C5a and CRP are all increased in the latter, and complement activation within the thrombi was further confirmed by immunohistological staining for CRP, C3d and C5b-9 (Distelmaier et al. 2009). Complement activation and the generation of C5a also resulted in the recruitment of neutrophils to the thrombus (Distelmaier et al. 2009). Several studies have compared plasma to plasma derived serum, with components of the coagulation cascade found to be depleted in serum compared to plasma. Interestingly, C3 and clusterin were found to have molecular weights matching cleavage products in serum with C3 cleavage resulting in C3b and C3dg (Misek et al. 2005). Analysis of the serum peptidome (low molecular weight peptides) found fibrinogen, prothrombin, albumin, apolipoprotein A1, collagen, C4, C3 and several cellular components (Tammen et al. 2005). Niessen et al (2011) initiated coagulation in plasma and whole blood by the addition of TF and calcium, removed the fibrin clot, and then monitored cleavage products of coagulation and complement proteins

(changes in molecular weight) in the supernatant compared to plasma controls. This method observed the majority of coagulation and complement proteins, however cleavage only occurring in FX, FV, prothrombin, protein S, fibrinogen, FXIII, complement C1r, C5, C6 and C9 (Niessen et al. 2011). These studies have highlighted the importance of complement and coagulation in plasma clot formation, in particular cleavage of coagulation and complement proteins as a result of plasma clot formation. These finding are relevant in the context of complement and coagulation cascades, which are derived form common ancestral genes (Krem and Di Cera 2002) and are activated to prevent blood loss and pathogen invasion and as a result are required to co-localise at the site of plasma clot formation.

In previous work carried out by Dr Howes within the Division of Cardiovascular and Diabetes Research (DCDR), perfused, solubilised plasma clots were analysed to determine the protein components of plasma clots. Although fibrin α, β and γ chains accounted for the majority of protein within the plasma clot; 20 additional proteins were identified, including proteins involved in haemostasis (FXIII, α2-macroglobulin, kininogen), inflammation (C3, factor H, carboxypeptidase N), protease inhibition (inter-alpha-trypsin inhibitor) and binding proteins (fibronectin) (Howes et al. 2012). Complement C3 and factor H identified within the FXIII deficient plasma clots were absent at their known molecular weights in plasma clots containing FXIII, suggesting they are cross-linked by FXIIIa during clot formation forming higher molecular weight complexes (Howes et al. 2012). These findings support a role for complement C3 and factor H within fibrin clots and were therefore selected for further functional *in vitro* analyses.

Chapter 2 Aims

Complement proteins C3 and factor H have been shown to play a role in T2DM, CVD risk and atherosclerosis progression. In previous work performed within the DCDR, complement proteins C3 and factor H were identified as novel plasma clot components using a proteomics based technique (Appendix A) (Howes et al. 2012). This study highlighted the potential of proteomics to reveal novel proteins involved in thrombosis. The aims of this study were therefore: to evaluate the influence of C3 and factor H on fibrin structure and function and to determine novel FXIII substrates using functional proteomics.

2.1. To evaluate the influence of C3 on fibrin structure and function

The influence of complement C3 on fibrin structure and function was evaluated by (a) a turbidimetric assay in a purified and plasma system to determine the effect of C3 on fibrin formation and fibrin density. (b) A turbidimetric assay in a purified and plasma system to determine the effect of C3 on time to 50% lysis. (c) Plate based BP incorporation assays and gel based cross-linking assays using purified proteins and plasma to determine whether C3 was a substrate for FXIII and undergoes FXIII-dependent cross-linking to fibrin during clot formation. (d) Gel based analysis of cleavage of purified C3 and C3 within purified fibrin clots to determine whether C3 was a substrate for thrombin and plasmin and was responsible for substrate competition with fibrin. (e) Gel based analysis of plasminogen cleavage in the presence of C3 and/or fibrin to determine whether C3 influences plasminogen cleavage. (f) Turbidimetric assays using purified proteins in the presence of either a t-PA or plasminogen dose response to determine whether the C3 influence on fibrinolysis was due to the concentration of t-PA and/or plasminogen. (g) Two chromogenic plate based plasmin generation asays to determine whether C3 influences t-PA and/or plasminogen binding and to determine whether FXIII-dependent cross-linking of C3 influences plasmin generation.

2.2. To evaluate the influence of factor H on CVD risk and fibrin structure and function

(1) The influence of complement factor H on CVD risk, evaluated in 119 healthy South Asian first degree relatives of patients with T2DM compared with 119 healthy South Asian control subjects with no personal or family history of T2DM by (a) analysing the relationship between factor H concentration measured by ELISA and conventional CVD risk factors. (b) The relationship between factor H concentration and zymogen and activated complement components. (c) The relationship between factor H concentration and ex vivo plasma fibrin structure and function measured by turbidimetric assay. (2) The influence of complement factor H on fibrin structure and function was evaluated by (a) measuring factor H in plasma and plasma-derived serum samples by ELISA to determine the percentage of factor H incorporated into plasma clots during fibrin clot formation. (b) A turbidimetric assay in a purified system to determine the effect of factor H on fibrin formation and fibrin density. (c) A turbidimetric assay in a purified system to determine the effect of factor H on time to 50% lysis. (d) Gel based cross-linking assays using purified proteins and plasma to determine whether factor H was a substrate for FXIII and undergoes FXIII-dependent cross-linking to fibrin during clot formation. (e) Gel based analysis of cleavage of purified factor H and factor H within purified fibrin clots to determine whether factor H was a substrate for thrombin and plasmin and was able to be cleaved during fibrin formation/lysis.

2.3. Determination of novel FXIII substrates using a functional proteomics approach

A functional proteomics technique has previously been used to identify the cellular substrates of tissue transglutaminase (Orru et al. 2003;Ruoppolo et al. 2003). In the present study this technique was adapted for the identification of potential novel substrates of FXIII in plasma. Based on (a) FXIII-dependent cross-linking of BP to plasma proteins. (b) Removal of excess BP from plasma. (c) Affinity chromatography of BP incorporated proteins using monomeric avidin and (d) MS analysis of BP incorporated protein. (e) Gel based analysis of perfused solubilised

plasma clots were performed as an alternative method for the identification of potential novel FXIII substrates.

Chapter 3 Methods

Unless stated otherwise, details of all buffers described in the following sections are provided in appendix B.

3.1. Protein Purification

The majority of functional analyses were carried out using purified proteins from whole blood. C3, plasminogen and fibrinogen were purified from blood samples from volunteers recruited from the LIGHT Laboratories. All volunteers gave informed consent according to a protocol approved by the University LIGHT/LIHS/LIMM research ethics committee (Appendix C). On each occasion, whole blood was taken from three healthy volunteers who had not receieved any anti-inflammatory or anti-platelet drugs. Sixty mls of whole blood from each volunteer was taken with a 19-guage needle into ethylenediaminetetraacetic acid (EDTA) on ice (C3 and plasminogen) or citrate (fibrinogen) and centrifuged at 3000 g at 4°C for 30 minutes. EDTA plasma from all three volunteers was removed, pooled and used immediately for C3 and plasminogen purification. Citrate pooled plasma, aliquoted and stored at -80°C for fibrinogen purification.

3.1.1. C3 Purification

C3 purification was performed based on method previously described (Tack and Prahl 1976).

3.1.1.1. Polyethylene glycol Precipitation

To the pooled EDTA plasma, 100 mM phenylmethylsulphonyl fluoride (PMSF) in isopropanol was added to a final concentration of 0.5 mM, along with polyethylene glycol (PEG) 4000 buffer I, to a final PEG concentration of 5%. The plasma sample was stirred for 30 minutes at 4°C, followed by centrifugation at 10,000 g, 4°C for 30 minutes (Sorvall). The supernatant was removed, PEG 4000 buffer II was added to a final PEG concentration of 12% and the sample was stirred at 4°C for 30 minutes prior to a second centrifugation at 10,000 g, 4°C for 30 minutes (Sorvall). The supernatant was discarded and the pellet was re-suspended in 20 ml of lysine sepharose buffer I by gentle agitation.

3.1.1.2. Lysine sepharose chromatography and plasminogen purification

Plasminogen was removed by passing the sample through a lysine sepharose (GE Healthcare Life Sciences) column (3 cm width by 8 cm height) equilibrated in lysine sepharose buffer I using an automated chromatography system (BioCad Sprint, Applied Biosystems). The sample was injected at a flow rate of 1 ml/min and the column was washed with three column volumes (CV) of lysine sepharose buffer I. The flow through was retained for diethylaminoethanol (DEAE) chromatography (section 3.1.1.3).

Plasminogen was eluted from the column using two CV of lysine sepharose buffer II. Five ml fractions were collected and the absorbance was monitored at 280 nm. The column was regenerated in lysine sepharose buffer I over two CV. Plasminogen containing fractions were pooled and concentrated in a 3 KDa molecular weight cut off Vivaspin column (Sartorius Stedim) at 5000g, 4°C until ~ 2 mls remained. The purity was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (section 3.2). Purified plasminogen was stored at -80°C in 20 µl aliquots.

3.1.1.3. DEAE Chromatography

The lysine sepharose flow-through (section 3.1.1.2) was diluted with 5 mM EDTA to a conductance of 3 mmho/cm using a conductance meter (Omega), before loading onto a DEAE cellulose column (3 cm width by 21 cm height, Sigma) equilibrated in ion exchange buffer I using the BioCad Sprint (Applied Biosystems). The sample was loaded at a flow rate of 2 ml/min and washed with one CV of ion exchange buffer I. C3 was eluted from the column using ion exchange buffers II containing 25 mM NaCl and ion exchange buffer III containing 300 mM NaCl, which produced a linear NaCl concentration gradient over 10 CV. Ten ml fractions were collected and the absorbance was monitored at 280 nm. The column was regenerated in ion exchange buffer I over two CV. The elution profile of C3 was established by C3 ELISA and shown to be reproducible between runs. C3 was pooled, solid PEG 4000 (Fisher) was added to a final concentration of 16% and the sample was stirred for 30 minutes at 4°C and underwent centrifugation at 10,000 g, 4°C for 30 minutes. The

supernatant was removed and the pellet was re-suspended in 5 ml of gel filtration buffer.

3.1.1.4. Gel Filtration Chromatography

The re-suspended C3 positive fraction (section 3.1.1.3) was injected onto a sepharose 6B column (3 cm width by 37 cm height, Sigma) equilibrated in gel filtration buffer using the BioCad sprint (Applied Biosystems). The sample was injected at a flow rate of 0.5 ml/min and fractions were eluted with one CV of gel filtration buffer. Five ml fractions were collected and the absorbance was monitored at 280 nm. The elution profile of C3 was established by C3 ELISA and shown to be reproducible between runs. C3 positive fractions were pooled.

3.1.1.5. Hydroxylapatite Chromatography

The pooled C3 positive fractions were dialysed using a 3 KDa molecular weight cut off dialysis membrane (Sigma) twice for 1 hour and then overnight in a total of two litres of potassium phosphate dialysis buffer with each buffer exchange. The dialysed sample was injected onto a hydroxylapatite column (3 cm width by 8 cm height, BioRad) equilibrated in potassium phosphate dialysis buffer using the BioCad Sprint (Applied Biosystems) at a flow rate of 0.9 ml/min and washed in one CV of potassium phosphate dialysis buffer. C5 eluted from the column over five CV using potassium phosphate dialysis buffer and C5 elution buffer I, which produced a linear potassium chloride gradient of 100 mM to 2 M. The column was washed with one CV of potassium phosphate dialysis buffer. C3 eluted from the column over six CV using potassium phosphate dialysis buffer and C3 elution buffer I, which produced a linear potassium phosphate gradient of 25 mM to 250 mM. Five ml fractions were collected and the absorbance was monitored at 280 nm. The column was washed in two CV of C3 elution buffer II, to remove any non-eluted proteins and regenerated in potassium phosphate dialysis buffer over two CV. C3 containing fractions were pooled and dialysed using a 3 KDa molecular weight cut off dialysis membrane (Sigma) overnight followed by two, one hour dialysis steps each in a total of 2 litres of tris buffered saline (TBS). The dialysed samples were concentrated in a 100 KDa molecular weight cut off Vivaspin column (Sartorius Stedim) at 5000g, 4°C until ~2

mls remained. Final C3 concentration was determined by ELISA (see section 3.3) and purity was determined by SDS-PAGE and immunoblotting for C3 and C5 (see section 3.2). Purified C3 was stored in 50 µl aliquots at -80°C.

3.1.1.6. Analysis of C3 purification products

A total of three C3 purifications were performed with each C3 purification resulting in ~10-30 mg of total protein. Following the C3 purification procedure the C3 and C5 containing fractions were analysed by SDS-PAGE and Western blotting to assess purity. Figure 7, Panels A and B show that the C3 fractions were pure with small amounts of C3 cleavage products. Panel C confirms there was no C5 contamination within the C3 preparation.

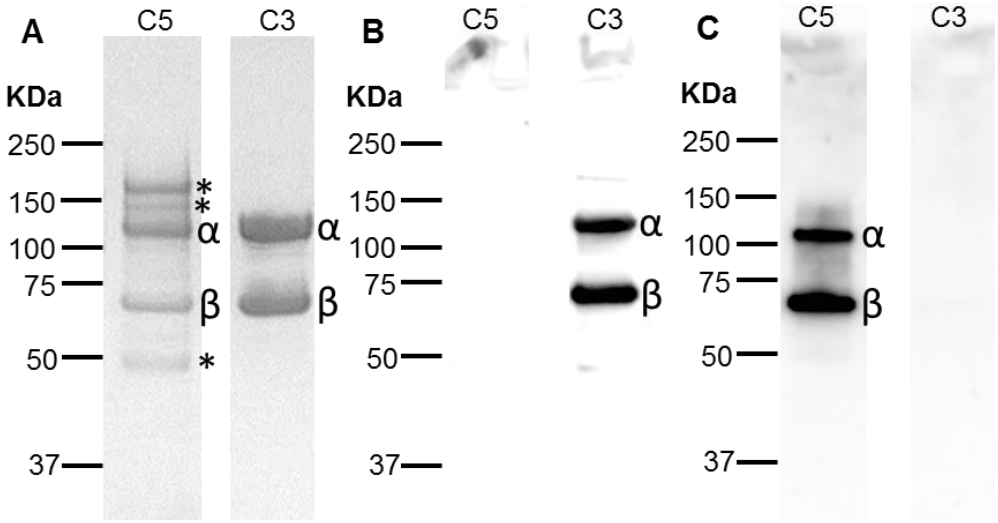


Figure 7: Analysis of C3 purification products by SDS-PAGE and Western blot

C3 purification products from the hydroxylapatite column were analysed by Coomassie stained SDS-PAGE (**Panel A**) and immunoblotting with anti-C3c antibody (**Panel B**) and anti-C5 antibodies (**Panel C**). * indicates contamination from other plasma proteins. Immunoblots show that the C3 is pure and contains no C5 contamination. Gels and Western blots representative of all C3 purifications performed.

3.1.2. Fibrinogen Purification

One aliquot (1.5 mls) of citrated plasma was defrosted at 37°C and mixed with 15 U/ml of heparin (Sigma). Fifty µl of 1 M $CaCl_2$ was added along with IF-1 equilibration buffer to a final volume of 5 mls. The plasma sample was filtered with a 0.2 µm syringe filter prior to injection onto an IF-1 column (1.5 cm width by 3 cm height) equilibrated in IF-1 equilibration buffer using the BioCad Sprint (Applied Biosystems). The IF-1 column is a calcium dependent monoclonal antibody (IF-1) coupled to sepharose 4B, previously described (Takebe et al. 1995). The IF-1 column was washed with IF-1 wash buffer I for six CV followed by a second wash with IF-1wash buffer II for six CV. Fibrinogen was eluted from the column using three CV of IF-1 elution buffer. Five ml fractions were collected and the absorbance was monitored at 280 nm. Fibrinogen positive fractions were concentrated using a 100 KDa molecular weight Vivaspin column (Sartorius Stedim) at 3000g, 4°C until ~ 5 ml remained. The concentration of fibrinogen positive fractions was determined by Nanodrop (Labtech International) using the extinction coefficient 15.1 $M^{-1}cm^{-1}$. Purified fibrinogen was assessed for purity by SDS-PAGE (see section 3.2) and 50 µl aliquots stored at -80°C.

3.1.3. Factor XIII Purification

Fibrogammin P (CSL Behring) contains a large number of contaminants including albumin that were removed by gel filtration. Two vials of fibrogammin P were reconstituted in 2 ml of water, giving an initial protein content of 5.48 mg/ml. This sample was injected onto a sepharose 6B column (3 cm diameter by 37 cm high, Sigma) equilibrated in TBS using the BioCad Sprint (Applied Biosystems) at a flow rate of 0.5 ml/min. The FXIII-A2B2 was eluted over one CV of TBS. Five ml fractions were collected and the absorbance was monitored at 280 nm. Purified FXIII-A2B2 was concentrated using a 100 KDa molecular weight cut off Vivaspin column (Sartorius Stedim) at 3000g, 4°C until ~ 3 ml remained. The purity of FXIII was assessed by SDS-PAGE (see section 3.2). The concentration of FXIII was determined by Nanodrop (Labtech International) using the extinction coefficient 13.8 $M^{-1}cm^{-1}$. Purified FXIII was stored at -80 °C in 20 µl aliquots.

3.1.4. Analysis of fibrinogen, FXIII and plasminogen purification products

As shown in Figure 8 fibrinogen and plasminogen were pure. Alternatively the FXIII purification products contain albumin (60 KDa) and a small amount of high molecular weight contaminants, therefore when determining the FXIII concentration the amount of albumin and high molecular weight contaminants were taken into consideration by dividing the total concentration by the percentage of FXIII within the sample as determined by densitometry.

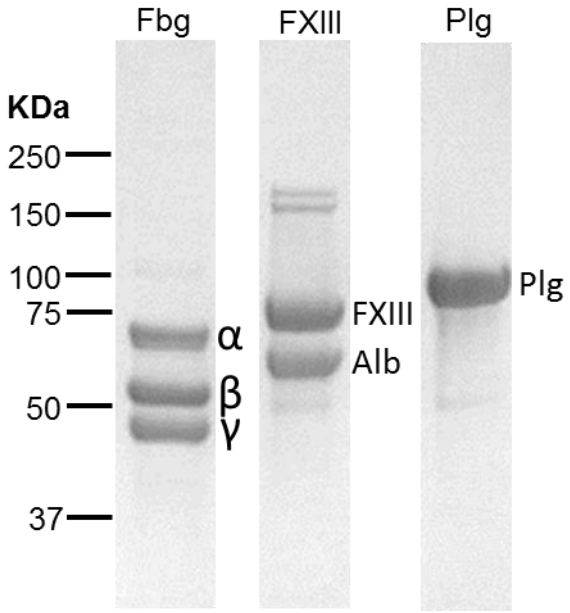


Figure 8: Analysis of fibrinogen, FXIII and plasminogen purification products by SDS-PAGE

Fibrinogen (Fbg) and plasminogen (Plg) were pure. Factor XIII (FXIII) contained contamination from albumin (Alb) and from high molecular weight contaminants. Coomassie stained SDS-PAGE were representative of all purifications performed.

3.2. Gel Electrophoresis and Immunoblotting

Gel electrophoresis and immunoblotting were used to assess purity of purified proteins, protein cleavage and protein cross-linking. Reduced proteins were run on either NuPAGE 4-12% Bis-Tris gels (Invitrogen) in 1x 2(N-morpholino)ethanesulfonic acid (MES) sodium dodecyl sulfate (SDS) running buffer (Invitrogen) or NuPAGE 3-8% Tris-Acetate gels (Invitrogen) in 1x Tris Acetate running buffer (Invitrogen).

3.2.1. Gel Electrophoresis

Samples were mixed with appropriate volumes of 4x loading buffer and 10x reducing agent (Invitrogen) and incubated at 95°C for 10 minutes in a heat block. Two NuPAGE gels were placed into Invitrogen gel running tanks and surrounded by 1x running buffer appropriate to the gel. Twenty µl of the sample were loaded into each well along with 3 µl of WesternC™ standard (BioRad). 4-12% Bis-Tris gels were run for 45 minutes at 200 volts, whereas 3-8% Tris-Acetate gels were run for 60 minutes at 150 volts using a PowerEase® 500 power supply (Invitrogen).

3.2.2. Coomassie staining of gels

To visualise proteins, NuPAGE gels were stained with coomassie. The NuPAGE gels were removed from their plastic covers and washed with distilled water three times each for 10 minutes. The water was removed and either ~50 mls of GelCode® Blue Stain Reagent (Pierce) or Coomassie stain reagent (BioRad) (Mass spectrometry gels only) were added to completely cover the gel. The gels were left in the stain reagents for 1 hour and de-stained by gentle agitation in distilled water until the bands became clear. An image was captured using a MultiImage™ Light Cabinet (Alpha Innotech Corporation).

3.2.3. Immunoblotting

For detection of specific proteins, proteins were transferred from non-stained gels onto a polyvinylidene fluoride (PVDF) membrane (Millepore) by conventional Western blotting. Prior to transfer, a 8 cm x 7.5 cm PVDF membrane was cut in the

top left hand corner to indicate orientation. The membrane was soaked in 100% methanol for 12 seconds, distilled water for 2 minutes and transfer buffer for 2 minutes. The Bio-Rad transfer cassette was prepared as shown in Figure 9, with filter paper placed on a sponge soaked in transfer buffer. The NuPAGE gel was removed from its plastic covers and washed in transfer buffer before placing on top of the filter paper. The PVDF membrane was positioned on-top of the gel with the cut corner to the top left hand side of the gel. Filter paper was placed on top of the PVDF membrane and the stack was finished with a sponge soaked in transfer buffer. The transfer cassette was clamped closed and transferred to a tank containing transfer buffer at 4°C and an ice block to maintain the temperature throughout the transfer. The tank was run at 100 volts for 60 minutes using a BioRad power pack.

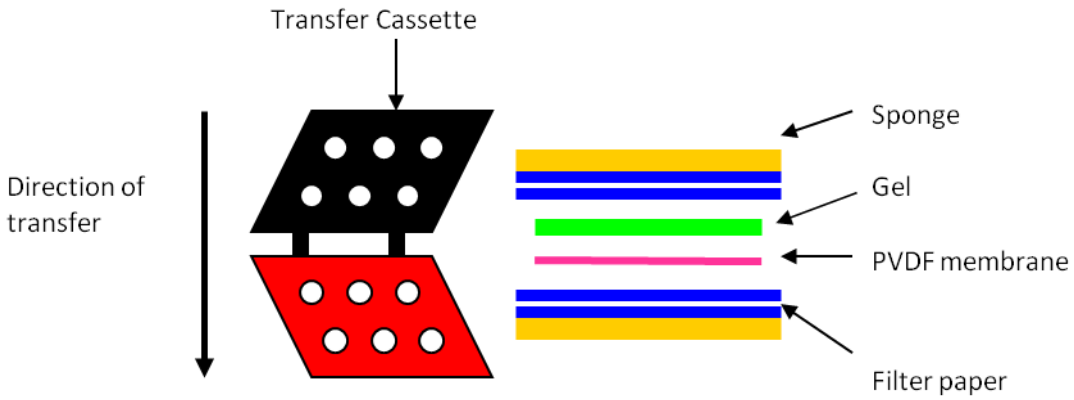


Figure 9: Preparation of the transfer cassette for immunoblotting.

The transfer cassette was prepared by placing filter paper on top of a sponge, followed by the gel. The PVDF membrane was placed on top of the gel with the cut corner to the top left hand side of the gel. Filter paper was placed on top of PVDF membrane and the stack was finished with a sponge. All components were soaked in transfer beffer before forming the stack.

After the transfer was complete, the filter paper and sponge on top of the PVDF membrane were removed. The resulting PVDF membrane was washed in TBS-Tween (TBS-T) three times each for 5 minutes followed by blocking in 5% non-fat dried milk in TBS-T for 1 hour or 3% BSA in TBS-T for 1 hour (For streptavidin HRP immunoblots only). The membrane was washed twice with TBS-T and incubated for one hour, unless specified otherwise, with the primary antibody at the appropriate dilution (See Table 3), whilst shaking. Following the incubation with the primary antibody the PVDF membrane was washed in TBS-T five times each for 10 minutes. The PVDF membrane was then incubated for one hour whilst shaking with a horseradish peroxidase (HRP)-conjugated secondary antibody at the appropriate dilution (See Table 3) and a 1 in 5000 dilution of Strep Tactin (Bio-Rad) to develop the marker. Following the incubation with the secondary antibody the PVDF membrane was washed in TBS-T five times each for 10 minutes. The PVDF membrane was developed by adding a 1 to 1 mix of Supersignal West Pico Luminol (Thermo scientific) and Supersignal West Pico Stable (Thermo scientific) onto the protein side of the membrane (cut corner to the right) and incubated for 5 minutes. Chemiluminescence was captured using an Image Station 2000R (Kodak).

Table 3: Primary and secondary antibodies used in immunoblotting.

Primary Antibody		Secondary Antibody	
Antibody	Dilution & incubation time	Antibody	Dilution & incubation time
Streptavidin HRP (Sigma)	1:500 1 hour		
Rabbit anti-human C3c (DAKO)	1:5000 Overnight	Goat anti-rabbit HRP (DAKO)	1:10000 1 hour
Mouse anti-human C3a (Technoclone)	1:2500 1 hour	Rabbit anti-mouse HRP (DAKO)	1:5000 1 hour
Goat anti-human α2-antiplasmin HRP (Enzyme Research Labs)	1:5000 1 hour		
Rabbit anti-human fibrinogen (DAKO)	1:5000 1 hour	Goat anti-rabbit HRP (DAKO)	1:2000 1 hour
Sheep anti-human factor XIII A subunit HRP (Enzyme Research Labs)	1:4000 1 hour		
Goat anti-human plasminogen HRP (Enzyme Research Labs)	1:5000 1 hour		
Goat anti-human Factor H (Quidel)	1:10000 1 hour	Rabbit anti-goat HRP (SIGMA)	1:10000 1 hour
Goat anti-human C5 (Quidel)	1:1000 Overnight	Rabbit anti-goat HRP (SIGMA)	1:2000 1 hour

3.3. C3 ELISA

An in-house sandwich ELISA established in the DCDR by Mrs May Boothby and Mrs Jane Brown (Technicians) was used to assess the concentration of C3 following C3 purification. A 96 well microtitre plate (Nunc) was coated with 100 µl of 1 in 10,000 diluted rabbit anti-human C3c antibody (DAKO) in phosphate buffered saline (PBS) for 1 hour at room temperature whilst shaking, before storing overnight at 4°C. On the day of assay the microtitre plate was brought to room temperature and washed four times with 200 µl of PBS-Tween (PBS-T) (No blocking step was included as described in section 3.3.1). A quality control sample (pooled normal human citrated plasma) and all test samples were diluted 1:100,000 in PBS-T using a MICROLAB 500 diluter. A standard curve was set up by diluting a different pooled normal human plasma sample calibrated against Liquichek immunology control level 3 (BioRad). Samples (100 µl) were added to the wells in duplicate before incubating for 2 hours at room temperature on a plate shaker. The microtitre plate was washed four times with 200 µl of PBS-T and 100 µl of 1 in 10000 diluted goat anti-human C3 antibody (Quidel) in PBS-T was added. The plate was incubated for 1 hour at room temperature whilst shaking and then washed four times with 200 µl of PBS-T. A 1 in 2000 diluted HRP conjugated rabbit anti-sheep (DAKO) in PBS-T (100 µl) was added and incubated for 1 hour at room temperature whilst shaking. The microtitre plate was washed four times with 200 µl of PBS-T. Activated o-Phenylenediamine (OPD) substrate was prepared by dissolving 4 OPD tablets (DAKO) in 12 ml of deionised water with the addition of 5 µl of 30% hydrogen peroxide (Sigma). The activated OPD substrate (100 µl) was added to the wells at 10 second intervals and allowed to develop for ~4 minutes whilst shaking. Once a suitable colour developed, the reaction was stopped by the addition of 100 µl of 1.5M sulphuric acid (Fisher) at 10 second intervals. The absorbance was measured at 490 nm on the DYNEX MRX microtitre plate reader and C3 concentrations in the samples were automatically read from the standard curve using Revelations software (version 2.02).

3.3.1. Evidence for the absence of a blocking step in the C3 ELISA

Previous optimisation steps including the need for a blocking step was performed by Mrs May Boothby and Mrs Jane Brown (Technicians). To determine whether C3 bound non-specifically to the ELISA plate in the absence of a blocking agent, a blocking step with 3% BSA in PBS-T was compared with no blocking step. As shown in Table 4 the background (PBS-T blank) absorbance and normal pooled plasma absorbances were higher in the presence of a blocking step compared to the absence of a blocking step, suggesting C3 does not bind non-specifically to the ELISA plate. These results suggest a blocking step was not required in the final optimised protocol.

Table 4: C3 ELISA performed in the presence and absence of a blocking step to determine non-specific binding to the ELISA plate

The ELISA plate was blocked with either PBS-T only or 3% BSA in PBS-T. The absorbance at 490 nm was measured in wells incubated with a PBS-T blank or normal pooled plasma. The PBS-T blank absorbance and normal pooled plasma absorbance was higher in the blocked compared to the non-blocked wells.

	No Blocking	Blocking
PBS-T Blank	0.179	0.206
Normal pooled plasma	0.629	0.770

3.4. Evaluation of the influence of C3 on fibrin structure and function

3.4.1. Turbidimetric analyses

The turbidimetric clotting assay is an optical method for monitoring fibrin formation and lysis. Parameters derived from turbidimetric measurements include lag time, maximum absorbance and 50% lysis time. Lag time represents the time required for sufficient formation of fibrin protofibrils to allow lateral aggregation (Figure 10, Panels A & C). Lateral aggregation of the fibrin protofibrils results in exponential increases in absorbance which plateaus at the maximum absorbance (Figure 10, Panels A & C) (Wolberg 2007). In plasma, maximum absorbance is a measure of clot density, whereas in a purified system, maximum absorbance is a measure of fibrin fibre thickness and pore size. 50% lysis time represents the time taken from the addition of the lysis activation mix (t-PA and plasminogen) to half the maximum absorbance, which in the concurrent turbidimetric assay is the time taken from the beginning of the assay period to half the maximum absorbance, whereas in the overlay turbidimetric assay it is from addition of t-PA and plasminogen after clot formation to half the maximum absorbance (Figure 10, Panels B & C).

3.4.1.1. Turbidity Assay

To a 96 well plate (Greiner), purified fibrinogen (final concentration of 0.5 mg/ml) was added in the presence and absence of purified FXIII (final concentration 0.0055 mg/ml). Purified complement C3 was added in duplicate at the following final concentrations: 0, 0.15, 0.35, 0.55 and 0.75 mg/ml along with TBS to a final reaction volume of 100 µl. Fifty µl of an activation mix containing 0.025 U/ml final concentration of thrombin and 5 mM final concentration of $CaCl_2$ in TBS were added to the fibrinogen mix to initiate fibrin clot formation. Immediately after the addition of the activation mix the plate was shaken for 2 seconds (intensity setting 3). The absorbance was measured every 12 seconds at 340 nm at 37°C for 1 hour in a ELx808 spectrophotometer (Bio-Tek). The resulting data was analysed for lag times and the maximum absorbance using customised software program (Carter et al.

2007) as illustrated in Figure 10, Panel A. Three independent experiments were carried out.

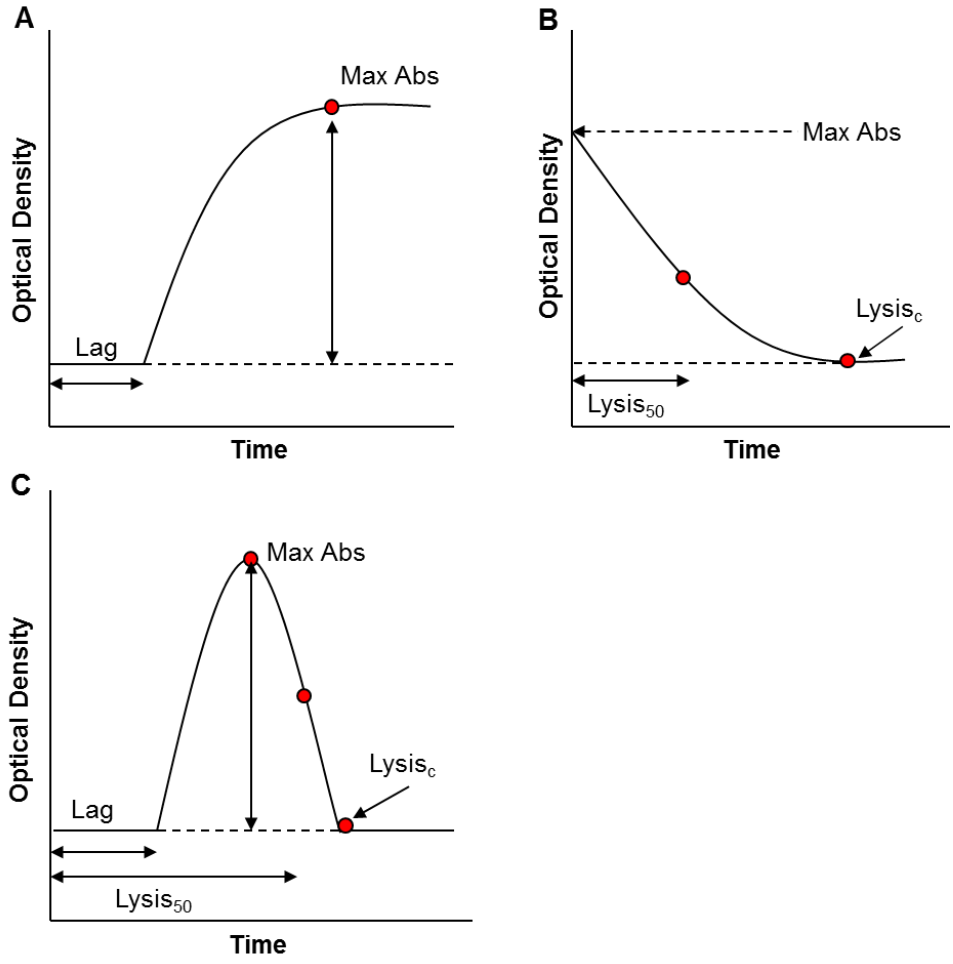


Figure 10: Illustration of the turbidity and lysis variables.

Panel A. Turbidimetric clotting assay parameters. **Panel B**. Lysis overlay assay parameters **Panel C**. Concurrent turbidity and lysis assay parameters: Lag time (Lag), Maximum Absorbance (Max Abs), Time to 50% Lysis ($Lysis_{50}$), Complete Lysis ($Lysis_c$).

3.4.1.2. Lysis Overlay Assay

Clots formed at the end of the turbidity assay (section 3.4.1.1) were overlaid with 100 µl of a lysis mix containing 100 ng/ml final concentration of t-PA (Technoclone) and 50 µg/ml final concentration of purified plasminogen in TBS. Following the addition of the lysis mix the plate was shaken on a plate shaker for 30 seconds and left to incubate for 1 hour. The plate was then placed into a ELx808 spectrophotometer (Bio-Tek), where the absorbance was measured every 108 seconds at 340 nm at 37°C for 9 hours. The resulting data was analysed for time to 50% lysis as illustrated in Figure 10, panel B. A minimum of 3 independent experiments were carried out.

Lysis products produced by the lysis overlay were stored in 4X loading and 10X reducing buffer at -20°C for analysis by gel electrophoresis and immunoblotting as described in section 3.2 and identification by MALDI-MS (see section 3.8).

3.4.1.3. Concurrent Turbidity and Lysis Assay

To a 96 well plate (Greiner), purified fibrinogen (final concentration of 0.5 mg/ml) and purified plasminogen (final concentration of 20 µg/ml) were added in the presence and absence of purified FXIII (final concentration of 0.0055 mg/ml). Purified complement C3 was added in duplicate to the following final concentrations: 0, 0.15, 0.35, 0.55 and 0.75 mg/ml, and TBS added to a final reaction volume of 100 µl. Fifty µl of an activation mix containing 0.025 U/ml final concentration of thrombin, 5 mM final concentration of $CaCl_2$ and 12.5 ng/ml final concentration of t-PA in TBS were added to the fibrinogen and plasminogen mix to initiate fibrin clot formation and lysis. The absorbance was measured every 12 seconds at 340 nm at 37°C for 2 hours in a ELx808 spectrophotometer (Bio-Tek). The resulting data was analysed for lag time, maximum absorbance and time to 50% lysis (Figure 10, Panel C) using a customised software program (Carter et al. 2007). A minimum of 3 independent experiments were carried out.

3.4.2. Concurrent turbidity and lysis assay modifications

3.4.2.1. Plasminogen and t-PA dose response

In some concurrent turbidity and lysis experiments the concentration of plasminogen and t-PA were modified to determine the influence of modified lysis time on the effect of C3 on fibrin structure and lysis. To a 96 well plate (Greiner), purified fibrinogen (final concentration of 0.5 mg/ml), purified C3 (final concentration of 0.5 mg/ml) were added in the presence of purified FXIII (final concentration 0.0055 mg/ml). Purified plasminogen was added in duplicate to the following final concentrations: 20, 2 and 0.2 μg/ml along with TBS to a final reaction volume of 100 μl. The activation mix (50 μl) containing 0.025 U/ml final concentration of thrombin, 5 mM final concentration of $CaCl_2$ and either 50, 10 or 5 ng/ml final concentration of t-PA in TBS was added to the fibrinogen mix to initiate fibrin clot formation and lysis. The absorbance was measured every 12 seconds at 340 nm at 37 °C for 2 hours in a ELx808 spectrophotometer (Bio-Tek). Three independent experiments were performed.

3.4.3. Plasma concurrent turbidity and lysis assay with spiked C3

To a 96 well plate, 25 μL of plasma, spiked with additional purified C3 to equivalent plasma concentrations of 0, 0.5, 1, 1.5 and 2 mg/ml. TBS was added to a final volume of 50 μL. Fifty μL of 12.5 ng/ml of t-PA in TBS were added at 10 second intervals to each column, followed by 50 μL of an activation mix containing 0.03 U/ml thrombin and 7.5 mM $CaCl_2$ in TBS at 10 second intervals for each column. The absorbance was measured every 12 seconds at 340 nm at 32°C for 1 hour in a ELx808 spectrophotometer (Bio-Tek). The resulting data was analysed for lag time and maximum absorbance using a customised software program (Carter et al. 2007).

At the end of the turbidity run, the plate remained in the ELx808 spectrophotometer (Bio-Tek) and the absorbance was measured every 108 seconds at 340 nm at 32°C for 9 hours. The resulting data was analysed for time to 50% lysis using a customised software program (Carter et al. 2007). Three independent experiments were carried out.

3.5. Analysis of FXIII-dependent cross-linking of C3 to 5-(biotinamido)pentylamine and fibrin

3.5.1. Analysis of FXIII-dependent cross-linking of 5-(biotinamido)pentylamine to C3

3.5.1.1. FXIII-dependent cross-linking of 5-(biotinamido)pentylamine into immobilised C3 in a plate based assay

To determine whether C3 is a substrate of FXIIIa, BP was cross-linked to immobilised C3. In a 96 well microtitre plate (Nunc), 100 µl of purified C3 (50 µg/ml) in TBS1, or 100 µl of purified fibrinogen in TBS1 (40 µg/ml) as a positive control, were incubated at room temperature whilst shaking for 1 hour. The plate was blocked with 200 µl of 1% BSA in TBS1 for 1 hour at room temperature whilst shaking. Wells were washed three times with 200 µl of TBS1-T. Purified FXIII (30 µl at final concentrations of 0, 0.5, 1 and 2 µg/ml) were added in duplicate. BP cross-linking was initiated by the addition of 70 µl of an activation mix containing final concentrations of 1 U/ml thrombin (Calbiochem), 10 mM $CaCl_2$, 1 mM BP (Pierce) and 0.5 mM dithiothreitol (DTT). As a negative control the activation mix contained final concentrations of 1 mM BP and 0.5 mM DTT only. The activation mixes were added to each well at 10 second intervals, the plate was incubated for 20 minutes at 37 °C and 200 µl of 200 mM EDTA were added to each well at 10 second intervals to stop the reaction. The plate was washed three times with 200 µl of TBS1-T and 100 µl of 2 µg/ml streptavidin-alkaline phosphatase (Sigma) in TBS1 were added to the plate and incubated at room temperature for 1 hour whilst shaking. The plate was washed with 200 µl of TBS1-T and 100 µl of 1 mg/ml p-nitrophenyl phosphate (Sigma) in 1M diethanolamine containing 0.5 mM $MgCl_2$, pH 9.8 were added to each well at 10 second intervals and incubated at 37°C until a suitable colour developed. The reaction was stopped by the addition of 100 µl of 4M NaOH at 10 second intervals. The plates were read at 405 nm on a DYNEX MRX microtitre plate reader. Three independent experiments were carried out.

3.5.1.2. Cross-linking of BP into C3 in solution using a plate based assay

To determine whether C3 is cross-linked to BP when incubated in solution, an alternative plate based assay was performed which captured C3 cross-linked products using a C3 antibody and detected BP cross-linking using streptavidin HRP. In a 96 well microtitre plate (Nunc) 100 µl of 1 in 10,000 rabbit anti-human C3c antibody (Dako) in TBS1 were incubated overnight at 4°C. The plate was brought to room temperature on the day of assay and was blocked with 200 µl of 1% BSA in TBS1 for 1 hour at room temperature whilst shaking followed by three washes with 200 µl TBS1-T. In separate eppendorf tubes 0.5 mg/ml final concentration of purified C3 was incubated with final concentrations of 5 mM BP, 0.022 mg/ml FXIII in the presence and absence of 1.0 U/ml thrombin and 5 mM $CaCl_2$. TBS1 was added to a final reaction volume of 110 µl. The above reactions were incubated at 37°C for 0, 5, 10, 30 and 60 minutes. At each time point the reactions were stopped by the addition of 110 µl of 200 mM EDTA. The reactions were transferred to the microtitre plate, 100 µl per well in duplicate, and incubated at room temperature for one hour whilst shaking. The plate was washed three times with 200 µl of TBS1-T. One hundred µl of 2 µg/ml streptavidin-alkaline phosphatase (Sigma) in TBS1 were added to the plate, which was incubated at room temperature for 1 hour whilst shaking prior to washing with 200 µl of TBS1-T. One hundred µl of 1 mg/ml p-nitrophenyl phosphate (Sigma) in 1M diethanolamine containing 0.5 mM $MgCl_2$, pH 9.8 were added to each column at 10 second intervals and incubated at 37°C until a suitable colour developed. The reaction was stopped in each column at 10 second intervals with 100 µl of 4M NaOH. The plates were read at 405 nm on a DYNEX MRX microtitre plate reader. Three independent experiments were carried out.

3.5.1.3. Cross-linking of BP into C3 in solution analysed by SDS-PAGE

To determine which chains of C3 become cross-linked by FXIIIa, BP was cross-linked to C3 in solution. Purified C3 (final concentration 0.5 mg/ml), FXIII (final concentration 0.022 mg/ml) and BP (final concentration 5 mM) were added to a 1.5 ml Eppendorf tube. Cross-linking was initiated by the addition of thrombin (final concentration 1 U/ml) and $CaCl_2$ (final concentration 5 mM) in TBS. The final

volume was 1 mls and the reactions were incubated at 37°C. Cross-linking was stopped by the addition of 5 µl of 200 mM EDTA at 30 minutes, 1 and 4 hours. As negative controls purified C3 was incubated in the absence of FXIII, thrombin, and $CaCl_2$ for 4 hours only. As a positive control purified fibrinogen (final concentration 0.5 mg/ml) was incubated with BP and pre-activated FXIII (to allow cross-link formation but prevent fibrin clot formation). FXIII was pre-activated with thrombin and $CaCl_2$ (concentrations as above) for 1 hour at 37°C. Thrombin was inactivated by the addition of 10 µl of 10 U/µl of hirudin at 37°C for 10 minutes. Pre-activated FXIII was incubated with purified fibrinogen for 4 hours at 37°C. Cross-link formation was stopped by the addition of 5 µl of 200 mM EDTA. The resulting BP cross-link products underwent gel electrophoresis under reduced conditions and were analysed by streptavidin HRP Western blot as described in section 3.2.

3.5.2. Cross-linking of C3 to fibrin in a purified system

Gel based cross-linking assays were performed to determine whether C3 is cross-linked to itself to form homodimers and cross-linked to fibrin during clot formation. To a 0.5 ml eppendorf, purified C3 (final concentration 0.5 mg/ml) or hydrolysed C3 (purified C3 dialysed in 1 litre of water each for 2 one hour incubations and a further week), purified fibrinogen (final concentration 1 mg/ml), purified FXIII (final concentration 0.022 mg/ml) and TBS were added to a final volume of 100 µl. Fibrin cross-linking was initiated upon the addition of 20 µl of an activation mix containing thrombin (final concentration of 0.025 U/ml), $CaCl_2$ (final concentration of 5 mM) and TBS. The reaction mixtures were vortex mixed and incubated at 37°C for the following time points; 0, 5, 10, 30, 60, 240 minutes and 24 hours. Cross-linking was stopped by the addition of 4x loading buffer (Invitrogen) and 10x reducing agent (Invitrogen) and incubation of the samples at 95°C for 10 minutes in a heat block. Twenty µl of the sample were loaded onto a NuPAGE 3-8% Tris-acetate gels (Invitrogen). The SDS-PAGE gels were either stained with GelCode® Blue Stain Reagent (Pierce) or immunoblotted, as described in section 3.2 with rabbit anti-human C3c antibody and rabbit anti-fibrinogen antibody (see Table 3).

3.5.3. Cross-linking of C3 to plasma clot components

Plasma clots were formed by the addition of 10 µl of an activation mix containing 5 U/ml thrombin, 15 mM $CaCl_2$ (final concentration) in TBS to 100 µl of human plasma. One hundred µl of the mix were pipetted into a burette and incubated in a humidified chamber for 4 hours at room temperature. Burettes were attached by tubing to a buffer reservoir (Figure 11). The plasma clots were washed by perfusing with either 2 M NaCl followed by a TBS wash each for 2 hours or a wash with TBS only for 4 hours. Cross-link formation was stopped by the addition of 4x loading buffer (Invitrogen) and 10x reducing agent (Invitrogen) as described in section 3.5.2 and clots were run on NuPAGE 3-8% Tris-Acetate gels, stained with GelCode Blue or immunoblotted as described in section 3.2 with rabbit anti-human C3c antibody and goat anti-human α2-antiplasmin antibody (see Table 3).

3.5.4. Cross-linking of C3 in a plasma based system over a time-course

Plasma clots were formed by the addition of 10 µl of activation mix containing 5 U/ml thrombin, 15 mM $CaCl_2$ (final concentration) in TBS to 100 µl of human plasma. Plasma clots were incubated at 37°C for 0, 5, 10, 30 minutes and 1, 2 and 4 hours. Plasma clot formation was stopped by the addition of 4x loading buffer (Invitrogen) and 10x reducing agent (Invitrogen) as described in section 3.5.2 and clots were run on NuPAGE 3-8% Tris-Acetate gels, stained with GelCode Blue or immunoblotted as described in section 3.2 with rabbit anti-human C3c antibody and rabbit anti-fibrinogen antibody (see Table 3).

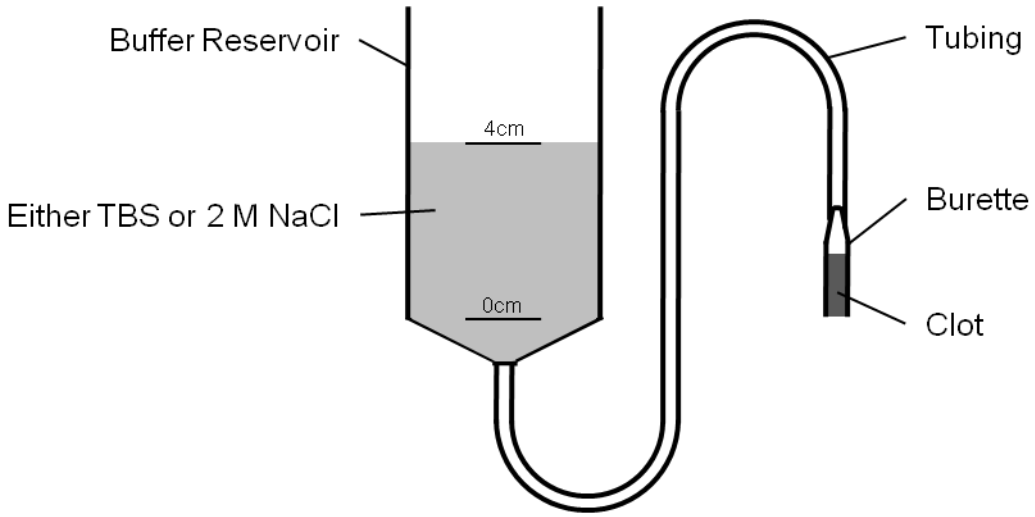


Figure 11: Method for perfusing plasma clots.

Plasma clots were formed in the end of a burette whilst the other end was filled with buffer and attached to the tubing containing buffer without the addition of any air bubbles. Buffer was added to the buffer reservoir until it reached the 4 cm mark.

3.6. Time dependent cleavage of C3

To evaluate the effect of t-PA mediated plasmin cleavage of C3 in the presence and absence of fibrin on the C3 cleavage products, a time course was set up. To a 0.5 ml eppendorf tube the reagents summarised in Table 5 were added (at final concentrations) to a total volume of 100 μL. Reactions 1-4 were incubated at 37°C for either 0, 30 minutes, 1, 2, 4 or 24 hours. Reactions 5-8 were initially allowed to form a clot by the addition of thrombin and calcium to fibrinogen in the presence and absence of C3 and FXIII for 1 hour at 37°C in a water bath. Plasminogen and t-PA were subsequently incubated on top of the clots at 37°C for either 0, 30 minutes, 1, 2, 4 or 24 hours. At each time point the reaction was stopped by the addition of 4x loading buffer (Invitrogen) and 10x reducing agent (Invitrogen) and heated to 95°C for 10 minutes in a heat block. Gel electrophoresis and immunoblotting were performed according to the methods described in section 3.2. Immunoblots were analysed for C3, fibrinogen and plasminogen (see Table 3).

3.7. Thrombin and plasmin cleavage of C3

To a 0.5 ml Eppendorf tube, purified C3 to a final concentration of 0.5 mg/ml was incubated with 0.025, 1, 5 or 18 U/ml final concentration of thrombin (Calbiochem) or 10, 22 or 44 μg/ml final concentration of plasmin (Calbiochem) for 4 hours at 37°C. TBS was added giving a final reaction volume of 60 μl. After 4 hours the reactions were stopped by the addition of 4x loading buffer (Invitrogen) and 10x reducing agent (Invitrogen) and heated to 95°C for 10 minutes in a heat block. C3 cleavage products were analysed by gel electrophoresis and immunoblotting as described in section 3.2. In addition, plasmin cleavage products of C3 were analysed by gel electrophoresis and coomassie stained for MS analysis (section 3.8).

Table 5: Reaction conditions used to analyse the time dependent cleavage of C3.

Reaction	C3 (0.5 mg/ml)	Fbg (0.5 mg/ml)	FXIII (5.5 µg/ml)	Thr (0.025 U/ml) Ca^{2+} (5 mM)	Plg (50 µg/ml) tPA (100 ng/ml)
1					√
2	√				√
3		√			√
4	√	√			√
5		√		√	√
6	√	√		√	√
7		√	√	√	√
8	√	√	√	√	√

Included in the reaction (√). Fibrinogen (Fbg), plasminogen (Plg), tissue plasminogen activator (t-PA), thrombin (Thr).

3.8. Analysis of the C3 cleavage products by MALDI-MS/MS and LC-MS/MS

Lysis products obtained in section 3.4.1.2 were run on 4-12% Bis Tris NuPAGE gels (section 3.2) and stained with Coomassie blue (section 3.2.2). Cleavage products of C3 were assessed by matrix assisted laser desorption/ionisation mass spectrometry (MALDI-MS) in the proteomics facility, University of Leeds, by Dr Jeffrey N Keen, employing standardised methods (Gage et al. 2009) or by MALDI-MS/MS and liquid chromatography tandem mass spectrometry (LC-MS/MS) in the proteomics facility, York University by Dr Adam Dowle.

3.8.1. Endoproteinase Asp-N and trypsin digestion of C3 cleavage products

Protein bands were manually excised from a Coomassie stained 4-12% Bis-Tris gel using a razor blade. Protein bands were cut into 2 mm^2 pieces and were washed and de-stained in 200 µL 50 mM ammonium bicarbonate (v/v) in acetonitrile for 10 minutes in a 37°C water bath. This process was repeated three times. The resulting gel pieces were dehydrated in 200 µL 100% acetonitrile for 5 minutes and allowed to completely dry in air (approximately 30 minutes). The dehydrated gel pieces were rehydrated with 2 µL 25 mM ammonium bicarbonate containing 0.02 mg/ml trypsin (Promega). Eight µL of 25 mM ammonium bicarbonate was added to cover the gel piece and digestion was allowed to proceed for 18 hours at 37°C.

In-gel combined Asp-N and trypsin digestion were performed after reduction with DTT and S-carbamidomethylation with iodoacetamide. Gel pieces were washed two times with 50% (v:v) aqueous acetonitrile containing 25 mM ammonium bicarbonate, then once with acetonitrile and dried in a vacuum concentrator for 20 min. Lyophilized Endoproteinase Asp-N from *Pseudomonas fragi* mutant strain (Sigma) was dissolved in the 100 µL 50 mM ammonium bicarbonate to give a final enzyme concentration of 0.02 µg/µL. Gel pieces were rehydrated by adding 10 µL of Asp-N solution and after 10 min enough 50 mM ammonium bicarbonate solution was added to cover the gel pieces. Digests were incubated overnight at 37°C. A 5 µL aliquot was taken for subsequent tryptic digestion. Sequencing-grade, modified

porcine trypsin (Promega) was dissolved in the 50 mM acetic acid supplied by the manufacturer, then diluted 5-fold by adding 25 mM ammonium bicarbonate to give a final trypsin concentration of 0.02 μg/μL. A 10 μL aliquot of trypsin solution was added to the peptide mixture. Digestion was allowed to proceed overnight at 37^{o}C.

Peptide mixtures were desalted and concentrated using 0.2 μL C18 ZipTips (Millipore) with elution into 3 mL 50% (v:v) aqueous acetonitrile containing 0.1% (v:v) trifluoroacetic acid. A 1 μL aliquot of the desalted peptide mixture was applied directly to a ground steel MALDI target plate, followed immediately by an equal volume of a freshly-prepared 5 mg/mL solution of 4-hydroxy-α-cyano-cinnamic acid (Sigma) in 50% aqueous (v:v) acetonitrile containing 0.1% trifluoroacetic acid (v:v).

3.8.2. MALDI-MS/MS analysis of Asp-N and tryptic peptides

Positive-ion MALDI mass spectra were obtained using a MALDI L/R mass spectrometer (Waters) in reflectron mode, equipped with a N_2 laser beam. MS spectra were acquired over a mass range of m/z 800-5000. Final mass spectra were externally calibrated against alcohol dehydrogenase and internally calibrated against trypsin autolysis products (m/z 2211.105 or 1045.564).

Positive-ion MALDI mass spectra were obtained using a Bruker ultraflex III in reflectron mode, equipped with a Nd:YAG smart beam laser. MS spectra were acquired over a mass range of m/z 800-5000. Final mass spectra were externally calibrated against des-Arg1-Bradykinin (m/z 904.681); Angiotensin I (m/z 1296.685); Glu1-Fibrinopeptide B (m/z 1750.677); ACTH (1-17 clip) (m/z 2093.086); ACTH (18-39 clip) (m/z 2465.198); ACTH (7-38 clip) (m/z 3657.929). Monoisotopic masses were obtained using a SNAP averaging algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) and a signal to noise threshold of 2. For each sample the ten strongest peaks of interest with a signal to noise greater than 30 were selected for MS/MS fragmentation. Further peaks were manually selected for fragmentation as required. Fragmentation was performed in LIFT mode without the introduction of a collision gas. Bruker flexAnalysis software (version 3.3) was used to perform the spectral processing and peak list generation for both the MS and MS/MS spectra.

3.8.3. LC-MS/MS analysis of Asp-N and trypsin peptides

The trypsin only and trypsin and Asp-N digested samples were loaded onto a nanoAcquity UPLC system (Waters) equipped with a nanoAcquity Symmetry C_{18}, 5 µm trap (180 µm x 20 mm Waters) and a nanoAcquity BEH130 1.7 µm C_{18} capillary column (75 µm x 250 mm, Waters). The trap wash solvent was 0.1% (v/v) aqueous formic acid and the trapping flow rate was 10 µL/min. The trap was washed for 5 min before switching flow to the capillary column which had a flow rate of 300 nL/min and a column temperature of 60°C. The peptide separation used a gradient elution of two solvents (solvent A: 0.1% (v/v) formic acid; solvent B: acetonitrile containing 0.1% (v/v) formic acid). Initial conditions used 5% solvent B (2 min), followed by a linear gradient to 35% solvent B over 20 min and a wash with 95% solvent B for 2.5 min. The column was returned to initial conditions and re-equilibrated for 25 min before subsequent injections.

The nanoLC system was interfaced with a maXis LC-MS/MS System (Bruker Daltonics) with a nano-electrospray source fitted with a steel emitter needle (180 µm O.D. x 30 µm I.D., Proxeon). Positive ESI- MS & MS/MS spectra were acquired using AutoMS/MS mode. Instrument control, data acquisition and processing were performed using Compass 1.3 SP1 software (microTOF control, Hystar and DataAnalysis, Bruker Daltonics). Instrument settings were: ion spray voltage= 1,500 V; dry gas= 6 L/min; dry gas temperature= 160 °C; ion acquisition range= *m/z* 50-2,200. AutoMS/MS settings were: MS= 0.5 s (acquisition of survey spectrum); MS/MS= Collision induced dissociation with N_2 as collision gas; ion acquisition range= *m/z* 350-1,400

3.8.4. MASCOT analysis

Tandem mass spectral data were submitted to database (Swissprot or NCBI) searching using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.3). Search criteria included: Enzyme= trypsin and Asp-N; Fixed modifications= Carbamidomethyl; Variable modifications= Oxidation, Deamidation. Peptide tolerance= 250 ppm; MS/MS tolerance= 0.5 Da; Instruments= MALDI-TOF, MALDI-TOF-TOF or ESI-MS/MS.

3.9. Plasmin Generation Assay

Plasmin generation assays were performed to determine whether C3 influenced the amount and rate of plasmin generated, using a plate based method previously described (Dunn et al. 2006) and a clot based method previously described (Longstaff and Whitton 2004).

3.9.1. Plate based plasmin generation assay

Plasmin generation was examined using a plate based assay monitoring absorbance at 405 nm as a result of S-2251 cleavage to form pNA (Chemical formula: H-D-Val-Leu-Lys-OH+pNA) production, which provides information relating to the final amount of plasmin and the rate of plasmin generation. The amount of t-PA required to allow complete plasmin generation within the two hour incubation period was optimised as described in section 3.9.1.1. A 96 well microtitre plate (Nunc) was coated with 100 µl of fibrinogen per well at a final concentration of 80 µg/ml in TBS2 for 40 minutes at room temperature. The coating solution was removed and the plate was washed 3 times with 300 µl per well TBS2-T. The wells were blocked with 300 µl of TBS2-blocking buffer for 90 minutes at 37°C. The blocking buffer was removed and the plate was washed 3 times with 300 µl per well with TBS2-T. The plate was incubated for 45 minutes at room temperature with 100 µl of 1 U/ml thrombin, 5 mM $CaCl_2$ in TBS2. Following incubation, the plate was initially washed 3 times with 300 µl per well with high salt buffer then 3 times with 300 µl per well of wash buffer. The plate was incubated for 90 minutes at 37°C with 50 µl per well of 0-700 ng/ml t-PA. Following incubation the plate was washed 3 times with 300 µl per well with TBS2-T. To the plate, 100 µl of 600 nmol/L plasminogen in the presence of 0.8 mmol/L S-2251 in TBS were added per well. The plate was incubated at 37°C for 60 minutes, during which time the increase in absorbance (monitoring cleavage of S-2251) was measured at 405 nm using an ELx808 spectrophotometer (Bio-Tek). The quantity of plasmin generated in each well was determined by calculating the rate of change in absorbance (production of pNA) between the start of the assay and the maximum absorbance and applying the standardisation provided by the manufacturer (Chromogenix): The increase in absorbance at 405 nm is proportional to the amount of plasmin generated. An

increase in absorbance of 0.05 units per minute at 37°C when S-2251 is 2x Km (0.8 mmol/L) of the reaction between S-2251 and plasmin equals a plasmin concentration of 0.4 mg/L (equation 1). Rate of plasmin generation between 0 and 2 hours was determined using equation 2.

Equation 1

Plasmin (mg/L) = (Abs change per minute / 0.05) x 0.4

Equation 2

Rate of plasmin generation = $\frac{\text{Plasmin generated}}{\text{Time taken}}$

(mg/L/min)

3.9.1.1. Optimisation of the plate based plasmin generation assay

The concentration of t-PA was optimised to determine the amount required to reach plateau in the presence of 600 mol/L plasminogen, and therefore the maximum plasmin generation. As shown in Figure 12 only t-PA concentrations above 350 ng/ml were capable of reaching plateau over the 2 hour incubation period. A t-PA concentration of 500 ng/ml was chosen for future experiments as the plateau was reached well within the 2 hour incubation and produced curves similar to those seen with 700 ng/ml t-PA.

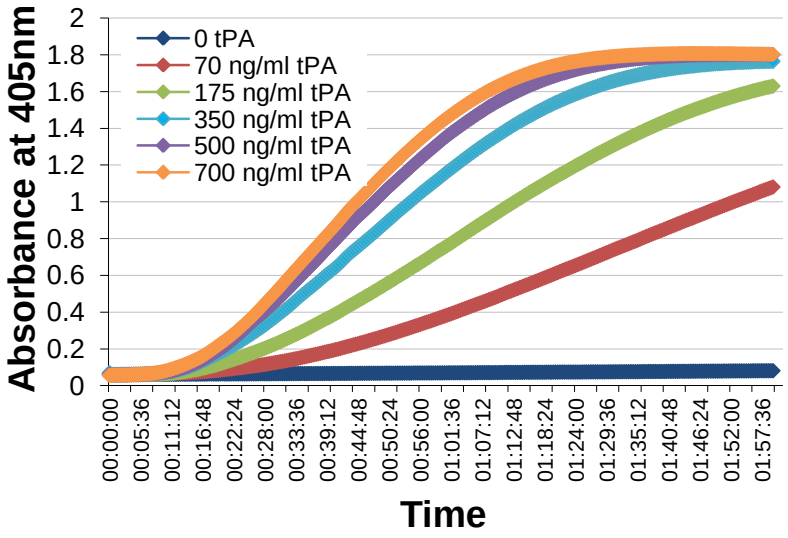


Figure 12: Optimisation of the concentration of t-PA in the plate based plasmin generation assay.

At higher t-PA concentrations the faster the maximum absorbance was reached. However only in the presence of 350 µg/ml or higher was the maximum absorbance reached within the assay period.

3.9.1.2. Final reaction conditions for the plate based plasmin generation assay

A 96 well microtitre plate (Nunc) was coated with fibrinogen, blocked with TBS2-blocking buffer and the fibrinogen was cleaved to form fibrin in the presence of 1 U/ml thrombin and 5 mM $CaCl_2$ as described in section 3.9.1. Following fibrin formation, the plate was initially washed 3 times with 300 µl per well with high salt buffer then 3 times with 300 µl per well of wash buffer. The plate was incubated for 90 minutes at 37°C with 50 µl per well of either TBS2, 500 ng/ml tPA, 0.5 mg/ml C3 or 600 nmol/L plasminogen in TBS2 as summarised in Table 6. Following incubation the plate was washed 3 times with 300 µl per well with TBS2-T prior to adding 50 µl per well of either 500 ng/ml tPA, 0.5 mg/ml C3 or 600 nmol/L plasminogen in TBS (Table 6) for 90 minutes at 37°C. To the plate, 100 µl of either 600 nmol/L plasminogen or 500 ng/ml t-PA in the presence of 0.8 mmol/L S-2251 in TBS were added per well (Table 6). Plasmin generation was measured using a

ELx808 spectrophotometer (Bio-Tek) as described in section 3.9.1. The amount of plasmin generated and rate of plasmin generation described in equations 1 and 2.

Table 6: Reaction conditions for the plate based plasmin generation assay.

Reaction	Incubation 1	Incubation 2	Activation
1	TBS	t-PA	Plg and S-2251
2	C3	t-PA	Plg and S-2251
3	t-PA	C3	Plg and S-2251
4	TBS	t-PA and C3	Plg and S-2251
5	TBS	Plg	t-PA and S-2251
6	C3	Plg	t-PA and S-2251
7	Plg	C3	t-PA and S-2251
8	TBS	Plg and C3	t-PA and S-2251

Tissue plasminogen activator (t-PA), tris buffer saline (TBS), plasminogen (Plg)

3.9.2. Clot based plasmin generation assay

To determine whether cross-linking affected plasmin generation, plasmin generation (production of pNA) was monitored in a solid clot at 405 nm. The amount of t-PA required to allow complete plasmin generation within the four hour incubation period was optimised as described in section 3.9.2.1. To a 96 well plate (Greiner), purified fibrinogen (final concentration of 0.5 mg/ml) and t-PA (final concentrations 0-172.8 ng/ml) was added in the presence and absence of purified FXIII (final concentration 0.0055 mg/ml). TBS was added to a final reaction volume of 70 µl. The activation mix (30 µl) containing 0.025 U/ml final concentration of thrombin and 5 mM final concentration of $CaCl_2$ in TBS were added to the fibrinogen mix to initiate fibrin clot formation. The absorbance was measured every 12 seconds at 340 nm at 37 °C for 1 hour in a ELx808 spectrophotometer (Bio-Tek). Following clot formation 50 µl of lysis mix containing 0.8 mmol/L of the chromogenic substrate S2251 (Chromogenix) and 20 µg/ml plasminogen in TBS were added. The plate was incubated at 37°C and

plasmin generation was monitored by the change in absorbance every 48 seconds for 4 hours at 405 nm using a ELx808 spectrophotometer (Bio-Tek). Plasmin generation and rate of plasmin generation were determined using equations 1 and 2 (section 3.9.1).

3.9.2.1. Optimisation of the clot based plasmin generation assay

The concentration of t-PA was optimised to determine the amount required to reach plateau and therefore the maximum plasmin generation. As shown in Figure 13 all the t-PA concentrations were capable of reaching plateau over the 4 hour incubation period whether this was in the presence (Figure 13, Panel A) or absence (Figure 13, Panel B) of FXIII. The rate of plasmin generation was unaffected by cross-linking (Figure 13, Panel C). A t-PA concentration of 21.6 ng/ml was chosen for future experiments as the maximum plasmin generation was reached by 2 hours, using higher concentrations the reaction was too fast.

3.9.2.2. Final conditions for the clot based plasmin generation assay

To a 96 well plate (Greiner), purified fibrinogen (final concentration of 0.5 mg/ml) was added in the presence and absence of purified FXIII (final concentration 0.0055 mg/ml) and in the presence and absence of purified C3 (final concentration 0.5 mg/ml). Either 21.6 ng/ml t-PA or 20 μg/ml plasminogen were added before clot formation. TBS was added to a final reaction volume of 70 μl. Thirty μl of activation mix containing 0.025 U/ml final concentration of thrombin and 5 mM final concentration of calcium in TBS were added to the fibrinogen mix to initiate fibrin clot formation. The absorbance was measured every 12 seconds at 340 nm at 37°C for 1 hour in a ELx808 spectrophotometer (Bio-Tek). Following clot formation 50 μl of lysis mix containing 0.8 mmol/L of the chromogenic substrate S2251 (Chromogenix) and either 21.6 ng/ml t-PA or 20 μg/ml plasminogen in TBS were added in the opposite orientations to the t-PA and plasminogen incubated within the clot. The plate was incubated at 37°C and plasmin generation was monitored by the change in absorbance every 48 seconds for 4 hours at 405 nm using a ELx808 spectrophotometer (Bio-Tek). Plasmin generation and rate of plasmin generation were determined using equations 1 and 2 (section 3.9.1).

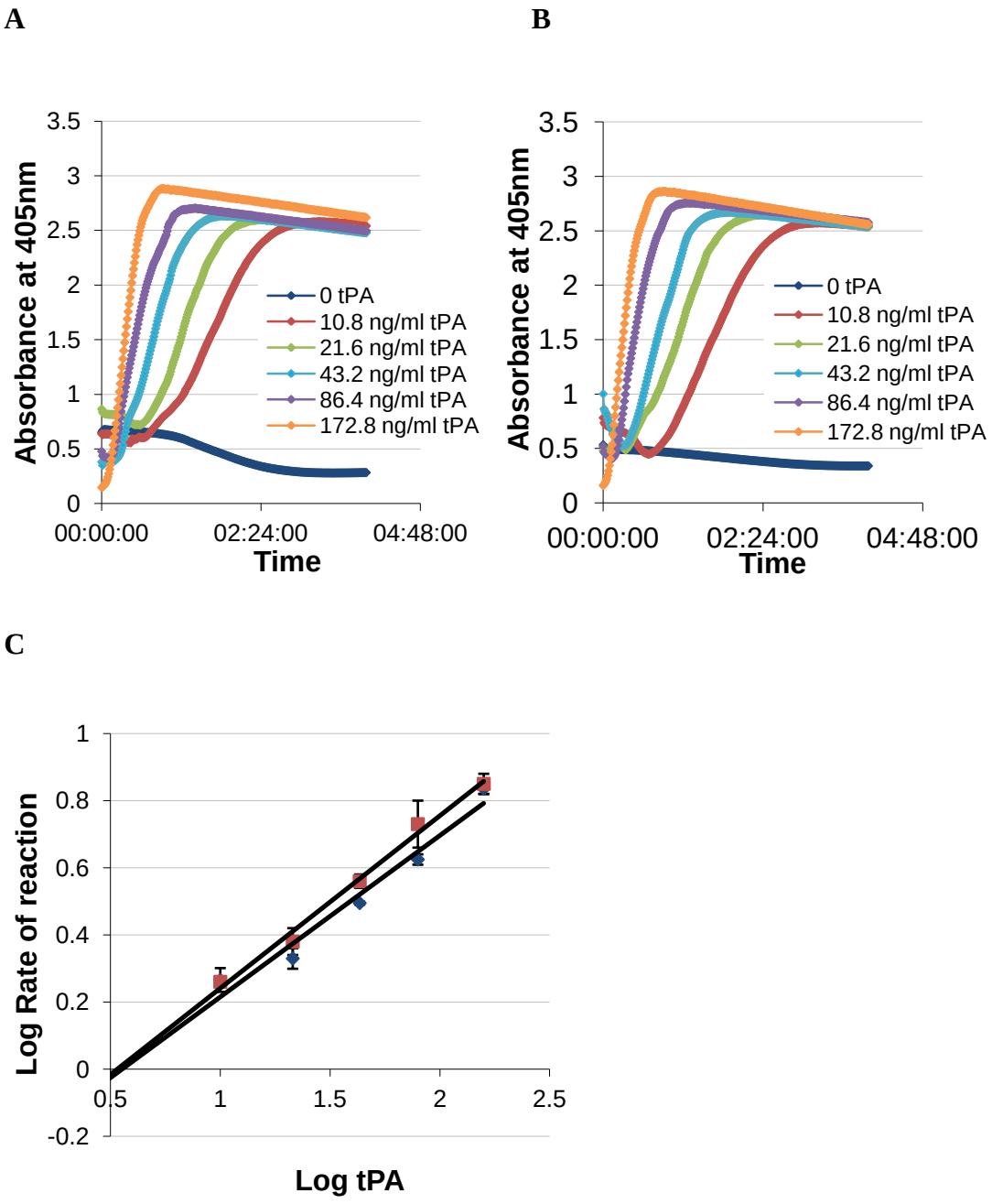


Figure 13: Optimisation of the amount of t-PA required for the clot based plasmin generation assay.

In the presence (**Panel A**) and absence (**Panel B**) of FXIII, t-PA at all concentrations reached the maximum absorbance in the assay period. (**Panel C**) Rate of plasmin generation was unaffected by the presence (■) or absence (♦) of FXIII.

3.10. Relationship between factor H, measures of fibrin structure and function and family history of T2DM in South Asians

Dr Riyaz Somani (former Clinical PhD student in DCDR) recruited all the subjects, processed all the samples and performed all of the other phenotyping. I analysed factor H levels in stored plasma from this study and carried out data analysis for factor H (Somani et al. 2012).

3.10.1. Subjects

Recruitment described in detail in Somani et al (2012). In brief, 119 healthy South Asian first degree relatives of patients with T2DM were recruited from West Yorkshire, along with 119 healthy South Asian control subjects with no personal or family history of T2DM. All subjects gave informed written consent according to protocols approved by the Leeds (West) and Bradford NHS research ethics committees (Somani et al. 2012).

3.10.2. Blood sampling and analysis of complement factors

Fifty millilitres of venous blood were taken with a 19-guage needle after a 10 hour fast and a 20 minute rest. Blood was taken into iced EDTA to assess the levels of C3a-desArg, sC5b-9 and fragment Bb, 0.109 M tri-sodium citrate (pH 8.8) on ice to assess the levels of C3, properdin, factor B and turbidimetric analyses and 0.109 M tri-sodium citrate (pH 8.8) at room temperature to assess the levels of factor H and fibrinogen. Samples were centrifuged at 2560g at 4°C for 30 minutes; the resulting plasma was stored at either at -80°C for EDTA plasma or -40°C for tri-sodium citrate plasma.

Complement proteins C3, factor B and properdin were measured using in-house ELISAs by Dr Somani (Somani et al. 2012). C3a-desArg (QUIDEL), sC5b-9 (QUIDEL) and fragment Bb (QUIDEL) were measured using commercial ELISAs by Dr Somani (Somani et al. 2012). Factor H was analysed by in house ELISA (section 3.12.2) by VR Richardson. Turbidimetric analyses were performed on

stored plasma using a method similar to that described in section 3.4.3 (with the omission of C3) by Dr Somani (Somani et al. 2012).

3.11. Evaluation of the influence of factor H on fibrin structure and function

3.11.1. Incorporation of Factor H in plasma clots

Plasma clots were formed by the addition of 50 µl of an activation mix containing 5 U/ml thrombin, 15 mM $CaCl_2$ (final concentration) in TBS to 250 µl of human plasma from 78 individuals who gave informed consent according to a protocol approved by the Leeds NHS Trust research ethics committee (Ajjan et al. 2005) and incubated at 37°C for 4 hours. Formed clots underwent centrifugation at 13,000 rpm for 5 minutes; the serum produced was removed and stored at -40°C. The concentrations of factor H in paired plasma and serum were determined by ELISA (section 3.12). Factor H incorporation into plasma clots was determined as the difference in factor H concentration between plasma and serum expressed as a percentage of plasma factor H concentration.

3.12. Factor H ELISA

An in-house factor H sandwich ELISA was established to analyse the concentration of factor H in plasma and serum samples. The factor H ELISA was optimised for antibody concentration, standard curve and blocking.

A 96 welled microtitre plate (Nunc) was coated with 100 µl of primary antibody in PBS as described in section 3.12.1.1 for hour at room temperature whilst shaking, before storing overnight at 4°C. On the assay day the microtitre plate was brought to room temperature and blocked with 3% BSA in PBS-T as described in section 3.12.1.2 followed by four washes with 200 µl of PBS-T and blotted onto absorbent paper. One hundred µl of PBS-T blank, human plasma diluted 1 in 3000 in PBS-T were added to the wells in duplicate along with a standard curve optimisation described in section 3.12.1.3 and incubated for 1 hour at room temperature whilst

shaking. The microtitre plates were washed four times with 200 µl of PBS-T and blotted onto absorbent paper. The plate was incubated with 100 µl of secondary antibody in PBS-T as described in section 3.12.1.1 for 1 hour at room temperature whilst shaking. The microtitre plates were washed four times with 200 µl of PBS-T and blotted onto absorbent paper. The plate was incubated with 100 µl of tertiary antibody in PBS-T as described in section 3.12.1.1 for 1 hour at room temperature whilst shaking. The microtitre plates were washed four times with 200 µl of PBS-T and blotted onto absorbent paper. Activated OPD substrate was prepared by dissolving 4 OPD tablets in 12 mls of distilled water and 5 µl of 30% hydrogen peroxide (Sigma). One hundred µl of activated OPD substrate were added to the wells and allowed to develop for 10 minutes whilst shaking. The reaction was stopped by the addition of 100 µl of 1.5 M sulphuric acid (Fisher). The absorbance was measured at 490 nm on the DYNEX MRX microtitre plate reader.

3.12.1. Optimisation of the Factor H ELISA

3.12.1.1. Antibody Optimisation

The factor H ELISA was initially developed using a grid experiment containing a range of capture and detection antibody dilutions using a polyclonal anti-human factor H antibody (Quidel) and mouse monoclonal anti-human factor H antibody (Quidel) (Figure 14).

In brief, the orientation of the polyclonal and monoclonal antibody against factor H was initially investigated to determine the optimal orientations for capture and detection antibodies (Figure 14, Panel A). The plate was coated with a 1:2500 to 1:20000 dilutions of either polyclonal or monoclonal factor H antibodies in PBS-T, followed by incubation with either PBS or room temperature normal pooled plasma at a 1 in 3000 dilution with PBS-T. Factor H was detected using a secondary detection antibody diluted from 1:2500 to 1:10000 (either the polyclonal or monoclonal factor H antibodies in the opposite orientation to the capture antibody) and a tertiary HRP conjugated antibody at 1:1000 (either rabbit anti-mouse HRP or rabbit anti-goat HRP) and the signal to noise ratio was monitored (equation 3). When the plate was coated with the polyclonal factor H antibody the signal to noise ranged from 7 – 10. When the plate was coated with the monoclonal factor H antibody using

the same antibody dilutions, the signal to noise ratio ranged from <1 – 2, indicating the polyclonal factor H antibody should be used as the capture antibody and the monoclonal as the detection antibody.

The antibody concentrations were further optimised to give the best signal to noise ratio for the least amount of antibody used (Figure 14, Panel B). The polyclonal capture antibody dilutions were kept the same and the monoclonal detection antibody was further diluted from 1:10000 to 1:40000. The signal to noise ranged from 5 – 20 across the plate. Higher signal to noise ratios were observed with the least amount of antibodies used; however, they gave poor overall absorbances. The optimum antibody dilutions were therefore chosen as a 1:10000 capture antibody and a 1:20000 detection antibody, as this had good overall absorbencies and a signal to noise ratio of 8.

Equation 3

$$\text{Signal to noise ratio} = \frac{\text{Absorbance of normal pool plasma}}{\text{Absorbance of the PBS blank}}$$

3.12.1.2. Blocking step optimisation

To determine whether factor H bound non-specifically to the ELISA plate in the absence of a blocking agent, a blocking step with 3% BSA in PBS-T was compared with no blocking step. As shown in Table 7 there was no difference in the background (PBS-T blank) absorbance between the blocked and non-blocked wells, whereas the absorbance of the normal pooled plasma was higher in the blocked wells compared to the non-blocked wells, suggesting factor H does not bind non-specifically to the ELISA plate. These results suggest a blocking step is not required in the final optimised protocol.

Table 7: Factor H ELISA performed in the presence and absence of a blocking step to determine non-specific binding to the ELISA plate.

The ELISA plate was blocked with either PBS-T only or 3% BSA in PBS-T. The absorbance at 490 nm was measured in wells incubated with a PBS-T blank or normal pooled plasma. There was no difference in the PBS-T blank absorbance between the blocked and non-blocked wells whereas there was reduced absorbance in the non-blocked compared with the blocked when incubated with normal pooled plasma.

	No Blocking	Blocking
PBS-T Blank	0.180	0.181
Normal pooled plasma	1.293	1.412

A	Capture 1:2500	Capture 1:5000	Capture 1:7500	Capture 1:10000	Capture 1:15000	Capture 1:20000
Detection 1:2500	0.175 (1.02)	0.172 (1.40)	0.159 (0.211)	0.160 (0.332)	0.144 (0.315)	0.117 (0.171)
	1.763 (1.076)	1.815 (1.510)	1.642 (0.223)	1.567 (0.316)	1.381 (0.339)	1.037 (0.180)
Detection 1:5000	0.144 (0.994)	0.165 (1.399)	0.151 (0.203)	0.161 (0.289)	0.150 (0.285)	0.115 (0.199)
	1.521 (1.031)	1.633 (1.458)	1.520 (0.220)	1.465 (0.321)	1.304 (0.298)	0.997 (0.191)
Detection 1:7500	0.146 (0.988)	0.162 (1.343)	0.153 (0.200)	0.166 (0.426)	0.144 (0.569)	0.132 (0.188)
	1.277 (1.052)	1.509 (1.145)	1.423 (0.231)	1.407 (0.426)	1.242 (0.592)	0.915 (0.230)
Detection 1:10000	0.109 (1.072)	0.173 (1.550)	0.152 (0.203)	0.163 (0.346)	0.149 (0.436)	0.122 (0.230)
	0.907 (1.564)	1.542 (1.689)	1.409 (0.220)	1.340 (0.333)	1.229 (0.451)	0.919 (0.221)

B	Capture 1:2500	Capture 1:5000	Capture 1:7500	Capture 1:10000	Capture 1:15000	Capture 1:20000
Detection 1:10000	0.256	0.214	0.208	0.156	0.144	0.132
	1.801	1.752	1.791	1.185	0.590	0.432
Detection 1:20000	0.22	0.192	0.198	0.151	0.095	0.111
	1.645	1.502	1.600	1.106	0.506	0.419
Detection 1:30000	0.246	0.212	0.192	0.140	0.092	0.149
	1.481	1.398	1.407	1.011	0.447	0.451
Detection 1:40000	0.219	0.203	0.192	0.144	0.107	0.095
	1.353	1.285	1.310	0.9535	0.442	0.433

Figure 14: Grid experiment for the optimisation of the factor H antibodies

A Grid experiment to determine the optimal orientation of antibodies. Data presented as absorbance at 490 nm when the polyclonal or monoclonal ( ) capture antibody were used against buffer (PBS-T only) (Purple) or normal pool plasma diluted 1 in 3000 in PBS-T (White) **B**. Grid experiment to determine the optimum concentrations of antibody for capture and detection. Data presented as absorbance at 490 nm when the polyclonal capture antibody and monoclonal detection antibody was used.

3.12.1.3. Standard Curve Optimisation

The standard curve was set up using six serial dilutions of normal pooled plasma calibrated against purified factor H (Quidel). The concentration of factor H in the normal pool plasma was 219 µg/ml therefore serial dilutions of the normal pool plasma resulted in a concentration range of 41.1 µg/ml to 1316.6 µg/ml when plasma samples are diluted 1 in 3000. The optimised normal pooled plasma standard curve was used in the final ELISA method.

3.12.2. Final conditions for the factor H ELISA

A 96 welled microtitre plate (Nunc) was coated with 100 µl of 1 in 10,000 diluted polyclonal goat anti-human factor H antibody (Quidel) in PBS for 1 hour at room temperature whilst shaking, before storing overnight at 4°C. The microtitre plates were washed four times with 200 µl of PBS-T and blotted onto absorbent paper. One hundred µl of human plasma or serum diluted 1 in 3000 in PBS-T were added to the wells in duplicate along with a standard curve diluted 1:500 to 1:16000 using pooled normal human plasma calibrated against purified factor H (Quidel) and incubated for 1 hour at room temperature whilst shaking. The microtitre plates were washed four times with 200 µl of PBS-T and blotted onto absorbent paper. The plate was incubated with 100 µl of 1 in 20,000 diluted mouse monoclonal anti-human factor H antibody (Quidel) in PBS-T for 1 hour at room temperature whilst shaking. The microtitre plates were washed four times with 200 µl of PBS-T and blotted onto absorbent paper. The plate was incubated with 100 µl of 1 in 1000 diluted rabbit anti-mouse HRP (DAKO) in PBS-T for 1 hour at room temperature whilst shaking. The microtitre plates were washed four times with 200 µl of PBS-T and blotted onto absorbent paper. The microtitre plates were developed with OPD substrate and stopped with 1.5 M sulphuric acid as described in section 3.12. The absorbance was measured at 490 nm on the DYNEX MRX microtitre plate reader and factor H concentrations in the samples were automatically read from the standard curve using Revelations software (Version 2.02).

3.12.2.1. Spiking and Recovery of Factor H

Purified factor H was spiked into diluted quality control normal pool plasma (195.9 µg/ml), to determine the recovery of factor H in the optimised ELISA. The spiking recovery results show between 97.5 and 110% recovery across a range of concentrations (Table 8).

Table 8: Spiking and recovery of factor H into quality control normal pool plasma.

Spiking of 100 to 350 µg/ml of purified factor H to normal pool plasma (195.9 µg/ml) resulted in 110 to 97.5% recovery.

Spike Level	Expected (µg/ml)	Observed (µg/ml)	Recovery %
100 µg/ml	295.9	327.3	110.6
200 µg/ml	395.9	415.7	105
300 µg/ml	495.9	511.3	103.1
350 µg/ml	545.9	532.3	97.5

3.12.2.2. Effect of freeze/thaw cycles on Factor H levels

To assess the suitablilty of this assay for measuring plasma samples undergoing freeze/thaw cycles, factor H concentration was determined in plasma samples having undergone several freeze-thaw cycles (Figure 15). Although the factor H concentration remained similar after 2 freeze-thaw cycles, the levels started to decrease after three-freeze thaw cycles; suggesting factor H denaturation prevents the recognition by the monoclonal secondary antibody. Only samples having undergone 1 or 2 freeze-thaw cycles were used for subsequent factor H analysis.

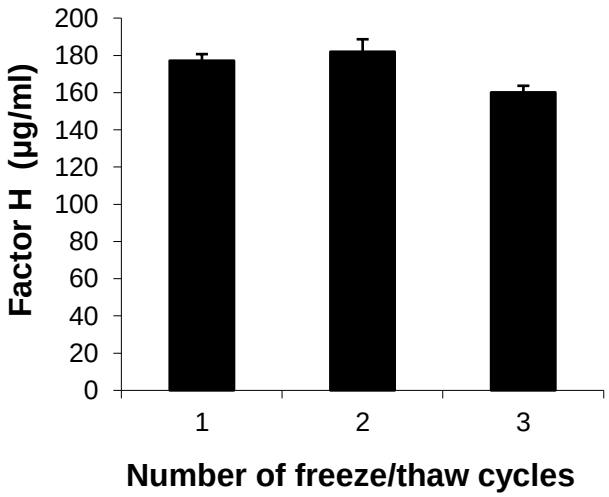


Figure 15: Factor H levels after repeat freeze/thaw cycles.

After three freeze/thaw cycles the factor H level begins to decrease. Mean (SEM) of 2 independent experiments.

3.13. Concurrent turbidity and lysis in the presence of factor H

Concurrent turbidity and lysis assay carried out as described in section 3.4.1.3, except with the addition of purified factor H (Quidel) to the following final concentrations: 0, 25, 50, 75, 100 and 500 µg/ml instead of C3.

Lysis products produced by the concurrent turbidity and lysis were stored in 4x loading buffer (Invitrogen) and 10x reducing agent (Invitrogen) for analysis by gel electrophoresis and immunoblotting as described in section 3.2.

3.14. Cross-linking of factor H to fibrin

3.14.1. Cross-linking of factor H in a purified system

Cross-linking of factor H to fibrin carried out as described in section 3.5.2 except with the addition of purified factor H (final concentration, 50 μg/ml) instead of C3. Cross-link products were analysed by gel electrophoresis and immunoblotting with a goat anti-human factor H antibody and a rabbit anti-fibrinogen antibody as previously described in section 3.2.

3.14.2. Cross-linking of factor H in plasma based system

Cross-linking of factor H to plasma clot components was carried out as described in section 3.5.3, except the PVDF membrane was immunoblotted with a goat anti-human factor H antibody instead of a rabbit anti-human C3c antibody.

3.15. Thrombin and plasmin cleavage of factor H

Cleavage of factor H by thrombin and plasmin was carried out as described in section 3.7, except with the addition of purified factor H (final concentration, 100 μg/ml) instead of C3. Factor H cleavage products were analysed by gel electrophoresis and immunoblotting with goat anti-human factor H antibody as described in section 3.2.

3.16. Identification of novel factor XIII substrates

A functional proteomics based technique was established to identify and characterise novel substrates of FXIII in plasma using a method previously established to identify substrates of tissue transglutaminase (Fleckenstein et al. 2004;Orru et al. 2003). BP was used due to its ability to identify and characterise cross-linking sites within α2-antiplasmin (Lee et al. 2000;Lee et al. 2001) and the ability to separate cross-linked (BP labelled) and non-cross-linked plasma proteins using avidin affinity chromatography.

3.16.1. Incorporation of BP into plasma proteins

To optimise the concentrations of BP, FXIII and incubation times, pooled normal plasma or fibrinogen depleted plasma (Diagnostic Reagents Ltd) at a final dilution of 1 in 100 was incubated with final concentrations of 0.022-0.22 mg/ml FXIII, 10-100 mM BP (Pierce), 5 mM $CaCl_2$ and 1 U/ml thrombin (Calbiochem) and TBS in an eppendorf tube, giving a final reaction volume of 1 ml. The sample was mixed and incubated for 2-4 hours at 37°C. The negative control was produced using the same reaction conditions described above except BP was excluded, whereas in the positive control 0.5 mg/ml final concentration of fibrinogen was incubated with 5 mM BP and preactivated FXIII as described in section 3.5.1.3.

3.16.2. Removal of excess BP

3.16.2.1. Removal of excess BP by dialysis

The 1 ml biotinylated plasma sample was dialysed in a 3.5 KDa molecular weight cut-off dialysis membrane against a total of 3-6 litres of TBS for two 1 hour incubations and overnight at 4°C.

3.16.2.2. Removal of excess BP by gel filtration

The biotinylated plasma sample was applied to either a 7 cm (height) by 0.5 cm (width) or a 7 cm (height) by 2.5 cm (width) Sephadex G25 course column equilibrated in TBS by gravity flow. Proteins and BP were eluted off the column in TBS and 1 ml fractions were collected. The absorbance (protein content) was monitored at 280 nm using the nanodrop, whilst free BP was monitored by FXIII activity assay (Section 3.16.3).

3.16.3. Factor XIII activity assay to monitor BP elutions from the gel filtration column

The FXIII activity assay used previously to assess the incorporation of BP into C3 (section 3.5.1.1) was adapted to assess BP elution from the gel filtration column (section 3.16.2.2). A 96 well microtitre plate (Nunc) was coated with 40 μg/ml

fibrinogen and blocked as previously described (section 3.5.1.1). Samples were loaded in quadruplicate with 10 µl of 0.022 mg/ml final concentration of purified FXIII, 30 µl of TBS1 and either 10 µl of each fraction eluted from the gel filtration column (section 3.16.2.2), 10 µl of 3 mM BP positive control or 10 µl TBS1 negative control. Incorporation of BP into fibrinogen was initiated upon the addition of 50 µl of activation mix containing 20 µl of TBS1, 10 µl of 5 mM final concentration of DTT, 10 µl of 10 mM final concentration of $CaCl_2$ and 10 µl of 1 U/ml final concentration of thrombin (Calbiochem). The reaction was incubated at room temperature for 3 or 10 minutes. Duplicate reactions were stopped with the addition of 200 µl of 200 mM EDTA. The plate was washed, incubated with streptavidin alkaline phosphatase, developed with p-nitrophenyl-phosphate and absorbance measured with a DYNEX MRX microtitre plate reader as previously described in section 3.5.1.1. Incorporation of BP was determined as the difference in absorbance between incubations for 3 and 10 minutes.

3.17. Purification of biotinylated plasma proteins by monomeric avidin affinity chromatography

A number of avidin technologies can be employed for the purification of biotinylated proteins as outlined in Table 9. Monomeric avidin was chosen as it has a high binding capacity with mild elution procedures allowing the re-use of the column. As the BP incorporation reaction was performed in TBS an in-house modified method was established to purify the BP labelled proteins, and was compared to the manufacturers recommended protocol.

3.17.1. The manufacturers recommended protocol for the purification of BP containing proteins by monomeric avidin affinity chromatography

A 3 cm (height) by 0.7 cm (width) monomeric avidin (Pierce) column was washed with 4 CV of PBS. The non-reversible binding sites were blocked with 3 CV of 2 mM biotin in PBS. Free biotin was removed from the reversible binding sites upon the addition of 6 CV of 0.1 M glycine pH 2.8. The column was equilibrated in PBS.

The BP labelled proteins were applied to the column under gravity and incubated on the column for 1 hour at room temperature. The column was washed with 6 CV of PBS to remove non-bound protein. The bound BP labelled proteins eluted from the column in 6 CV of 2 mM biotin in PBS. The column was regenerated with 4 CV of 0.1 M glycine pH 2.8 and finally equilibrated for storage in PBS. Two ml fractions were collected during all steps except for elution fractions which were 1 ml. The protein content of the fractions was monitored at 280 nm using a nanodrop (Labtech International).

3.17.2. The in-house modified method for the purification of BP containing proteins by monomeric avidin affinity chromatography

A 3 cm (height) by 0.7 cm (width) monomeric avidin (Pierce) column was washed with 4 CV of TBS1. The non-reversible binding sites were blocked with 3 CV of 2 mM biotin in TBS1. Free biotin was removed from the reversible binding sites upon the addition of 6 CV of 0.1 M glycine pH 2.8. The column was equilibrated in TBS1. The BP labelled proteins were applied to the column under gravity and incubated on the column for 1 hour at room temperature. The column was washed with 6 CV of TBS1 to remove non-bound protein, followed by a 4 CV 2M salt wash and a 6 CV wash in TBS1. The bound BP labelled proteins eluted from the column with 6 CV of 2 mM biotin elution buffer. The column was regenerated with 4 CV of 0.1 M glycine pH 2.8 and finally equilibrated in TBS1. Two ml fractions were collected during all steps except for elution fractions which were 1 ml. The protein content of the fractions was monitored at 280 nm using a nanodrop (Labtech International).

3.17.3. Optimisation of the monomeric avidin affinity chromatography method using biotinylated BSA

3.17.3.1. Comparison of the elution profiles using the manufacturers recommended protocol and an in-house modified method for monomeric avidin purificationof BP containing proteins

The differences in elution profiles between the manufacturer recommended protocol and in-house modified methods were examined using commercially available

biotinylated BSA, and protein concentrations were determined at 280 nm using a nanodrop (Labtech International). The majority of the biotinylated BSA eluted from the column during the elution step with a small amount eluting during the regeneration step using both methods (Figure 16, Panels A & B). In the in-house method a small amount of biotinylated BSA was seen during the initial wash steps (Figure 16, Panel B). Fractions from the wash and elution steps were concentrated by acetone precipitation and analysed by SDS-PAGE confirming that the majority of protein was present in the elution step with small amounts present in the wash fractions from both methods (Figure 16, Panels C and D).

3.17.3.2. Identification of the monomeric avidin affinity column binding capacity

As a small amount of biotinylated BSA was eluted from the column during the wash step (Figure 16), the column capacity was determined using biotinylated BSA. Biotinyated BSA (2.6 mg) was incubated on the monomeric avidin column and the elution profiles of the manufacturers and in-house methods were monitored at 280 nm using a nanodrop (Labtech International). The amount of protein in each elution fraction was combined to give a total binding capacity of the column. The binding capacity equalled 1.3 mg for both the manufacturers recommended protocol and the in-house modified method, suggesting 2 mls of monomeric avidin can accommodate 1.3 mg of biotinylated BSA, which was half the binding capacity specified by the manufacturer. As there were no obvious differences in elution profiles between the two methods and the buffers are compatible with acetone precipitation (section 3.18.2.1) the in-house modified method containing TBS would be used for further purifications of the BP labelled proteins.

Table 9: Advantages and disadvantages of avidin affinity chromatography

Information taken form (Molecular Probes Inc 2001;Thermo fisher scientific Inc 2009;Thermo fisher scientific Inc 2011)

Isolation Method	Binding affinity	Elution Methods	Re-usability of the column/beads	Non-specific binding	Binding Capacity	Extra Information
Avidin	High	Guanidinium chloride or SDS-PAGE	Unable to re-use	High	>29 µg biotin/ml of gel	Stable at pH 2-11 and over a range of temperatures
Streptavidin	High	Guanidinium chloride or SDS-PAGE	Unable to re-use	Lower than avidin	1.3 mg biotinylated BSA/ml of resin	Less soluble in water compared to avidin. Stable at pH 2-11 and is leach resistant
Neutravidin	High	Guanidinium chloride or SDS-PAGE	Unable to re-use	Low	≥ 20 µg of biotin/ml of resin	Stable at pH 2-11 and is leach resistant
Captavidin	Lower than avidin	Increasing the pH to 10	Can be used multiple times	Low	10 µg of biotin / mg protein	Biotin binding site altered by tyrosine nitration. Free biotin is added to eliminate binding to non-nitrated tyrosines

Isolation Method	Binding affinity	Elution Methods	Re-usability of the column/beads	Non-specific binding	Binding Capacity	Extra Information
Monomeric Avidin	Lower than avidin	2mM free biotin or 0.1M glycine	Maximum of 10 times, with 2.5% decrease in binding capacity per regeneration	Low	≥ 1.2 mg biotinylated BSA/ml of resin	Binding is reversible allowing non-harsh elution conditions to be used
Coated Magnetic Beads	High	SDS-PAGE boiling (harsh conditions)	Single use only	Lower than avidin	55 µg biotin / mg of beads	Can separate the biotinylated proteins within 5-10 minutes. Contains streptavidin coating

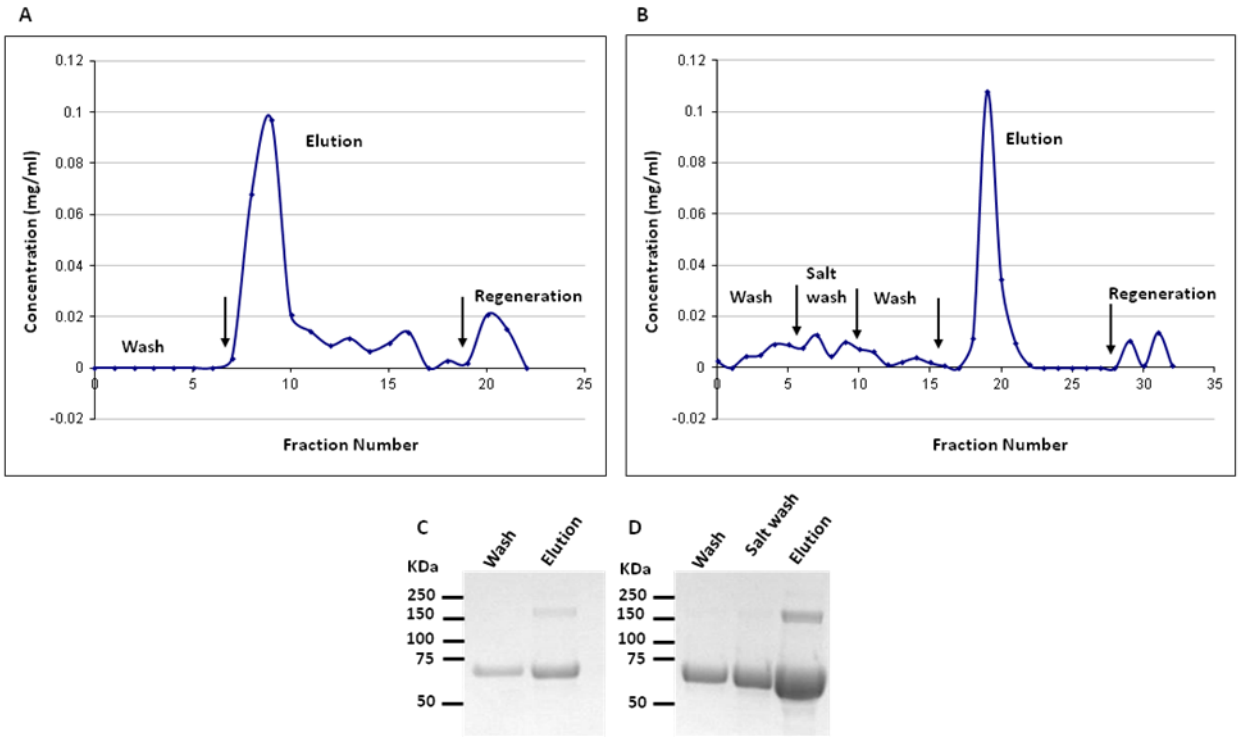


Figure 16: Purification of biotinylated BSA by monomeric avidin affinity chromatography.

Purification of biotinylated BSA using (**Panel A**) Manufacturers recommended method (**Panel B**) In-house modified method showed that the majority of the biotinylated eluted from the monomeric avidin column during the elution step. Analysis of pooled purification products from the manufactures recommended method (**Panel C**) and in-house modified method (**Panel D**) by coomassie stained gel, confirmed elutions of biotinylated BSA in the elution step but also shows biotinylated BSA in the wash steps using both methods.

3.18. Acetone Precipitation

Acetone precipitation was performed to concentrate the eluted protein sample and remove any residual salt from the sample prior to LC-MS/MS.

3.18.1. Acetone precipitation of proteins following monomeric avidin affinity chromatography

Four times the sample volume of acetone (Fisher) cooled to -20°C was added to each fraction. The sample was mixed and incubated overnight at -20°C. Following incubation the sample was spun at 13,400 rpm for 10 minutes, the supernatant was removed and the pellet was left to air-dry for 30 minutes. Pellets were stored at -40°C.

3.18.2. Optimisation of the acetone precipitation method

3.18.2.1. Buffer compatibility with the acetone precipitation method

Following avidin affinity chromatography, the fractions required concentration and desalting by acetone precipitation. To optimise acetone precipitation, the buffers used in the monomeric avidin purification methods were analysed for compatibility with the acetone precipitation method. The buffers in the absence of any protein were mixed with ice cold acetone and the formation of a precipitate was monitored. Glycine formed a precipitate, preventing acetone precipitation of samples containing this buffer. In contrast TBS and TBS containing free biotin did not form a precipitate, suggesting acetone precipitation can be performed on proteins containing these buffers.

3.18.2.2. Protein recoveries after acetone precipitation

To determine whether protein from the avidin column would be lost during acetone precipitation, assuming yields of 210 to 60 µg/ml from the avidin column, plasma samples were diluted to appropriate concentrations and were acetone precipitated (Table 10). The resulting pellet was air dried to remove remaining acetone; however,

the length of time the sample was air dried affected the ability to re-suspend the pellet and therefore the recovery of the protein. As expected the protein recoveries after acetone precipitation were highest with a higher starting concentration of protein and decreased with decreasing initial protein concentrations in all three air drying conditions. The percentage protein recovery was highest after air drying for 30 minutes. Although the protein recoveries after 20 minutes and 40 minutes were slightly lower, there was very little difference between the three time points assuming a protein recovery of 110 or 60 µg/ml from the avidin column. Air drying for thirty minutes was used for future acetone precipitation methods.

Table 10: Protein recoveries after acetone precipitation

Assumed yields of biotinylated plasma proteins from the avidin column were incubated in ice cold acetone and the resulting protein recoveries were determined after different air drying times. Protein recovery after acetone precipitation was improved with increasing initial protein concentration with the optimum air drying time of 30 minutes. Mean of three independent experiments.

Assumed protein recovery (µg/ml)	**% recovery after acetone precipitation**		
	20 minutes	**30 minutes**	**40 minutes**
210	44.8 %	64.5 %	47.9 %
110	29.3 %	29.5 %	34.8 %
60	7.9 %	10.4 %	7.2 %

3.19. Identification of Factor XIII substrates by SDS-PAGE

Plasma clots produced from normal pool plasma and FXIII deficient plasma (Affinity Biologicals Inc) were formed and perfused as described in section 3.5.3. Cross-link formation was stopped with NuPAGE reducing agent and NuPAGE loading buffer as described in section 3.2 and clots were heated to either 95°C for 15 minutes or 37°C for 1 hour. Reduced clots were run on NuPAGE 3-8% Tris-Acetate gels for 4 hours at 150 volts (see section 3.2.1). NuPAGE 3-8% Tris-Acetate gels were stained with silver staining (see section 3.19.1) or Coomassie (see section 3.2.2).

3.19.1. Silver staining of plasma clot components

The 3-8% tris-acetate gels were removed from their plastic covers and immediately fixed for one hour whilst shaking in fixing solution. The fixing solution was removed and the gel was washed three times with 30% ethanol each for 20 minutes whilst shaking. The gels were incubated with 100 ml of 0.02% sodium thiosulphate for 90 seconds. The gels were washed three times with distilled water, each for 20 seconds, and incubated for 20 minutes with 100 ml of silver nitrate solution whilst shaking. The silver nitrate solution was removed, gels were washed three times with distilled water, each for 20 seconds, and incubated with 100 ml of sodium nitrate developing solution for between 2-5 minutes or until a suitable colour had developed. The development solution was removed and the gels were washed three times with distilled water, each for 20 seconds. The development was finally stopped by the addition of 100 ml of 0.05% glycine for 10 minutes whilst shaking. The stopping solution was removed and the gels were washed three times with distilled water each for 20 seconds. An image was captured using a MultiImage™ Light Cabinet (Alpha Innotech Corporation).

3.20. Statistical Analysis

3.20.1. Analysis of clinical data

Data was tested for normality using the Shapiro-Wilk test and examination of histograms. Normality was assumed if p was >0.05. Data which was not normally

distributed was Log10 transformed. Difference in mean values between two groups was assessed by the independent sample t-test. Partial correlation was used to assess the relationship between factor H, turbidimetric parameters and other complement proteins after accounting for age and sex in the South Asian study group. A linear regression was performed to determine whether factor H was a predictor of CVD risk factors, complement activation after accounting for age and sex and changes in turbidimetric parameters, after accounting for age, sex, fibrinogen and PAI-1. Statistical significance was determined as a *P value* of <0.05. All statistical analyses were performed in SPSS version 19.

3.20.2. Analysis of in vitro functional study data

Data was tested for normality using the Shapiro-Wilk test and examination of histograms. Normality was assumed if p was >0.05. Data which was not normally distributed was Log10 transformed. Difference in mean values between two groups was assessed by the independent sample t-test after bonferroni adjustment for multiple comparisons. Analysis of the affect of t-PA and plasminogen dose responses on fibrin parameters was assessed by one-way ANOVA. Statistical significance was determined as a P value of <0.05. All statistical analyses were performed in SPSS version 19.

Chapter 4 Evaluation of the influence of C3 on fibrin structure and function

C3 has previously been identified in perfused solubilised plasma clots. In FXIII-depleted plasma clots C3 was present whereas in plasma clots containing FXIII, C3 was reduced (Appendix A). Plasma clots were also found to contain ~25% of plasma C3 and C3 binds to fibrin with high affinity (Howes et al. 2012). These findings may suggest that C3 influences fibrin formation and structure and potentially fibrinolysis via cross-linking and binding interactions. Therefore to evaluate the influence of complement C3 on fibrin structure and function a series of turbidimetric experiments were initially performed to evaluate the effect of C3 on fibrin formation, fibrin structure and fibrinolysis.

4.1. Influence of C3 on fibrin formation and fibrinolysis

To assess whether complement C3 influences fibrin formation and time to 50% lysis in a purified system, a turbidimetric method was performed. A turbidimetric assay measures the lag time, a measure of the time taken to form protofibrils and maximum absorbance, a measure of lateral aggregation and final fibrin structure (Wolberg 2007). Fibrinolysis was measured as the time to 50% lysis following the addition of the lysis mix (containing t-PA and plasminogen) overlayed over the previously preformed fibrin clot.

4.1.1. Influence of C3 on fibrin clot formation and lysis assessed by turbidimetric assay

As shown in Figure 17, there was no significant difference (P= NS) in lag time (Panel A) or maximum absorbance (Panel B) comparing cross-linked fibrin clots containing no C3 and those containing increasing concentrations of C3. As shown in panel C, increasing C3 concentrations significantly prolonged time to 50% lysis by 1.5 fold in the presence of 0.55 mg/ml and 0.75 mg/ml C3. These results suggest C3 does not influence fibrin formation and structure but does prolong lysis.

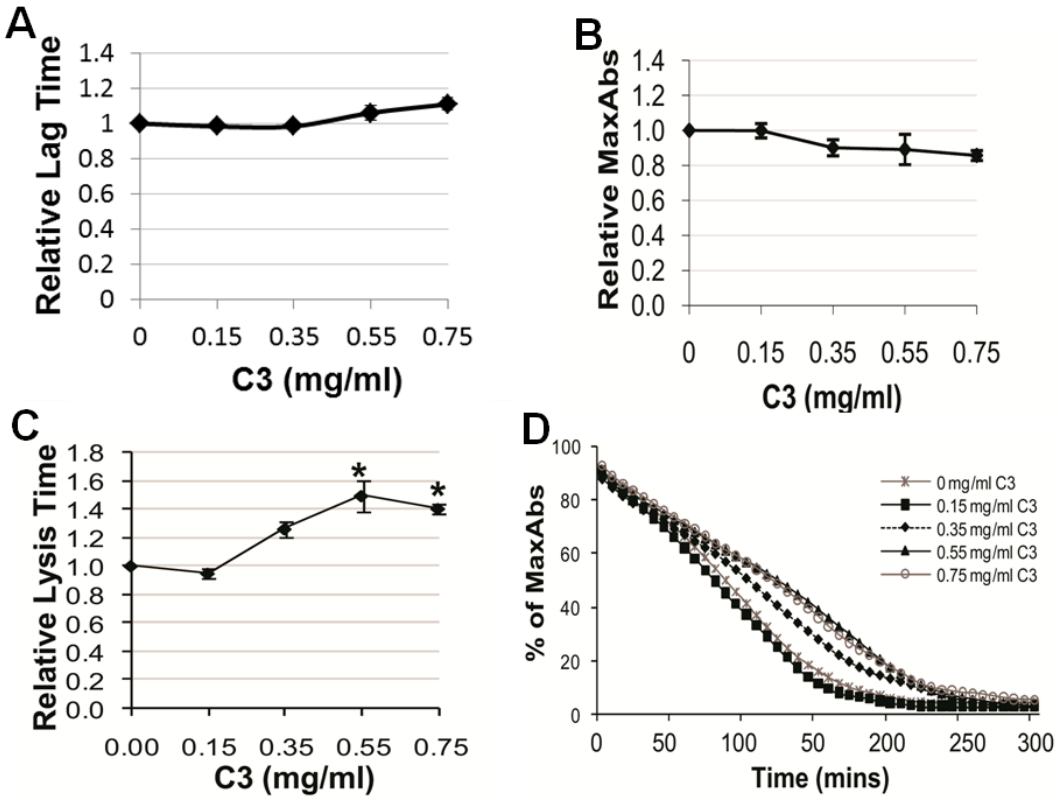


Figure 17: Influence of C3 in the turbidimetric clotting assay followed by overlay of t-PA and plasminogen to assess fibrinolysis.

Using purified proteins, turbidity and lysis overlay were measured in a turbidimetric assay at 340 nm. There was no significant difference in relative lag time (**Panel A**) and relative maximum absorbance (MaxAbs) (**Panel B**) comparing cross-linked fibrin clots containing no C3 and those containing increasing concentrations of C3. Time to 50% lysis (**Panel C, D**) was significantly increased comparing cross-linked fibrin clots containing no C3 and those containing increasing concentrations of C3. Data normalised relative to the values obtained in the presence of no C3. Mean of 3 independent experiments (SEM). * indicates a P value of <0.05 between +/- C3 after Bonferroni adjustment. (Howes et al. 2012)

Fibrinolysis was significantly prolonged in the presence of increasing concentrations of C3. To determine whether the influence of C3 on fibrinolysis was dependent on FXIII or protein-protein interactions within fibrin two turbidimetric methods were established. The first method was the lysis overlay method which was analogous to ex vivo fibrinolysis (Figure 18), whereby pre-formed fibrin clots were lysed by t-PA and plasminogen. As shown in Figure 18, Panels A and B, there was no significant difference in lag times or maximum absorbance between clots formed in the presence of FXIII compared to clots formed in the absence of FXIII. Additionally there was no significant difference in lag time and maxiumum absorbance with increasing concentrations of C3 in either the presence or absence of FXIII. As shown in Figure 18, Panel C, 50% lysis times were significantly longer in clots formed in the presence of FXIII compared to clots formed in the absence of FXIII, at C3 concentrations of 0.55 and 0.75 mg/ml (P=0.02, P=0.01 respectively). Additionally there was a significant difference in 50% lysis times with increasing concentrations of C3 in the presence (2.3-fold increase) of FXIII but not in the absence of FXIII, which was in agreement with the original turbidity overlay experiment which saw a 1.5 fold increase in fibrinolysis rate in the presence of C3. These findings suggest there were large batch variations between purified C3 even though the reaction conditions and methods were the same. Combined these results confirm that C3 does not influence fibrin formation and structure (lag time and maximum absorbance) but does prolong fibrinolysis, and the prolongation of fibrinolysis was greater in the presence of FXIII compared to in the absence of FXIII.

The second turbidimetric method was the concurrent fibrinolysis method which was analogous to in vivo fibrinolysis (Figure 19), whereby fibrin formation (thrombin cleavage of fibrinogen) occurs at the same time as fibrinolysis (Plasmin cleavage of fibrin(ogen)). As shown in Figure 19, Panel A, lag times were longer in clots formed in the presence of FXIII compared to clots formed in the absence of FXIII, at all concentrations of C3, although not significant (P=NS). This finding suggests there was a difference between the time taken by thrombin to cleave fibrinogen only and fibrinogen and FXIII. Shemirani et al (2006) showed that FPA cleavage occurs first with the generation of protofibrils, followed by a short lag phase whereby FXIII binds the fibrin protofibrils and was subsequently cleaved by

thrombin. Finally FPB was cleaved to initiate lateral aggregation and leading to an increase in turbidity (Shemirani et al. 2006). However as a difference in lag time in the presence and absence of FXIII was not observed in the turbidimetric and lysis overlay experiment (Figure 18) it suggests the interactions between thrombin with fibrinogen and FXIII were affected by the presence of fibrinolytic proteins (t-PA and plasminogen). As shown in Figure 19, Panel A there was no significant difference in lag time with increasing concentration of C3 in either the presence or absence of FXIII, confirming the previous results which suggest C3 does not influence lag time. As shown in Figure 19, Panel B, there was no significant difference in maximum absorbance between clots formed in the presence or absence of FXIII or between clots containing increasing concentrations of C3 in either the presence or absence of FXIII, thus confirming the previous results which suggest C3 does not influence the final fibrin structure. These findings may be surprising, as C3 is a relatively large plasma protein (185 KDa) and although it was unlikely to affect fibrin fibre thickness, it may have affected the pore size within the fibrin clot. In other work carried out within DCDR, it was found that in the presence of C3 in a purified system, the maximum absorbance was increased, suggesting thinner fibres (Hess et al. 2011), this was in contrast to the present study and may be accounted for by the use of double the amount of fibrinogen (1 mg/ml vs 0.5 mg/ml in the present study and a plasma concentration of 2 mg/ml) and a reduced amount of plasminogen (6 μg/ml vs 20 μg/ml in the present study and a plasma concentration of 200 μg/ml) by Hess et al (2011). An increase in fibrinogen concentration has previously been found to form a fibrin clot with a dense structure which contains thinner fibrin fibres (Carr and Carr 1995). Alternatively, increases in maximum absorbance could have occurred to the differences in plasminogen concentration. A previous study found that a reduced t-PA concentrations prevented plasmin generation before protofibril formation and lateral aggregation, thus prompting an increase in maximum absorbance (Bauer et al. 1994). A similar affect on protofibril formation and lateral aggregation may occur with a reduced plasminogen concentration leading to an increased maximum absorbance.

As shown in Figure 19, Panel C, 50% lysis times were longer in the clots formed in the presence of FXIII (2-fold increase) compared to clots formed in the absence of FXIII (1.5-fold increase) (P=NS). Although not significant these findings

reflect the FXIII-dependent effect on fibrinolysis in the presence of C3 observed in the turbidity and lysis overlay experiment (Figure 18). However as an increase in fibrinolysis time was not observed in the absence of FXIII in the turbidimetric and lysis overlay experiment (Figure 18) it suggests protein-protein interactions between C3, t-PA, plasminogen and plasmin may also influence fibrinolysis.

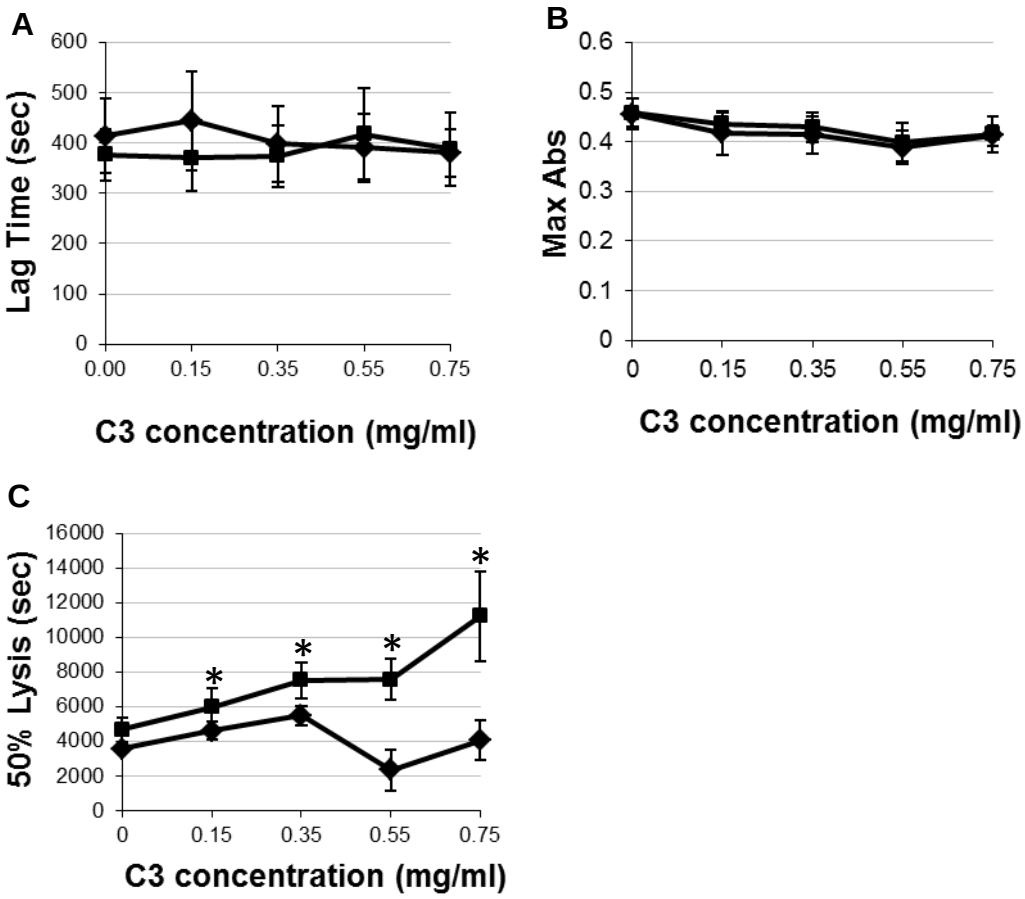


Figure 18: Evaluation of the influence of C3 in fibrin formation and lysis using the turbidity and lysis overlay assay.

Using purified proteins turbidity and lysis was measured in a turbidimetric assay at 340 nm in the presence (■) and absence (♦) of FXIII. Three independent experiments were performed and error bars are SEM. **Panel A:** Lag time was not significantly different in the presence or absence of FXIII and/or C3. **Panel B:** Maximum absorbance (MaxAbs) was not significantly different in the presence or absence of FXIII and/or C3. **Panel C:** 50% lysis time was significantly longer in the presence of FXIII compared to the absence of FXIII at 0.55 and 0.75 mg/ml only (P=<0.02) and was significantly increased with increasing C3 concentration in the presence of FXIII only. * indicates a P value of <0.05 between + and - C3 after Bonferroni adjustment in the presence of FXIII.

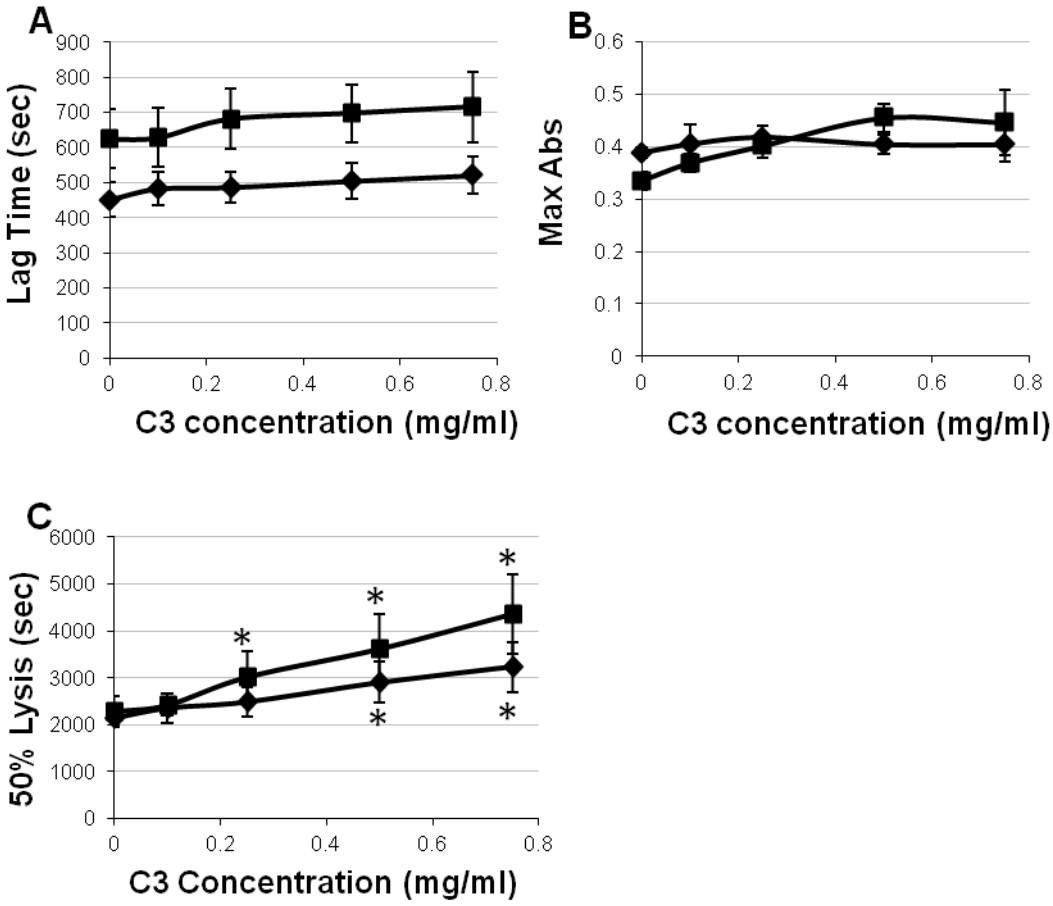


Figure 19: Evaluating the influence of C3 in fibrin formation and lysis using the concurrent turbidity and lysis assay.

Using purified proteins turbidity and lysis were measured in a concurrent turbidimetric assay at 340 nm in the presence (■) and absence (♦) of FXIII. Three independent experiments were performed and error bars are SEM. **Panel A:** Lag time was longer in the presence of FXIII at all C3 concentrations compared to the absence of FXIII, with increased C3 concentration not affecting lag time. **Panel B:** Maximum absorbance (MaxAbs) was not significantly different in the presence and absence of FXIII and/or with increasing concentrations of C3. **Panel C:** 50% lysis time was longer in the presence of FXIII with increasing C3 compared to in the absence of FXIII. 50% lysis time was significantly longer with increasing C3 concentration in both the presence and absence of FXIII. * indicates a P value of <0.05 between + and- C3 in the presence of FXIII and between + and – C3 in the absence of FXIII after Bonferroni adjustment.

4.2. Evaluation of C3 as a substrate for activated FXIII

Previous work from the DCDR demonstrated that C3 was present in FXIII-depleted plasma clots but was reduced in normal pool plasma clots containing FXIII (Howes et al. 2012), suggesting C3 was cross-linked. Furthermore the turbidimetric data shows that in the presence of C3, cross-linking affects fibrinolysis times, which suggests C3 may be cross-linked to fibrin. To determine whether C3 was a substrate for FXIIIa, plate based and gel based assays were performed using the lysine analogue, BP. To determine whether C3 was cross-linked to itself or fibrin during clot formation, gel based cross-linking assays were set up using either purified proteins or plasma.

4.2.1. Analysis of cross-linking of BP to purified C3

BP is a lysine analogue which can be cross-linked into the reactive glutamine residues within substrates of FXIIIa and tissue transglutaminase. Previous studies have used the small primary amine to determine reactive glutamine residues of FXIII in α2-antiplasmin (Lee et al. 2000;Lee et al. 2001). Therefore to determine whether C3 was a substrate for FXIIIa, a plate-based cross-linking assay was performed involving C3 coated plates incubated with BP in the presence of FXIIIa or FXIII-A2B2. As shown in Figure 20, Panel A, in the presence of FXIIIa, BP was cross-linked to immobilised C3. As a positive control BP was cross-linked to immobilised fibrinogen by FXIIIa. The amount of BP cross-linked to C3 and fibrinogen increased with increasing FXIII concentration, however, more BP incorporated into fibrinogen than C3. When C3 and fibrinogen were incubated with FXIII-A2B2, no incorporation of BP was observed. These results suggest C3 has FXIIIa susceptible glutamine residues.

BP was cross-linked into immobilised C3, however to check that immobilisation did not expose non-specific glutamine residues; BP was cross-linked to C3 in solution using an alternative assay where C3 was incubated with BP and either FXIIIa or FXIII-A2B2, then C3 containing products were captured on anti-C3 coated plates and BP incorporation was detected by streptavidin HRP. As shown in Figure 20, Panel B, in the presence of FXIIIa, there was increased cross-linking of BP into C3 over a time course of 1 hour. No time-dependent incorporation of BP

into C3 was observed in the presence of FXIII-A2B2, however the background incorporation of BP was greater in solution compared to immobilised C3, suggesting immobilisation promotes conformational changes which would prevent BP non-specific binding to C3. These results indicate C3 has FXIIIa susceptible glutamine residues which were cross-linked by FXIII in solution.

To determine which C3 chain contained FXIIIa susceptible glutamine residues, cross-linking of BP to C3 in solution was carried out and incorporation was analysed by Western blotting with streptavidin HRP. As shown in Figure 20, Panel C, BP was incorporated into both the C3 α and β chains and increased in intensity over a 4 hour period, with the greatest amounts of BP cross-linking occurring in the C3α chain. Cross-linking of another primary amine, dansylcadaverine into C3 has recently been reported (Nikolajsen et al. 2012), thus confirming that C3 was a substrate for FXIIIa. The authors further identified glutamine cross-linking sites at Gln652 in the C3 β chain and Gln686, 936, 967 and 1317 within the C3 α chain using BP. As only one reactive glutamine was present in the C3 β chain compared to four in the C3 α chain, it may account for the weaker reaction in the C3 β chain on the streptavidin-HRP Western blot in the present study.

As a negative control, C3 was incubated with BP in the absence of FXIIIa, thrombin and calcium; however, incorporation was still observed. As no bands were observed when C3 was incubated alone, it suggests that streptavidin HRP did not cross-react with C3 (Figure 20, Panel C). One possibility for cross-linking in the negative controls could be that the C3 preparation contained small amounts of FXIII contamination; however, none were observed by FXIII Western blot (data not shown). This finding might suggest that BP incorporates non-specifically into C3 (via binding) at this concentration of BP (5 mM) and confirms the high background observed when C3 was incubated with BP in the presence of FXIIIA2B2 in the plate based assay (Figure 20, Panel B). A small amount of dansylcadaverine was previously observed in the absence of FXIII-A by Nikolajsen et al (2012) after 1 hours incubation, however as the negative controls in the present study were incubated for 4 hours, it suggests greater incubation times allows greater non-specific incorporation of BP into C3.

To confirm whether BP incorporates non-specifically at 5 mM BP, a positive control in the form of fibrinogen was used. In the positive control samples, BP was incorporated into the α and γ chains of fibrinogen (Figure 20, Panel D), which were mainly present as α-polymers and γ-dimers, suggesting that fibrin-fibrin cross-links were not prevented by BP under these conditions. A small amount was also cross-linked to the fibrinogen β chain. No cross-linking of BP into fibrinogen was observed in the absence of FXIII, suggesting BP does not incorporate non-specifically into fibrinogen in the absence of cross-linking, however BP was incorporated into the fibrinogen β chain in the presence of FXIII, even though no cross-linking sites have previously been characterised in the fibrinogen β chain. Cross-linking of the fibrinogen β chain has not been previously reported and as the fibrinogen β chain remains at 55 KDa after fibrin cross-linking in previous studies (Standeven et al. 2007), it suggests that BP was most likely non-specifically incorporated into the β chain.

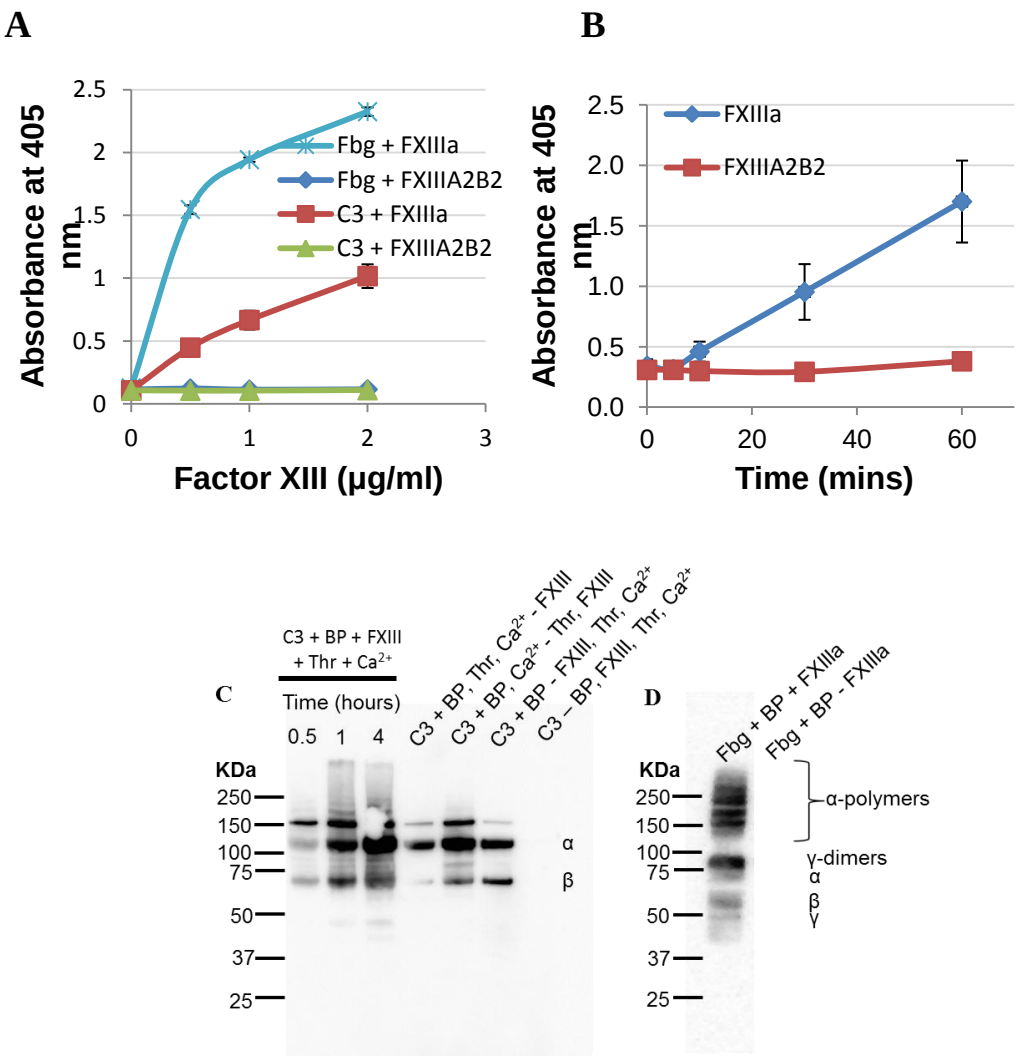


Figure 20: Cross-linking of BP into purified C3.

Panel A: Incorporation of BP into immobilised C3 and fibrinogen (Fbg) was observed in the presence of FXIIIa and not in the presence of FXIII-A2B2 in a plate based FXIII activity assay. **Panel B**: Incorporation of BP into C3 was observed in the presence of FXIIIa in solution as analysed by an ELISA based assay. Mean of three independent experiments (SEM). **Panel C**: BP incubated with C3 in the presence of FXIIIa for 0.5-4 hours resulted in increased BP incorporation into the C3 α and β chains as analysed by streptavidin HRP Western blot, with BP incorporation also occurring in the absence of FXIII, thrombin (Thr) and calcium. **Panel D**: BP incubated with fibrinogen for 4 hours resulted in BP incorporation in the presence of FXIIIa and not in the absence of FXIIIa as analysed by Western blot with streptavidin HRP. Western blots representative of two independent experiments. (Richardson et al. 2012)

Another possibility could be the non-enzymatic cross-linking of BP to the internal glutamyl thioester of cysteine within C3. Lorand (1983) previously showed that small primary amines (methylamine and dansylcadaverine) could be non-enzymatically cross-linked to the internal glutamyl thioester in α2-macroglobulin, complement C3 and C4 via a nucleophillic attack by ε-lysine amines. In the case of α2-macroglobulin both FXIII-dependent (Mosher 1976;Sottrup-Jensen et al. 1983) and non-enzymatic cross-linking (Lorand 1983) was known to occur in the same protein, however it was not known whether this can occur simultaneously. The mechanisms of action of FXIII-dependent cross-linking and non-enzymatic cross-linking are illustrated in Figure 21. As the thioester domain is present in the C3 α chain this can only account for incorporation in this chain in the absence of FXIIIa.

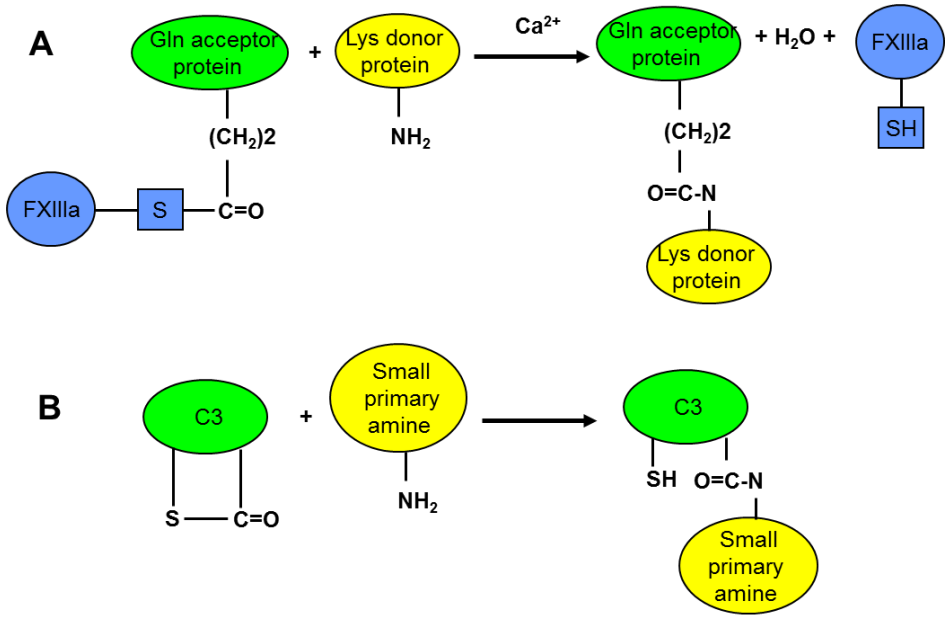


Figure 21: Formation of enzymatic and non-enzymatic cross-links.

Panel A. Cross-linking of glutamine and lysine donor proteins by FXIII. **Panel B**. Non-enzymatic cross-linking of the internal thioester of C3 to a small primary amine.

4.2.2. Evaluation of cross-linking of complement C3 to fibrin in a purified system

BP was a small substrate of FXIIIa, therefore to determine the physiological relevance of C3 cross-linking, C3 cross-linking to itself and cross-linking to fibrin was evaluated using purified proteins. To determine whether C3 forms cross-linked homodimers, C3 was incubated for 4 hours with thrombin and $CaCl_2$ in the presence and absence of FXIII and cross-linked bands were monitored by SDS-PAGE and Western blot. As shown in Figure 22, Panels A and B there were no additional high molecular weight bands in the presence of FXIIIa compared to without. In addition no lower molecular weight bands were observed in the presence of thrombin and calcium, suggesting C3 was not cleaved by thrombin under these reaction conditions. As a positive control, fibrinogen was analysed under the same reaction conditions and as shown in Figure 22, Panels A and C, in the presence of FXIIIa fibrinogen was cross-linked to form γ-dimers and numerous α-polymers, with additional high molecular weight products present in the loading wells. Fibrin and C3 incubated together in the absence of FXIIIa did not produce high molecular weight complexes (Figure 22, Panels A-C). These results indicate that C3 did not form cross-linked homodimers and therefore does not contain reactive lysine residues for the cross-linking reaction by FXIIIa.

Fibrin is the main substrate for FXIIIa and several plasma proteins are cross-linked to fibrin (See Table 1), therefore to determine whether C3 was cross-linked to fibrin during clot formation, C3 was incubated with fibrin in the presence of FXIIIa over a time course and cross-linked bands were monitored by SDS-PAGE and Western blot. As shown in Figure 23, Panel B, six C3 containing higher molecular weight bands (bands a to f) appeared after 5 minutes; two of these same bands cross-reacted with an anti-fibrinogen antibody (bands a and c) (Figure 23, Panel C) and remained over the 24 hour period (Figure 23, Panels B and C). A C3 containing band (band h- most likely C3b) was present at time point 0 but was absent at 5 minutes, suggesting this C3 containing product could be cross-linked to form one or all of the higher molecular weight bands that appeared after 5 minutes. Several of the bands (bands a, d, e and f) were not observed at time points later than 5 minutes, suggesting they became cross-linked to form higher molecular weight products. A seventh higher molecular weight band (g) was observed in the anti-C3c Western blot at the

24 hour time point (Figure 23, Panel B). This band did not cross-react with the anti-fibrinogen antibody (Figure 23, Panel C), suggesting C3 may have cross-linked to itself but only after considerable lengths of time. High molecular weight smearing (parantheses) and C3 containing products were also observed in the loading wells after 4 hours and increased in intensity after 24 hours, suggesting cross-linked C3 was incorporated into very high molecular weight complexes. Although small amounts of C3 were cross-linked to fibrin, the majority of C3 remained non-cross-linked under these conditions (Figure 23, Panel B), whereas the majority of fibrin γ and α chains were cross-linked under these conditions (Figure 23, Panel C). These findings suggest that C3 was cross-linked to fibrin within the first 5 minutes, however only small amounts of C3 were cross-linked by FXIIIa to fibrin.

It is known that C3b can non-enzymatically cross-link (intramolecular acyl-imidazole bond) hydroxyl and amine groups on bacterial surfaces via its internal thioester (Law et al. 1997), suggesting the possibility that FXIII-dependent and non-enzymatic cross-linking of proteins was likely. Therefore to confirm FXIII-dependent cross-linking of C3 to fibrin, the internal thioester was hydrolysed by dialysing purified C3 for 2 one hour incubations and a further week into water, since native C3 hydrolyses slowly (186 hours) and C3b spontaneously hydrolyses (30 psec) (Pangburn and Mullereberhard 1983). This process will prevent the ability of C3 to form intramolecular acyl-imidazole bonds and only allow FXIII-dependent cross-linking. Time dependent cross-linking of hydrolysed C3 (C3(H_2O)) was performed as before and C3(H_2O) cross-linked products were monitored by SDS-PAGE and Western blot. As shown in Figure 24, Panel B, C3(H_2O) was cross-linked within 5 minutes to form two C3(H_2O) containing high molecular weight products (bands b and c); these same bands cross-reacted with anti-fibrinogen antibodies and remained over 24 hours (Figure 24, Panel C). These two cross-reactive bands were previously observed in the presence of C3 (Figure 23). A fourth band (band g) appeared after 10 minutes and remained over 24 hours and a fifth band (band i) appeared after 1 hour and increased after 24 hours. High molecular weight C3 containing complexes were observed in the loading wells after 5 minutes and increased over 24 hours. Unlike C3 (Figure 23) band h remained constant throughout the assay period. Although small amounts of C3(H_2O) were cross-linked to fibrin, the majority of C3(H_2O) remained non-cross-linked under these conditions (Figure

24, Panel B), whereas the majority of fibrin γ and α chains were cross-linked under these conditions (Figure 24, Panel C). This observation was the same as in the presence of C3 (Figure 23). These results suggest C3(H_2O) was as readily cross-linked to fibrin as C3, however less high molecular weight bands were observed in the presence of C3(H_2O), suggesting that the C3 containing band (C3b- band h) present in the C3 cross-link experiment arose due to intramolecular acyl-imidazole bonds with fibrin. Interestingly, these C3b high molecular weight products were only observed after the initiation of cross-linking, which suggests that cleavage of the FPA and FPB from fibrinogen were required for C3b attachment, possible suggesting free hydroxyl or amine groups were exposed following fibrin formation which would allow C3b attachment. These findings also confirm that C3 can undergo FXIII-dependent cross-linking and non-enzymatic cross-linking outlined in Figure 21 simultaneously within fibrin clots.

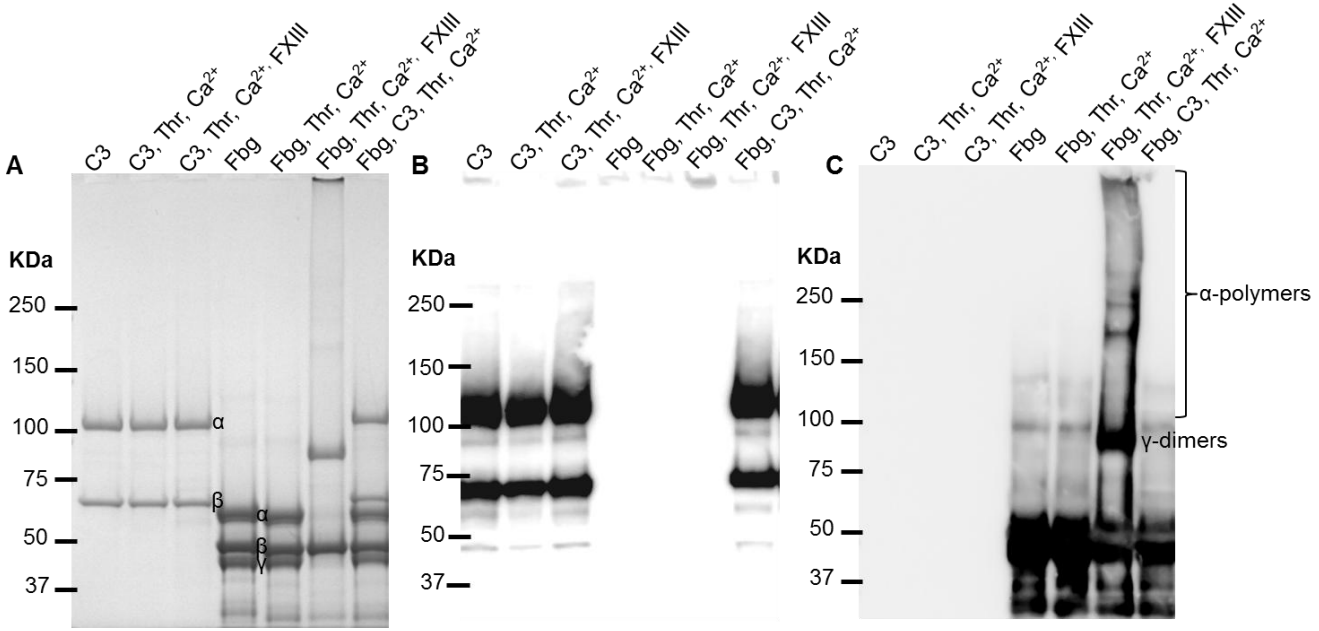


Figure 22: Analysis of C3 homodimer formation in the presence of FXIIIa.

C3 and fibrinogen (Fbg) were incubated for 4 hours with combinations of thrombin (Thr), calcium (Ca^{2+}) and FXIII and analysed by Coomassie stained SDS-PAGE (**Panel A**) and Western blot with anti-C3c (**Panel B**) or anti-fibrinogen (**Panel C**) antibodies. C3 does not form cross-linked homodimers, whereas fibrin was fully cross-linked under the same reaction conditions. Representative of two independent experiments. (Richardson et al. 2012)

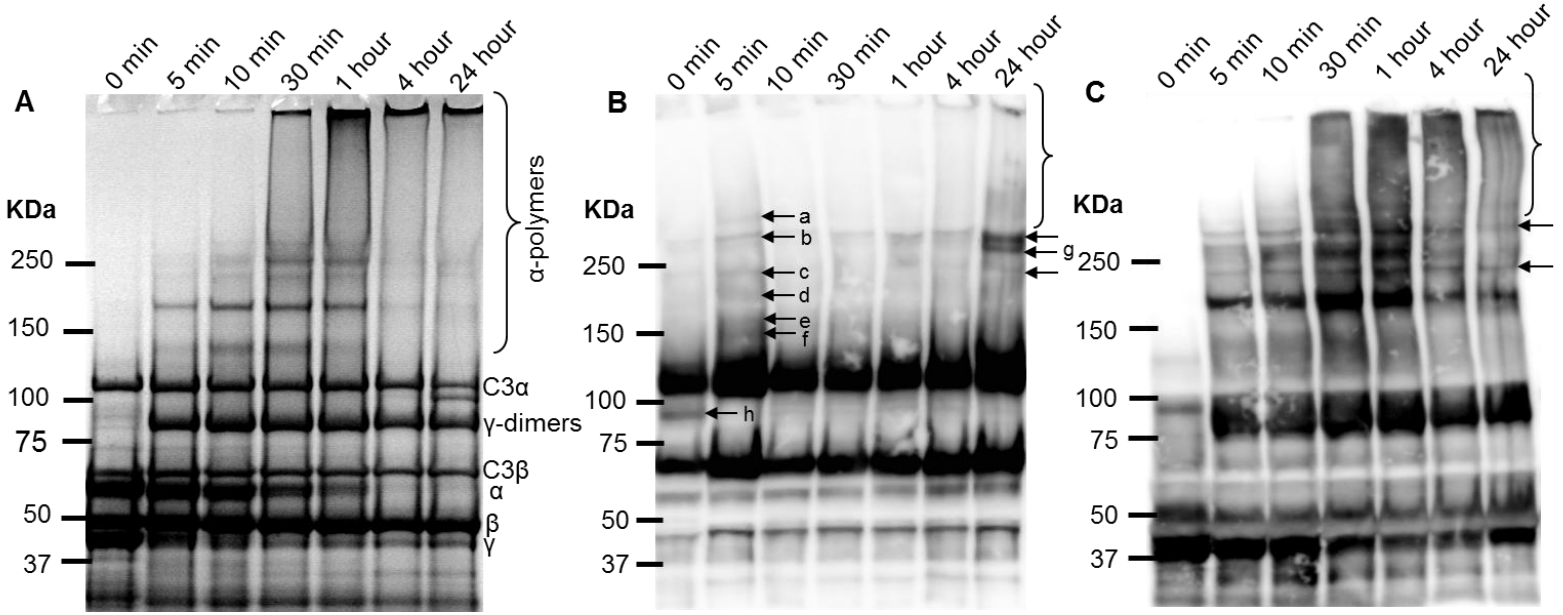


Figure 23: Cross-linking of C3 to fibrin over a time course.

C3 and fibrinogen were incubated with FXIII, thrombin and calcium over a 24 hour period and analysed for cross-link formation by Coomassie stained SDS-PAGE (**Panel A**) and Western blot with anti-C3c (**Panel B**) and anti-fibrinogen (**Panel C**) antibodies. Arrows a-g and parentheses represent C3 containing cross-linked products. Bands a-f appear after 5 minutes, whilst band g appears after 24 hours, with bands b and c cross-reacting with the anti-fibrinogen antibody. Band h was present at 0 minutes and disappears at 5 minutes suggesting it was cross-linked into higher molecular weight products. Representative of two independent experiments. (Richardson et al. 2012)

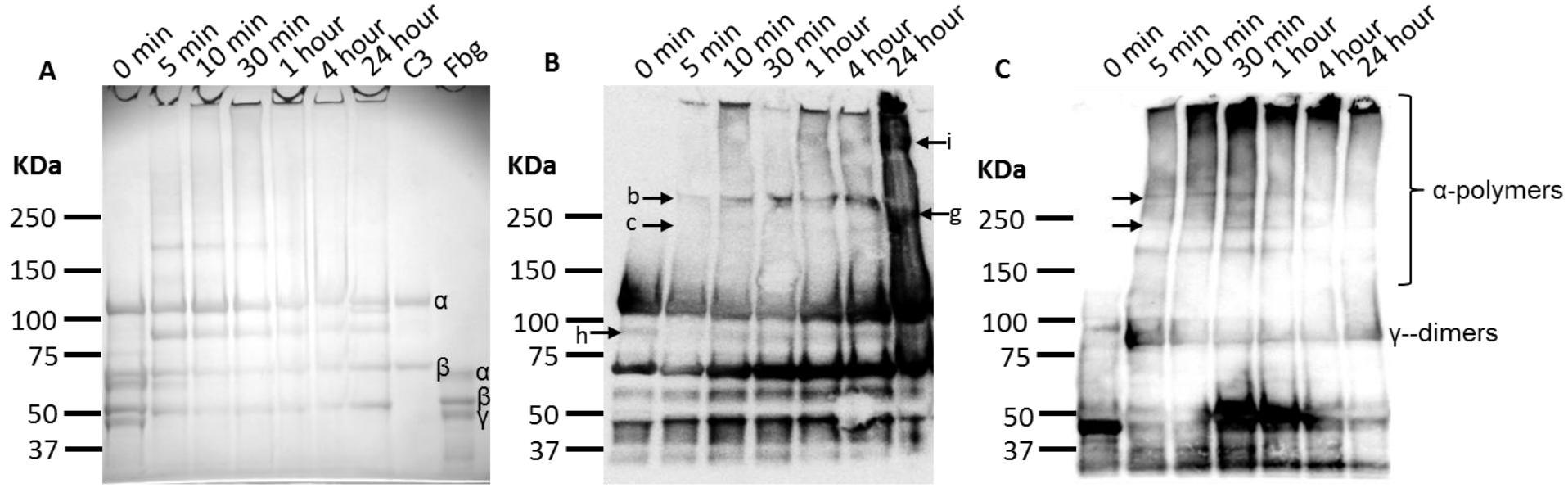


Figure 24: Cross-linking of hydrolysed C3 to fibrin over a time course

Hydrolysed C3 and fibrinogen were incubated with FXIII, thrombin and calcium over a 24 hour period and analysed for cross-link formation by Coomassie stained SDS-PAGE (**Panel A**) and Western blot with anti-C3c (**Panel B**) and anti-fibrinogen (**Panel C**) antibodies. Bands b, c, g, i represent C3 containing cross-linked products. Bands b and c appear after 5 minutes, whilst band g appears after 10 minutes, with bands b and c cross-reacting with the anti-fibrinogen antibody. Band i appears after 1 hour. Band h remains constant over the 24 hours. Representative of one experiment.

4.2.3. Influence of plasma C3 incorporation into plasma clots on fibrinolysis and cross-linking

In a purified system C3 did not influence fibrin structure but did prolong lysis and this was found to be FXIII dependent. Therefore to determine whether C3 influences fibrin formation and fibrinolysis in a plasma milieu purified C3 at concentrations between 0.5 and 2 mg/ml were spiked into plasma and plasma clot formation and lysis were analysed using the concurrent turbidity/lysis assay. As shown in Figure 25, Panel A, lag time was significantly decreased with increasing C3 concentration, at all C3 concentrations, suggesting the time required for protofibril formation was shorter. As shown in Figure 25, Panel B maximum absorbance was significantly decreased with increasing C3 concentration, at all C3 concentrations, suggesting the final fibrin structure was less dense. As shown in Figure 25, Panel C lysis time was significantly increased with increasing C3 concentration. At the highest C3 concentration lysis time was 2.7 fold longer than in the absence of added C3. This was significantly lower than the 9-fold increase in lysis time in the presence of the known plasmin inhibitor α2-AP (Fraser et al. 2011). Since C3 prolonged fibrinolysis in the presence of known fibrinolysis inhibitors which are cross-linked, such as α2-AP, it suggests C3 may play a key role in prolonging fibrinolysis in plasma clots. In the plasma clots a decrease in maximum absorbance was also observed in the presence of C3, suggesting the fibrin structure has a higher fiber density with thinner fibers, which would normally promote fibrinolysis in a plasma system (Carr and Alving 1995), suggesting the effect of C3 on fibrinolysis was important in plasma clots. Several previous studies in DCDR have observed an association between complement C3 and prolonging lysis time in plasma clots (Hess et al. 2011;Schroeder et al. 2010). These findings were consistent with the increase in fibrinolysis times observed in the present study. To fully appreciate the effect of C3 and FXIII on plasma fibrinolysis rates future experiments need to compare fibrinolysis rates in normal pool plasma and FXIII depleted plasma spiked with C3 to determine the influence of FXIII on lysis. Alternatively fibrinolysis rates could be compared between C3 depleted plasma and FXIII depleted plasma to determine the importance of both proteins in fibrinolysis.

In a purified system C3 was cross-linked to fibrin, however during plasma clot formation a number of other plasma proteins may compete with C3 for cross-

linking to fibrin, therefore a series of experiments were established to determine whether C3 was cross-linked in plasma clots, and whether C3 was cross-linked to fibrin during plasma clot formation. Plasma clots were formed by the addition of thrombin and $CaCl_2$ to plasma and left to clot in a humidified incubator for 4 hours. Non-incorporated proteins were removed by perfusion with either TBS to remove all non-bound proteins or a 2M NaCl wash to remove any bound proteins and C3 incorporation/cross-linking was assessed by Western blot (Figure 26, Panels A). C3 α and β chains were observed in TBS washed plasma clots, but were absent in NaCl washed plasma clots. However higher molecular weight products reactive with anti-C3 antibodies were observed in the loading wells of both TBS and NaCl washed plasma clots (Figure 26, Panel A). As a positive control, α2-AP was analysed in the same washed plasma clots. No bands were observed at the molecular weight of α2-antiplasmin; however, numerous higher molecular weight bands were observed in both the TBS and NaCl washed plasma clots characteristic of α2-AP incorporation into fibrin α-multimers (Figure 26, Panel B). Band intensity in the α2-AP Western blot was the same in the presence of both the TBS and NaCl washed plasma clots suggesting the differences observed in the C3 Western blot was not the result of loading differences between the lanes. These findings suggest C3 binds and cross-links to plasma clot components, whereas α2-antiplasmin was only cross-linked. This data was also consistent with the presence of high molecular weight complexes containing C3 in the purified cross-linking experiments. This data was also consistent with previous findings that show C3 binds to fibrinogen with high affinity, with 2 binding sites on fibrin(ogen) for C3 (Howes et al. 2012) which was replicated by (Hess et al. 2011). Furthermore, in unpublished data generated in collaboration with Dr Kerrie Smith (Post-doc in DCDR) a recombinant fragment of the αC domain of fibrinogen was found to bind to immobilised C3 (Appendix D, Figure 69).

To evaluate plasma C3 cross-linking over a time course, non-perfused plasma clots were reduced with SDS buffers and run on 3-8% tris acetate gels before Western blotting with anti-C3 and anti-fibrinogen antibodies (Figure 26, Panels C and D). Four C3 containing higher molecular weight products were observed after 5 minutes (Figure 26, Panel C, arrows) and remained present over the four hour period (bands b-e). Bands b to d present in the C3 Western blot cross-reacted with similar molecular weight bands present in the anti-fibrinogen Western blot (Figure 26, Panel

D, arrows). C3 reactive high molecular weight products were observed in the loading wells and in weak higher molecular weight products after 10 minutes, suggesting C3 was actively cross-linked to plasma proteins in particular fibrin. Although similar fibrinogen containing high molecular weight products were observed in the plasma cross-linking assay, cross-linking to other plasma proteins cannot be ruled out. Nikolajsen et al (2012) also found that C3 was cross-linked in plasma clots but did not specify any plasma proteins which were cross-linked to C3, whereas in the present study fibrin was identified as a substrate for FXIII dependent cross-linking to C3 (Richardson et al. 2012). Similar to the purified system the majority of C3 remained non-cross-linked after 4 hours. This observation was in agreement with Nikolajsen et al (2012) who found that only 5% of C3 was cross-linked to plasma clots proportional to α2-AP cross-linking. Several possibilities could account for the low amount of C3 cross-linking within fibrin clots. For example, C3 may be incorporated into fibrin via alternative mechanisms such as binding which was previously observed in the perfused plasma clots (Figure 26, Panel A) and has previously been observed using surface plasmon resonance (Howes et al. 2012; Hess et al. 2011). Alternatively the small amount of C3 cross-linking to fibrin may be because FXIII has a higher substrate specificity for other plasma proteins such as fibrin and α2-AP, thus FXIII promotes fibrin-fibrin and fibrin-α2-AP cross-linking instead of C3-fibrin cross-linking. This was supported by the immobilised plate based assay, in which more BP was cross-linked to fibrinogen than C3. Substrate specificity of FXIII has been found to be dependent on not only the reactive glutamine but also the surrounding amino acids which bind to the active site of FXIII (Cleary and Maurer 2006), suggesting that C3 has weaker interactions between C3 and FXIII-A compared to fibrin. These results in both purified proteins and plasma suggest C3 was actively incorporated into plasma clots through FXIII-dependent cross-linking to fibrin. The most likely site for C3 cross-linking on fibrin was within the alpha chain, as highlighted in Table 1, previous plasma proteins α2-AP, TAFI, PAI-2, von Willebrand factor, thrombospondin-1 and fibronectin have been found to cross-link this region of fibrin. A recent study found that fibrin α–α cross-linking occurs between four glutamine residues (Gln223, Gln237, Gln366, Gln563) and five lysine residues (Lys 418, Lys508, Lys539,Lys556, Lys601) in the formation of nine cross-link combinations (Wang 2011), with previous studies identifying many more available lysine residues for cross-linking including; Lys580, Lys448, Lys606,

Lys427, Lys429, Lys208, Lys224 and Lys219 (Matsuka et al. 1996;Sobel et al. 1996), which provides the opportunity for C3 to cross-link the fibrin α-chain. Previous studies have shown that α chain cross-linking was important in protecting against fibrinolysis (Siebenlist et al. 1994) and cross-linking of fibrinolysis inhibitors such as α2-AP to the α chain further prevents the ability of the clot to be broken down by plasmin (Fraser et al. 2011). If C3 cross-linking to the fibrin α-chain were to occur it might suggest a possible mechanism for prolonging fibrinolysis, due to the ability of α-chain cross-linking to protect against fibrinolysis. Further experiments need to be performed to confirm cross-linking of C3 to the fibrin α-chain and to determine the potential cross-linking sites on fibrin. Another important mechanism for C3 cross-linking to fibrin may be in localising C3 to the site of fibrin clot formation where complement activation is required to combat invading pathogens as part of the innate immune response.

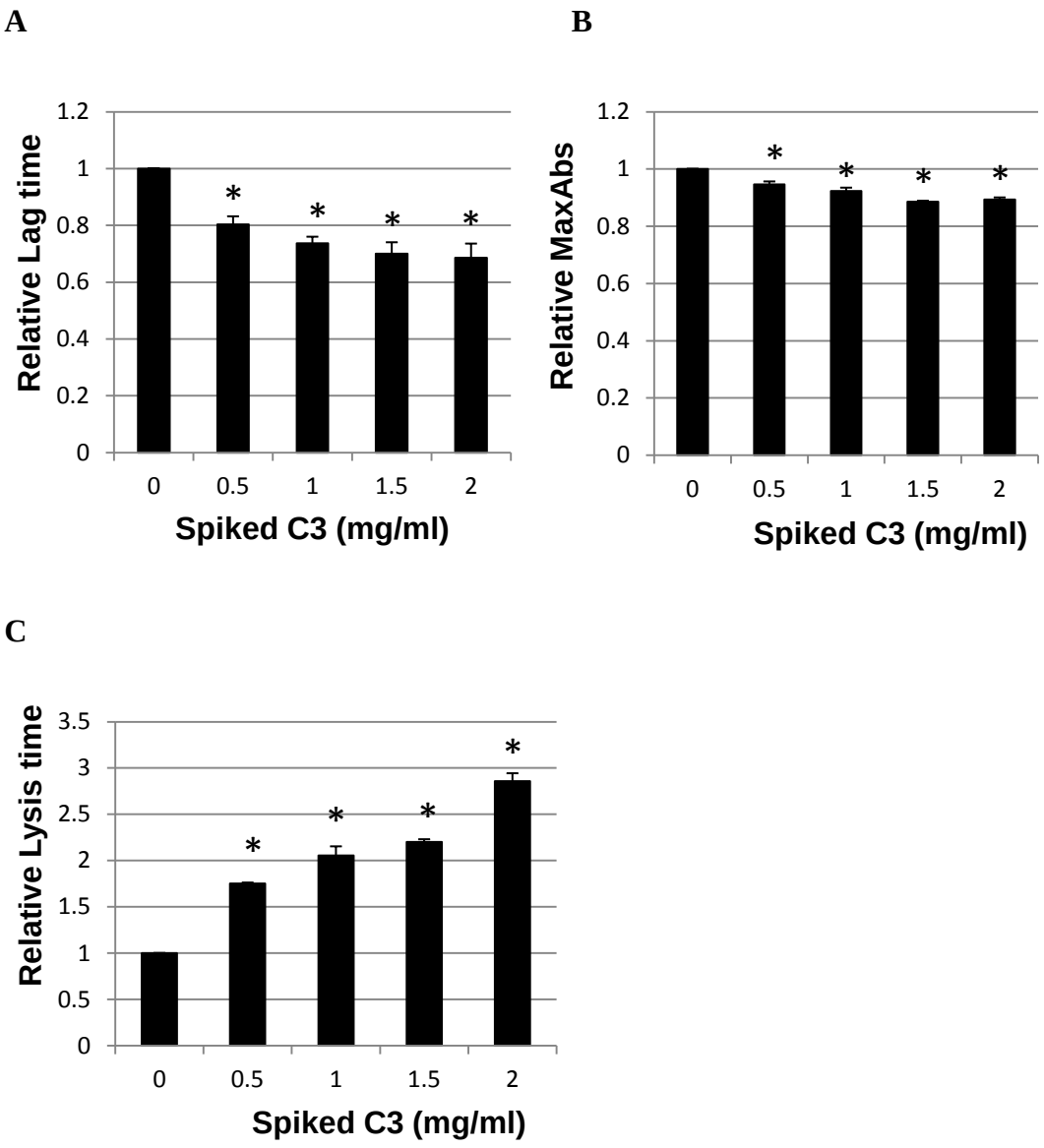


Figure 25: Evaluation of the influence of C3 in plasma clot formation and lysis.

Purified C3 at 0.5 to 2 mg/ml, was spiked into plasma (C3 concentration of 1 mg/ml) and analysed by concurrent turbidimetric assay at 340 nm for relative lag time (**Panel A**) which showed a significant decrease with increasing C3 concentration, suggesting a shorter time for protofibril formation; relative maximum absorbance (MaxAbs) (**Panel B**) which showed a significant decrease with increasing C3 concentration, suggesting the fibrin clots were less dense; relative lysis time (**Panel C**) which showed a significantly increased with increasing C3 concentration, suggesting fibrinolysis was prolonged. Mean of three independent experiments (SEM). Data normalised relative to the values obtained in the presence of no added C3. * indicates a P value of <0.05 comparing +/- C3 after Bonferroni adjustment.

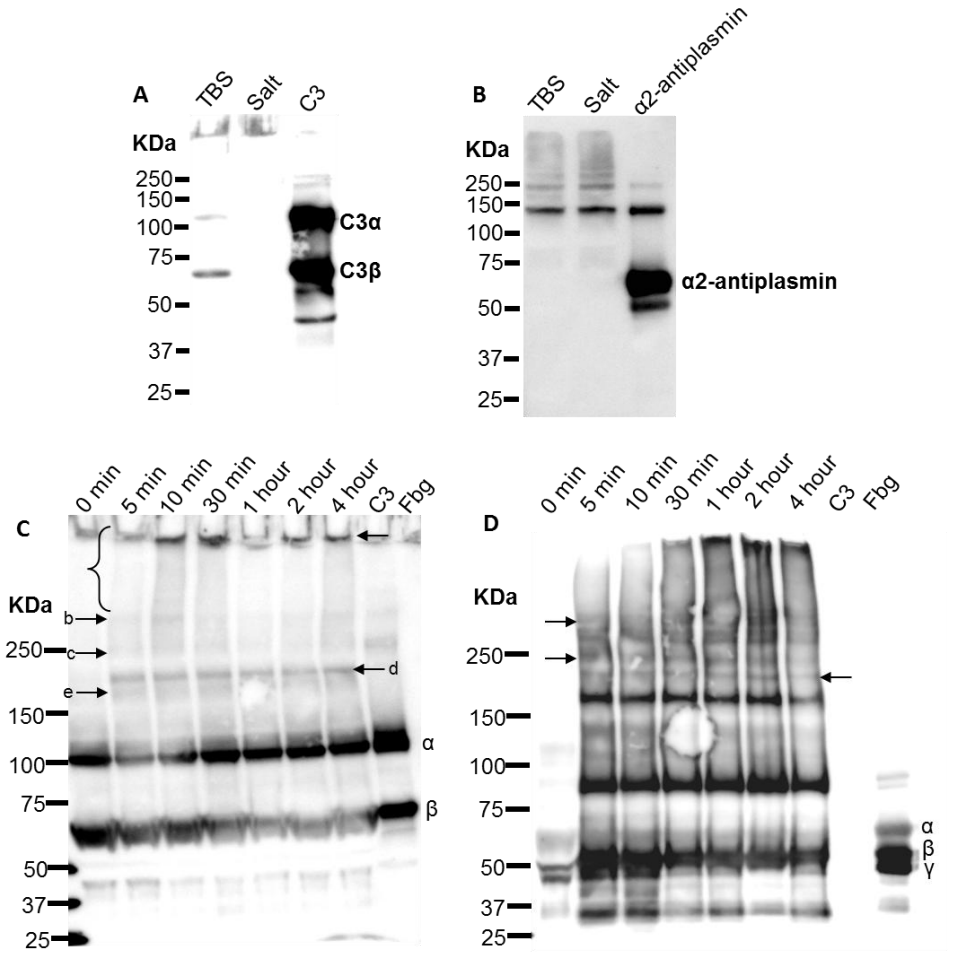


Figure 26: Cross-linking of C3 to plasma clot components.

Panels A & B Plasma clots formed in a humidified chamber for 4 hours were perfused with tris buffered saline (TBS) or 2 M NaCl, solubilised and analysed by Western blot with anti-C3c (**Panel A**) and anti-α2-APantibodies (**Panel B**). C3 was incorporated into TBS perfused clots and C3 containing high molecular weight products in the loading wells in TBS and salt perfused clots. α2-AP containing high molecular weight products were observed in both TBS and salt perfused clots. **Panels C & D** Non-perfused plasma clot formation stopped at time points between 0-4 hours by SDS buffers were analysed by Western blot with anti-C3c (**Panel C**) and anti-fibrinogen (**Panel D**) antibodies. Four C3 containing higher molecular weight products were observed at 5 minutes and C3 containing products in the loading wells were observed at 10 minutes. Bands b-d cross-reacted with similar molecular weight products present in the anti-fibrinogen Western blot. Representative of two independent experiments. (Richardson et al. 2012)

4.3. The influence of C3 on protein-protein interactions involved in fibrinolysis

In the turbidimetric and lysis overlay assay C3 was found to prolong lysis in the presence of FXIII only, and C3 was confirmed to be a substrate for FXIII and was cross-linked to fibrin. Whereas in the concurrent turbidimetric assay C3 prolonged lysis in the presence and absence of FXIII, suggesting C3 interacts with other fibrin clot components to prolong lysis. Therefore the interactions between C3 and thrombin, plasmin, t-PA and plasminogen were investigated. To determine whether C3 was a substrate for proteolysis, C3 influenced the interactions between t-PA and plasminogen on the fibrin surface and C3 influenced plasmin generation.

4.3.1. Proteolysis of C3 within cross-linked fibrin clots

To determine whether C3 undergoes proteolysis by thrombin and plasmin during fibrin formation and lysis, clots formed and lysed in the overlay experiments (Section 4.1.1) were analysed by SDS-PAGE and Western blot for the presence of possible C3 cleavage products (Figure 27, Panels A-D). Fibrin cleavage in the presence and absence of C3 resulted in the same cleavage products at all C3 concentrations (Figure 27, Panels A). This suggests that C3 does not influence the final cleavage products of fibrin, but this experiment was unable to determine whether C3 influenced cleavage of fibrin over time.

A number of additional bands were observed (labelled a to l) when comparing clots containing no C3 and those containing increasing concentrations of C3. The majority of these bands were confirmed as C3 containing bands by Western blotting with an anti-C3c antibody (Figure 27, Panel B). The C3 cleavage products also increased in intensity with increasing concentration of C3. To confirm the identity of the C3 cleavage products bands a to l were excised from a Coomassie stained SDS-PAGE gel (Figure 27, Panel C) and analysed by MALDI-MS. Table 11 shows that a number of the C3 cleavage products were consistent with known cleavage products of the C3 α chain generated by conventional complement activation/inhibition (a; C3b, f; C3c α chain fragment 2, g; C3d, h; C3c α chain fragment 1) with bands d, j and k containing un-known cleavage of the C3 β chain. Nagasawa and Stroud (1977) had previously shown that C3b and not zymogen C3

was cleaved by plasmin in the β chain, resulting in two products of 58KDa and 17KDa. These products reflect the approximate molecular weights observed by SDS-PAGE seen in the present study. Several of the bands (b and i) produced peptide patterns consistent with known C3 products (Table 11); however, their molecular weight by SDS-PAGE suggests they underwent additional cleavage resulting in minor products. These results suggest C3 was cleaved within lysed fibrin clots, with the generation of known cleavage products of the C3 α chain and several un-known cleavage products of the C3 β chain. A recent study found that C3a was produced by thrombin and plasmin cleavage of C3 and that the C3a produced was functionally active and capable of chemoattraction of mast cells and neutrophils (Amara et al. 2010), suggesting that protease cleavage of C3 results in fragments that are still capable of complement specific functions. However, Amara et al (2010) failed to identify the additional thrombin and plasmin cleavage products of C3 observed in the present study. As this experiment evaluating concentration dependent cleavage of C3 contained two serine proteases, thrombin and plasmin, capable of cleaving C3, cleavage of C3 by thrombin and plasmin was evaluated further.

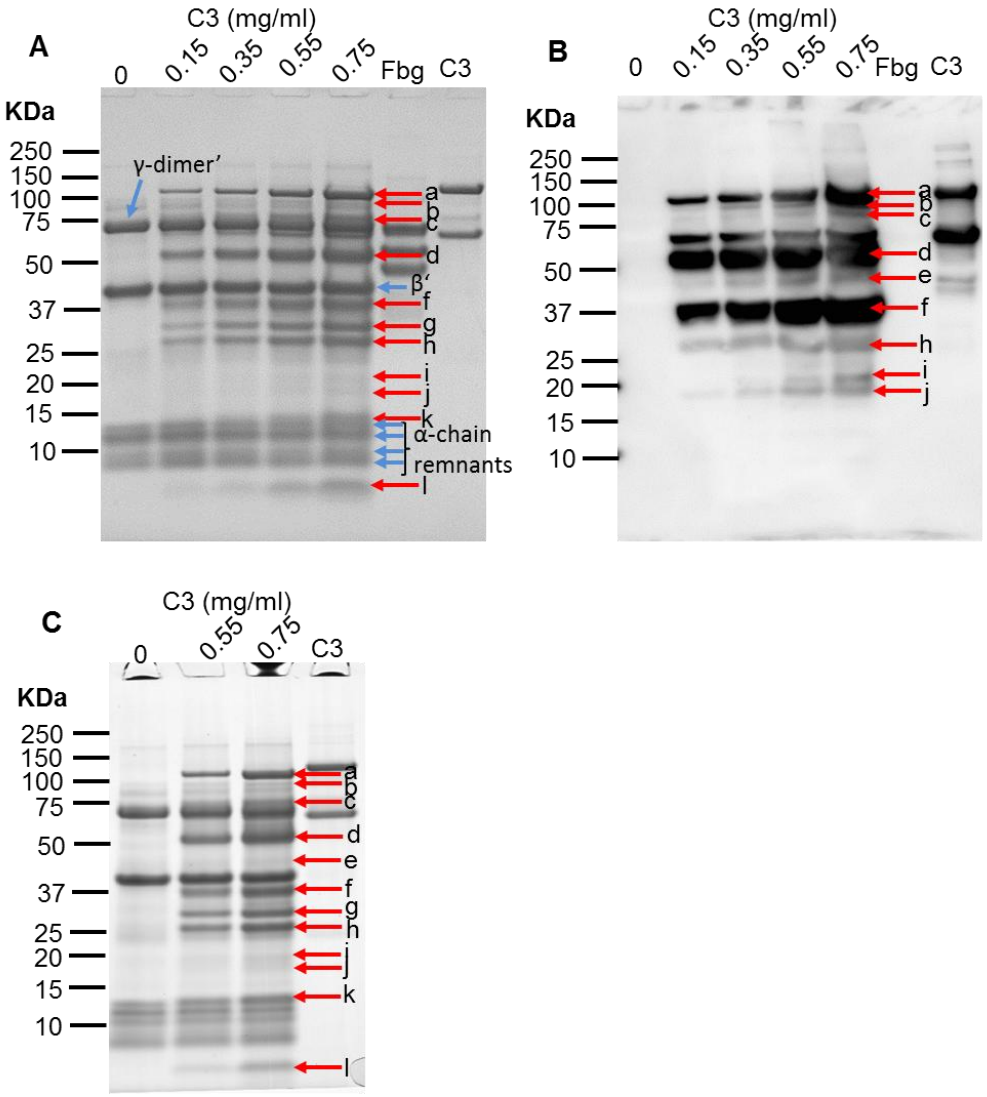


Figure 27: Concentration dependent cleavage of C3 within fibrin clots.

Clots formed and lysed in overlay experiments (Figure 17) were analysed for C3 cleavage by Coomassie stained SDS-PAGE (**Panel A**) and Western blotting with an anti-C3c antibody (**Panel B**). Fibrin cleavage products were the same at all C3 concentrations. Whereas C3 was cleaved resulting in bands a-l, which increased in intensity with increasing C3. Bands a to l were excised from a Coomassie stained gel (**Panel C**) and analysed by MALDI-MS (identity in Table 11). Representative of three independent experiments.

Table 11: Characterisation of complement C3 lysis products analysed by MALDI-TOF-MS peptide mass fingerprinting

Band	Protein Identified	Accession number	pI	MW (KDa)	MOWSE score*	Expected value	Number of peptide matches	Sequence coverage (%)
a	Complement C3b α chain	gi 118137965	5.18	103.8	173†	1.2e-12	47	46
b	Complement C3b α chain	gi 118137965	5.18	103.8	104†	9.3e-06	37	36
c	Complement C3 α chain	gi 78101268	5.5	112	59†	0.31	32	28
d	Complement C3 β chain	gi 78101267	6.82	71.1	117†	4.7e-07	33	55
e	Complement C3	P01024	6.02	187	94	7.5e-06	47	28
f	Complement C3c α chain fragment 2	gi 78101271	4.79	39.4	75†	0.0079	26	62
g	Complement component C3d	gi 157830512	6.34	32.8	69†	0.032	16	41
h	Complement C3c α chain fragment 1	gi 78101270	5.84	21.4	94†	0.0001	14	59
i	Complement C3c α chain fragment 1	gi 78101270	5.84	21.4	62†	0.13	11	52
j	Complement C3 β chain	gi 78101267	6.82	71.1	109†	3e-06	32	58
k	Complement C3 β chain	gi 78101267	6.82	71.1	93†	0.00013	25	48
l	None identified							

*MASCOT MOWSE search score is -10 log (*p*), where *p* is the probability that the observed match is a random event. Search scores >56 indicate identity at the $p< 0.05$ level. pI and MW are theoretical values.

†Analysis using NCBI database indicated splice variants and truncated polypeptides of relevance with highly significant scores.

4.3.2. Proteolysis of complement C3 by thrombin

In the previous experiment evaluating concentration dependent cleavage of C3 the reaction contained two serine proteases, thrombin and plasmin which were possibly capable of C3 cleavage. Previous studies have also shown that C3 was cleaved by thrombin at 1 U/ml (Amara et al. 2010) and 18 U/ml (Clark et al. 2008). Therefore to determine whether thrombin at 0.025 U/ml was capable of cleaving C3 and to confirm cleavage of C3 at higher thrombin concentrations, purified C3 was incubated with thrombin at different concentrations for 4 hours. In the presence of thrombin (Figure 28) purified C3 was only cleaved when 5 U/ml thrombin or greater was present, with the generation of C3b (band a), C3a (band l) and a small amount of C3c (band h). Cleavage products of C3 by thrombin are illustrated in Figure 30. Since the turbidimetric assays were performed using 0.025 U/ml of thrombin it suggests that C3 was not cleaved by thrombin during fibrin formation or lysis. This result confirms the previous observation in the cross-linking experiment which found no lower molecular weight products after purified C3 was incubated with thrombin and calcium. In the context of in vivo fibrin clot formation, this data suggests that C3 may be cleaved by thrombin during the thrombin burst when the greatest amount of thrombin was generated at around 7.2-28.8 U/ml (Brummel-Ziedins et al. 2005).

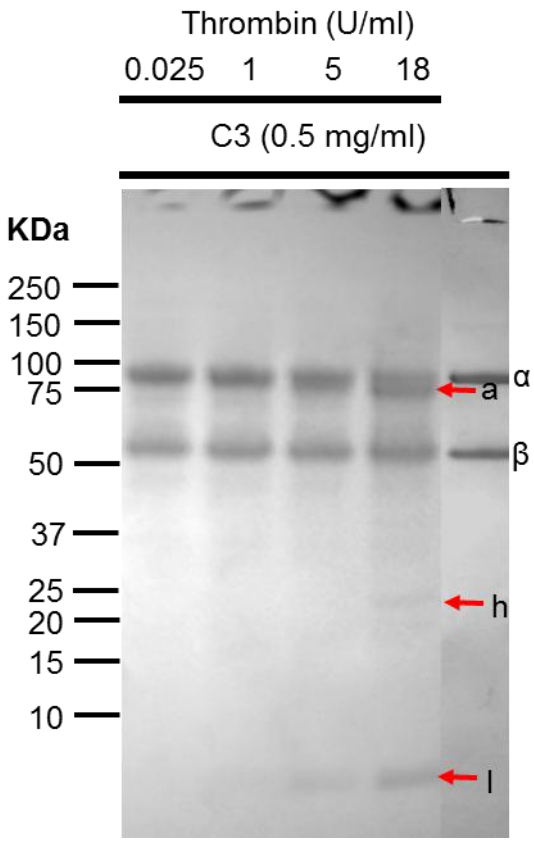


Figure 28: Thrombin cleavage of complement C3

Complement C3 was incubated with different concentrations of thrombin for 4 hours and C3 cleavage products were analysed by Coomassie stained SDS-PAGE. C3 was cleaved by thrombin at 5 U/ml or above resulting in three products (bands a, h, l), labels based on Table 11 products.

4.3.3. Proteolysis of complement C3 by plasmin

In the previous experiment evaluating concentration dependent cleavage of C3 the reaction contained two serine proteases, thrombin and plasmin. As shown in the previous experiment thrombin did not cleave C3 at the concentration used in the turbidimetric assay, which suggests plasmin might be cleaving C3. Amara et al (2010) previously observed C3 cleavage with the generation of C3a at 2 μg/ml plasmin, whereas Nagasawa and stroud (1977) observed cleavage of C3b by plasmin at a plasmin to C3 ratio of 15:1 (w/w). To determine whether C3 was a substrate for plasmin in the turbidimetric assay (complete conversion of plasminogen to plasmin

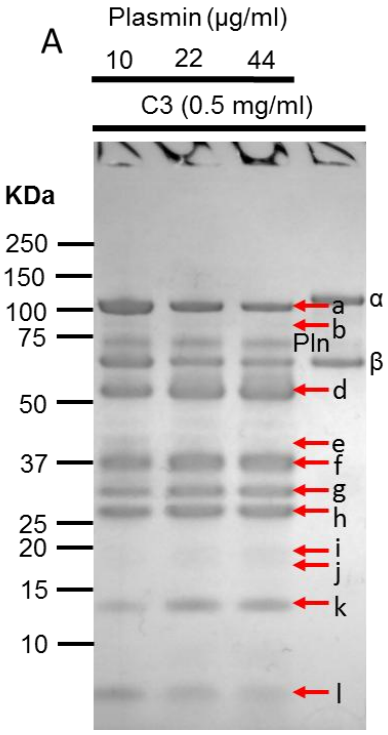
in the turbidimetric assay would result in 17 μg/ml plasmin), plasmin was incubated with purified C3 for 4 hours at different concentrations. In the presence of 10-44 μg/ml plasmin (Figure 29, Panel A) the same cleavage products were observed as those in the concentration dependent experiment (bands a-l, labels based on Table 11 products). Cleavage products of C3 by plasmin are illustrated in Figure 30. These results suggest C3 was a substrate for both thrombin and plasmin however in the concentration dependent experiments C3 was cleaved by plasmin only, as the cleavage products reflect those seen when purified C3 was cleaved by plasmin and since no cleavage of C3 by thrombin was observed at 0.025 U/ml thrombin (the concentration used in the concentration dependent experiment).

To analyse the plasmin-generated C3 β chain cleavage products further, bands d and k were excised from a Coomassie stained SDS-PAGE (Figure 29, Panel A) and were analysed by MALDI-MS/MS and LC-MS/MS to localise the plasmin cleavage site (Figure 29, Panel B). MALDI-MS/MS and LC-MS/MS confirmed that the cleavage products were indeed C3 β chain with peptide coverage of band d at the carboxyl terminus of the C3 β chain and peptide coverage of band k at the amino terminus of the C3 β chain, suggesting the site of cleavage was between residues 60 and 109, with possible plasmin cleavage sites at residues 65, 66, 73, 97, 100 and 104. Trypsin cleavage of the C3 β chain was previously observed at position Lys100 (Eggertsen et al. 1985). Backes et al (2000) found that plasmin requires an aromatic amino acid at position 2 amino terminally from the cleavage site by screening a peptide library (Backes et al. 2000). Taking into consideration this study, a possible candidate cleavage site in the C3 β chain based on this principle could be at Lys97. Both Lys100 and Lys97 lie within the region of residues 60-109, which was observed by MALDI-MS/MS and LC-MS/MS in the present study, suggesting they are likely candidate sites for plasmin cleavage of the β chain. In the present study, peptide coverage by MALDI-MS/MS and LC-MS/MS show that the cleavage site was most likely at the amino terminus, leading to the production of two peptides. However, as complete peptide coverage at the carboxyl terminus was not achieved by either MALDI-MS/MS or LC-MS/MS, additional cleavage at the carboxyl terminus cannot be ruled out.

In the present study MALDI-MS/MS and LC-MS/MS identified the region of plasmin cleavage within the β chain between 60 and 109. This region lies within

macroglobulin domain 1 (Janssen et al. 2005). Nagasawa and Stroud (1977) found that C3b and not zymogen C3 could be cleaved within the β chain, suggesting structural changes are required to allow access to the cleavage site within macroglobulin domain 1. Indeed large conformational changes occurred in the generation of C3b, with the thioester containing domain dropping down to come into contact with macroglobulin domain 1 (Janssen et al. 2006). Very few roles have been identified for the C3 β chain, however free C3 β chain has been shown to have eosinophil cytotoxic inhibitor activities (Minkoff et al. 1991), which suggests the C3 β chain supports a feedback mechanism to prevent further inflammation.

In summary, this series of results show that thrombin and plasmin cleave C3, however cleavage of C3 within fibrin clots was most likely the result of plasmin and not thrombin. These experiments were unable to determine whether C3 and fibrin compete for plasmin mediated cleavage over time and therefore act as competitive substrates to prolong fibrinolysis.



B

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  1 MGPTSGPSLL LLLLTHLPLA LGSPMYSIIT PNILRLESEE TMVLEAHDAQ
 51 GDVPVTVTVH DFPGKKLVLS SEKTVLTPAT NHMGNVTFTI PANREFKSEK
101 GRNKFVTVQA TFGTQVVEKV VLVSLQSGYL FIQTDKTIYT PGSTVLYRIF
151 TVNHKLLPVG RTVMVNIENP EGIPVKQDSL SSQNQLGVLP LSWDIPELVN
201 MGQWKIRAYY ENSPQQVFST EFEVKEYVLP SFEVIVEPTE KFYYIYNEKG
251 LEVTITARFL YGKKVEGTAF VIFGIQDGEQ RISLPESLKR IPIEDGSGEV
301 VLSRKVLLDG VQNPRAEDLV GKSLYVSATV ILHSGSDMVQ AERSGIPIVT
351 SPYQIHFTKT PKYFKPGMPF DLMVFVTNPD GSPAYRVPVA VQGEDTVQSL
401 TQGDGVAKLS INTHPSQKPL SITVRTKKQE LSEAEQATRT MQALPYSTVG
451 NSNNYLHLSV LRTELRPGET LNVNFLLRMD RAHEAKIRYY TYLIMNKGRL
501 LKAGRQVREP GQDLVVLPLS ITTDFIPSFR LVAYYTLIGA SGQREVVADS
551 VWVDVKDSCV GSLVVKSGQS EDRQPVPGQQ MTLKIEGDHG ARVVLVAVDK
601 GVFVLNKKNK LTQSKIWDVV EKADIGCTPG SGKDYAGVFS DAGLTFTSSS
651 GQQTAQRAEL QCPQPAAR
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Figure 29: Plasmin cleavage of purified complement C3

Panel A, Complement C3 was incubated with plasmin for 4 hours and C3 cleavage products were analysed by SDS-PAGE. C3 was cleaved by plasmin (at all plasmin concentrations) resulted in numerous products (bands a-l) labels based on Table 11 products. Bands d and k were excised (as they were unknown cleavage products of the C3 β chain), digested with Asp-N and trypsin and analysed by MALDI-TOF-MS/MS and LC-MS/MS. **Panel B**, Sequence coverage of band d (red) and band k (green) show that the possible cleavage site in the C3 β chain lies between residues 60-109 with possible lysine cleavage sites highlighted (yellow).

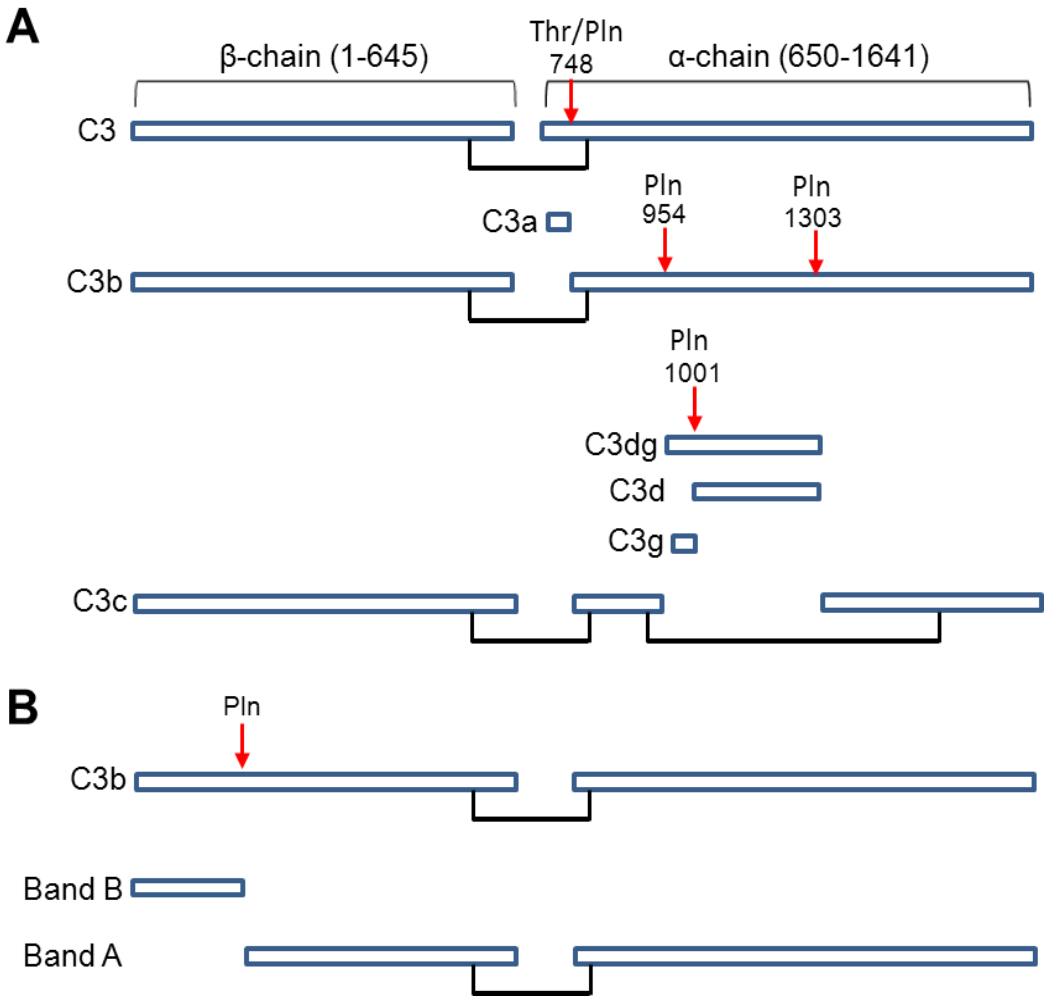

Figure 30: Diagrammatic representation of C3 cleavage by plasmin and thrombin

Panel A. Cleavage of the C3 alpha chain by thrombin and plasmin results in the generation of C3b, C3c, C3d and C3g. **Panel B**. Cleavage of the C3 beta chain by plasmin resulting in two products. Arrows indicate cleavage sites by thrombin (Thr) and plasmin (Pln) including the amino acid residues resulting from cleavage during complement activation.

4.3.4. Time-dependent cleavage of purified C3 and fibrin clots containing C3 by plasmin generated as a result of t-PA cleavage of plasminogen

In the previous series of experiments C3 was found to be cleaved by plasmin and plasmin cleavage of C3 occurred in cross-linked fibrin clots. In addition the final fibrin degradation products were unaffected by C3. However previous experiments were unable to determine whether C3 alters fibrin cleavage products during fibrinolysis and would therefore act as a competitive substrate for plasmin to prolong fibrinolysis. In the first instance time-dependent cleavage of purified C3 in the absence of fibrin by plasmin generated as a result of t-PA cleavage of plasminogen was monitored over 24 hour period by SDS-PAGE and Western blotting to determine the rate of C3 cleavage. As shown in Figure 31, Panels A-C, at 2 hours, C3b (band a) and C3c (bands f and h) were generated and increased in intensity by 24 hours; cleavage of the C3 β chain was also observed (band d) at 2 hours and increased in intensity over the 24 hours. Band e also appeared at 2 hours but was nearly absent at 24 hours, suggesting that this band was further cleaved, possibly to produce the minor lower molecular weight bands i and j which appeared at 24 hours (Figure 31, Panel B). These results suggest in the absence of fibrin, C3 was cleaved by plasmin by 2 hours; the cleavage products produced were the same as in the previous experiment where fibrin was present.

To determine the effect of t-PA mediated plasmin generation on C3 and fibrin cleavage over time, purified C3 was incubated with fibrinogen, thrombin and $CaCl_2$ in the absence (Figure 32) and presence (Figure 33) of FXIII for 1 hour to allow clot formation. Lysis was initiated following the addition of t-PA and plasminogen, and cleavage products of C3 and fibrin were monitored over a time course by SDS-PAGE and Western blot. In non-cross-linked fibrin clots containing C3 (Figure 32, Panel C), the C3 α chain was cleaved by 30 minutes generating C3b (band a) and C3c α chain fragment 2 (bands f) along with cleavage of the C3 β chain (band d), all of which increased in intensity over time; however, C3b and C3c α chain fragment 2 were nearly absent after 24 hours, suggesting they were additionally cleaved. C3c α chain fragment 1 (band h and band i) was generated after 1 hour; however, only band h increased in intensity over time. C3d (band g) and lower molecular weight C3 β chain cleavage products (bands j and k) were present at

24 hours only. These results suggest in the presence of non-cross-linked fibrin cleavage of C3 was quicker than in the presence of purified C3 alone. One possibility for this was that increased plasmin generation occurred due to the presence of fibrin, since fibrin has previously been found to be cofactor for plasminogen cleavage by t-PA (Fredenburgh and Nesheim 1992;Horrevoets et al. 1997;Hoylaerts et al. 1982).

The banding patterns for fibrin cleavage were similar in the presence and absence of C3 (Figure 32, Panels A and B), with complete cleavage of the non-cross-linked fibrin chains (α, β, γ) by 4 hours in both the presence and absence of C3 to form α', α-chain remnants, β' and γ'. These results suggest fibrin cleavage was un-affected by the presence of C3 and confirms the previous observation in the turbidimetric and lysis overlay experiment which found no significant difference in fibrinolysis in non-cross-linked fibrin clots in the presence of C3.

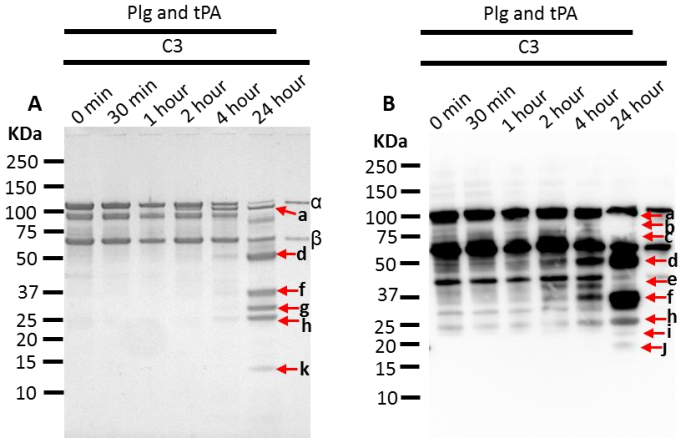

Figure 31: Time-dependent cleavage of purified C3 in the absence of fibrin by plasmin generated as a result of t-PA cleavage of plasminogen.

Purified C3 was incubated with t-PA and plasminogen (to generate plasmin) over a time course and cleavage products were monitored by Coomassie stained SDS-PAGE (**Panel A**) and Western blotting with anti-C3c antibody (**Panel B**). C3 was cleaved (bands a-k) by plasmin within 2 hours and C3 cleavage products increase over the 24 hour period. Labels a-k based on Table 11 products. Representative of two independent experiments.

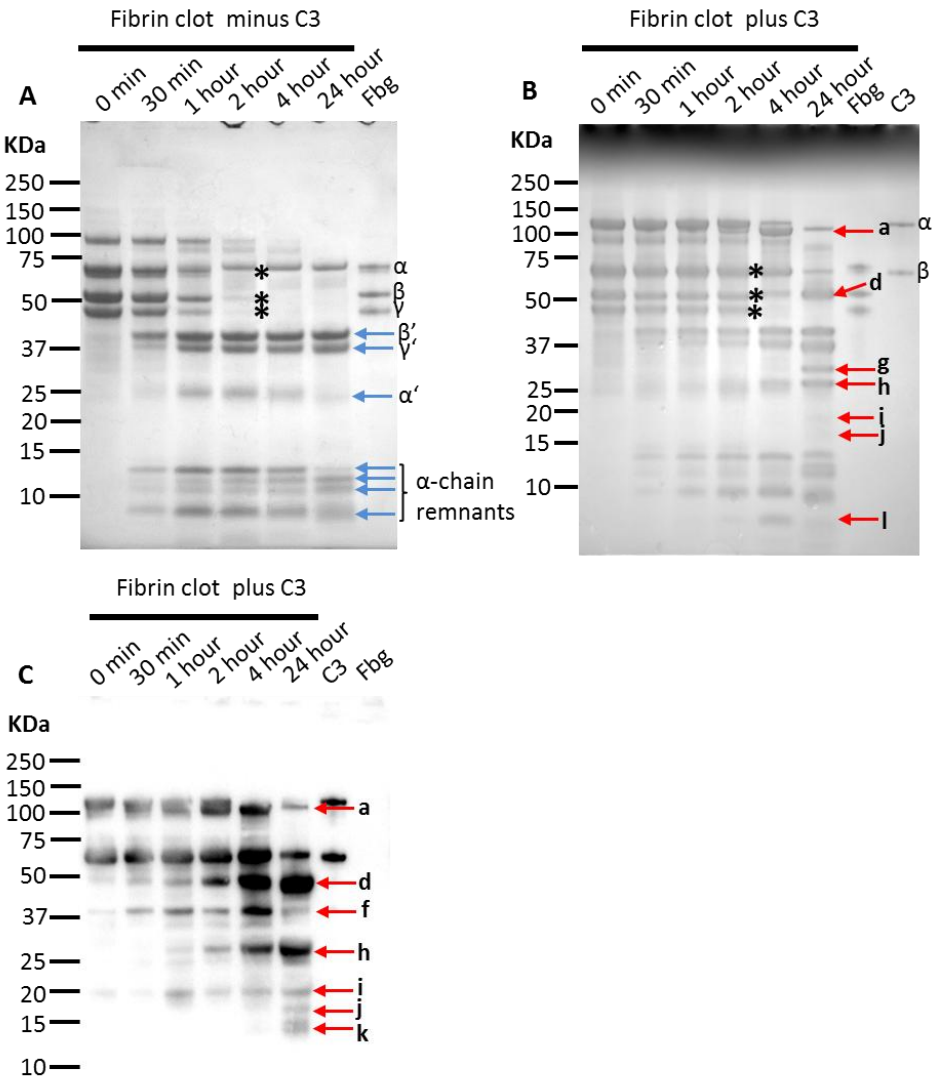

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Figure 32: Time dependent cleavage of non-cross-linked fibrin clots containing C3 by plasmin generated as a result of t-PA cleavage of plasminogen.

Non-cross-linked fibrin clots formed in the absence (**Panel A**) and presence (**Panel B & C**) of C3 for 1 hour were overlayed with t-PA and plasminogen over 24 hours and cleavage products were monitored by Coomassie stained SDS-PAGE (**Panels A & B**) and Western blot with anti-C3c antibody (**Panel C**). Arrows indicate C3 cleavage products (bands a-l based on Table 11 products), with C3 cleavage occurring by 30 minutes. * indicates complete cleavage of fibrinogen chains in the presence and absence of C3 by 4 hours. Representative of two independent experiments.

In cross-linked fibrin clots containing C3 (Figure 33) the C3 α chain and C3 β chain were cleaved within 30 minutes, resulting in C3b and β chain cleavage product (bands a and d). Full length C3 α and β chains were completely cleaved after 24 hours, resulting in lower molecular weight products. C3c α chain fragment 2 (band f) was generated after 30 minutes and increased in intensity over time, whereas C3c α chain fragment 1 (band h) decreased over time. C3d (band g) was present after 4 hours and increased at 24 hours. Lower molecular weight bands i and j were present after 4 hours and remained constant at 24 hours. These results suggest cleavage of C3 by plasmin in the presence of cross-linked fibrin clots was faster than in the presence of purified C3 alone but not different to cleavage of C3 within non-cross-linked fibrin clots.

As shown in Figure 33, at time point 0 minutes all the γ chains were cross-linked forming γ-dimers, however some fibrinogen α chains remained in both the presence and absence of C3. This finding was consistent with the previous cross-linking assay which found fibrinogen α chains remained after 1 hour and was consistent with a previous study which found that un-cross-linked fibrinogen α chains remained after 2 hours (Pizzo et al. 1973). Interestingly fewer higher molecular weight α-polymers were observed in the presence of C3, however it was possible that very high molecular weight fibrin products may be present in the loading wells, as the previous cross-linking experiments contained fibrin high molecular weight complexes in the loading wells after 1 hour incubation. Analysis of the production of cross-linked fibrin degradation products by t-PA mediated plasmin generation (Figure 33, Panels A and B) over the time course showed many of the same cleavage patterns in the presence and absence of C3; however, in the presence of C3 fewer higher molecular weight fibrin cross-links (above 150 KDa) were formed, therefore allowing these to be cleaved at a faster rate (within 1 hour) compared to cross-linked fibrin in the absence of C3, where high molecular weight fibrin cross-linked products remained at 2 hours. Cleavage of the γ dimers was similar between fibrin clots in the presence and absence of C3. Cleavage of the fibrin β chain to β' was faster in the presence of C3 with complete cleavage by 2 hours compared to 24 hours in the absence of C3. In the presence of C3 non-cross-linked α-chains were cleaved to form α' at a similar rate compared to in the absence of C3, however after 24 hours this product was further cleaved to form α-chain remnants in

the absence of C3, whereas the majority of this chain remained at 24 hours in the presence of C3, which may account for why there were fewer α-chain remnants (bands 4 and 5) in the presence of C3. A number of additional bands were observed in the presence of C3 which were not observed in the absence of C3 (bands 1-3) these most likely represent α-polymer fragments that contain cross-linked C3. As previously discussed cross-linking of C3 to fibrin was most likely to occur in the fibrin α-chain as many lysine residues are available for FXIII-dependent cross-linking. These results suggest C3 may be important in protecting α chain cleavage products from further plasmin cleavage. Previous studies have shown that FXIII-dependent cross-linking may prevent plasmin mediated degradation of fibrin by reduced the numbers of carboxyl terminal lysine residues required for the amplification of plasmin generation (Fleury and Anglescano 1991). Furthermore, resistance of the fibrin clot to plasmin-mediated fibrinolysis was found to be dependent on the formation of γ-dimers or highly complex α-multimers which conferred increased resistance to fibrinolysis (Siebenlist et al. 1994). These results were not expected, as it would be expected that adding another plasmin substrate in the form of C3 would decrease the effective plasmin concentration available for fibrin cleavage. It is possible that the plasmin concentration generated as a result of t-PA dependent cleavage of plasminogen was sufficient in these clots to not have a substrate competition effect between fibrin and C3. These findings were also the opposite to what was expected, based on the results of the turbidimetric overlay experiment, which observed prolonged fibrinolysis in the presence of C3 and FXIII compared to in the absence of C3.

To compare the rate of C3 chain cleavage in the presence and absence of fibrin and FXIII, C3 chain cleavage was analysed by densitometry of Western blots. As shown in Figure 34, both C3 α and β chains were cleaved over the 24 hour period; however, the C3 α-chain was cleaved to a greater extent than the C3 β-chain (Figure 34, Panels A and B) in the presence of purified C3 alone, cross-linked and non-cross-linked fibrin. In the presence of cross-linked fibrin clots the C3 α and β chains were nearly completely cleaved after 24 hours. In the presence of non-cross-linked fibrin approximately 25% and 60% of the C3 α and β chains remained intact after 24 hours (P= NS comparing cross-linked and non-cross-linked fibrin clots), similar to the cleavage of purified C3 alone with approximately 30% and 40%

respectively. These results confirm that C3 was a substrate for plasmin and further show that the C3 β chain appeared to be more resistant to cleavage over the first 2 hours in cross-linked and non-cross-linked fibrin clots.

In summary, these results show that C3 was cleaved by plasmin at a similar rate between cross-linked and non-cross-linked fibrin clots and cleavage of C3 occured at the same time as cleavage of fibrin, which suggests C3 was not a competitive substrate for plasmin under these reaction conditions, however as fewer lower molecular weight fibrin α-chain remnants were observed in the presence of C3 in cross-linked fibrin clots it suggests C3 might protect cleavage of fibrin α-chains by plasmin.

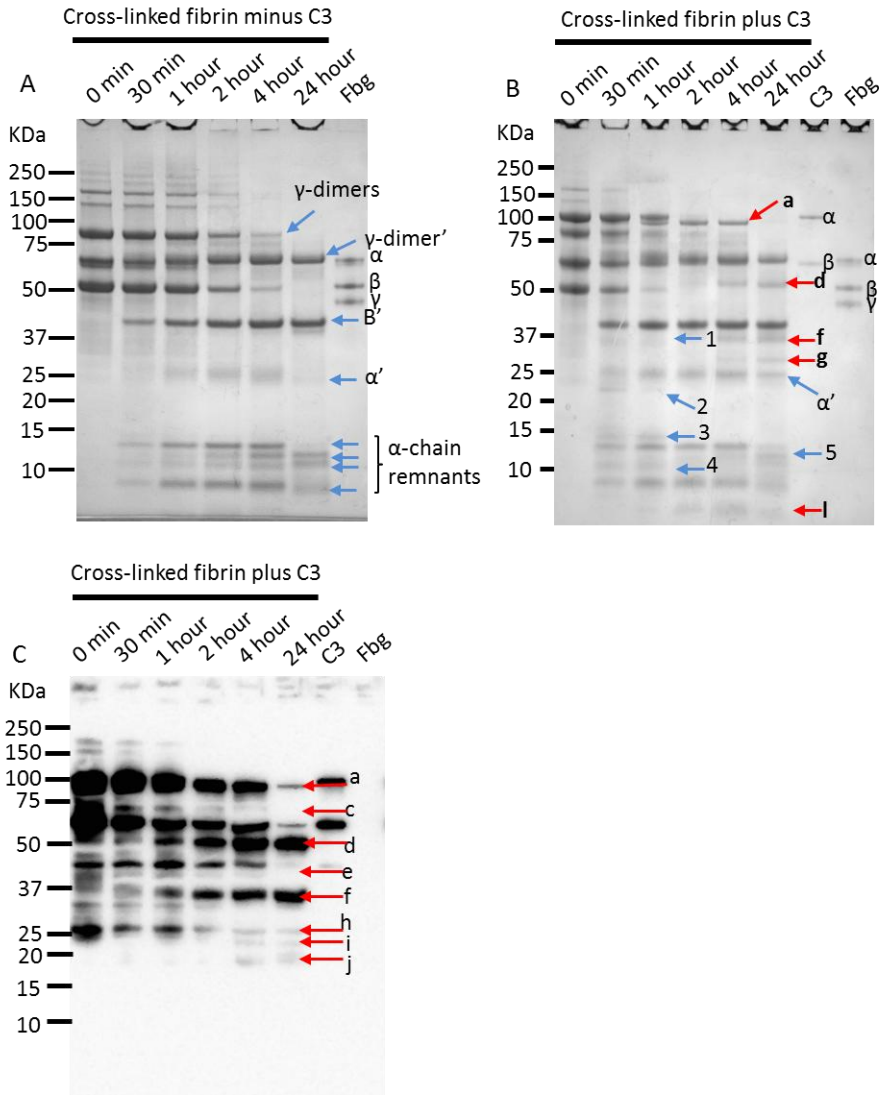

Figure 33: Time dependent cleavage of cross-linked fibrin clots containing C3 by plasmin generated as a result of t-PA cleavage of plasminogen.

Cross-linked fibrin clots formed in the absence (**Panel A**) and presence (**Panel B & C**) of C3 for 1 hour were overlayed with t-PA and plasminogen over 24 hours and cleavage products were monitored by Coomassie stained SDS-PAGE (**Panels A & B**) and Western blot with anti-C3c antibody (**Panel C**). Arrows indicate C3 cleavage products (bands a-l based on Table 11 products), with C3 cleavage occurring by 30 minutes (**Panels B & C**). High molecular weight cross-linked fibrin α polymers were cleaved faster in the presence of C3 (**Panel B**) compared to the absence of C3 (**Panel A**), with low molecular weight bands (bands 1-5) differentially produced in the presence of C3 compared to in the absence of C3. Representative of two independent experiments.

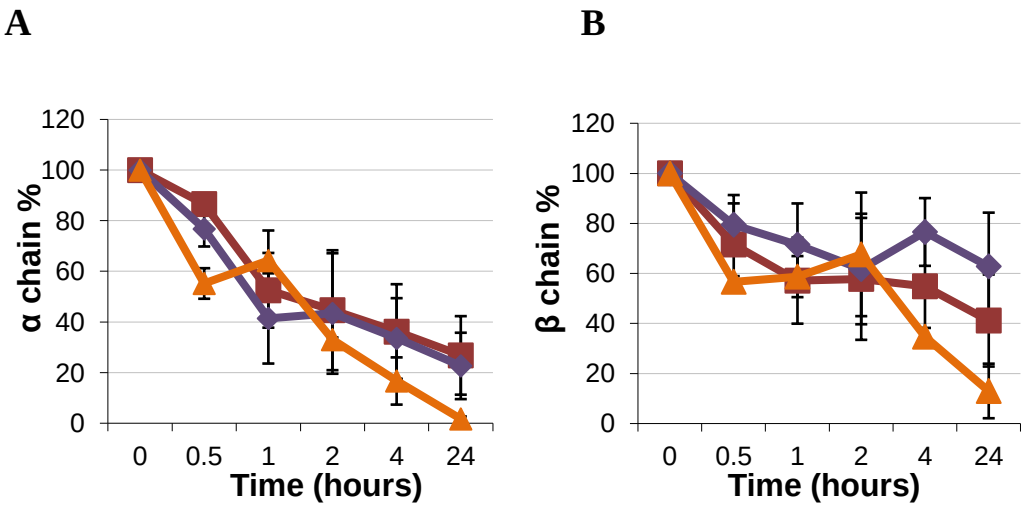

Figure 34: Densitometry analysis of C3 α and β chain cleavage over time.

Band intensity of the intact C3 α (**Panel A**) and C3 β (**Panel B**) chains measured by densitometry from Western blots, blotted with an anti-C3c antibody over a 24 hour period in purified C3 (■), non-cross-linked fibrin clot containing C3 (♦) and cross-linked fibrin clot containing C3 (▲) which have undergone t-PA mediated plasminogen cleavage. Band intensity was converted to a percentage of intact C3 α and β chains at time point 0. At 24 hours the C3 α and β chains were nearly completely cleaved in cross-linked fibrin clots, however in non-cross-linked fibrin clots and in purified C3, 25 and 30% of the C3 α chain remained and 60 and 40% of the C3 β chain remained. Mean of two independent experiments (SEM).

4.3.5. The influence of C3 on t-PA and plasminogen interactions involved in plasmin generation

In the concurrent turbidimetric assay C3 was found to significantly prolong fibrinolysis in the presence and absence of FXIII, suggesting C3 may interact with proteins involved in fibrinolysis. Furthermore C3 was found to interact with plasmin and was cleaved by plasmin within fibrin clots at the same rate as fibrin cleavage by plasmin, suggesting C3 was not a competitive substrate for plasmin. Therefore to determine whether t-PA and plasminogen interact with C3 to prolong fibrinolysis a

series of experiments were established to determine the effect of C3 on plasminogen cleavage, fibrinolysis in response to plasminogen/t-PA concentration and plasmin generation.

4.3.5.1. Analysis of the influence of C3 on plasminogen cleavage

To determine whether C3 influences plasminogen cleavage in the absence of fibrin, purified C3 and/or purified fibrinogen were incubated with t-PA and plasminogen over a time course and plasminogen cleavage products were monitored by Western blot. As shown in Figure 35, Panel A, when t-PA and plasminogen were incubated alone, plasminogen was not converted to plasmin over the 24 hour time course. As shown in Figure 35, Panel B, when t-PA and plasminogen were incubated with purified C3 a small amount of plasminogen was converted to plasmin with the majority remaining as plasminogen after 24 hours. Two additional lower molecular weight products (bands a and b) were present at 24 hours only. This finding suggests that C3 forms a surface for t-PA mediated plasmin generation. As shown in Figure 35, Panel C, when t-PA and plasminogen were incubated with purified fibrinogen, plasminogen was converted to plasmin as a decrease in band intensity was observed over the 24 hours, with the majority of plasminogen cleaved by 24 hours. Two additional lower molecular weight products (bands a and b) were present at 24 hours only. When purified C3 and fibrinogen were incubated together with t-PA and plasminogen (Figure 35, Panel D) plasminogen was converted to plasmin as a decrease in band intensity was observed over 24 hours, with the majority of plasminogen cleaved by 24 hours. Three additional lower molecular weight products (bands a-c) were present at 24 hours only. Plasminogen was cleaved by t-PA to a greater extent in the presence of fibrin(ogen) compared to in the absence of fibrin(ogen), thus confirming previous studies which found that fibrinogen and fibrin promoted plasmin generation greater than t-PA and plasminogen alone (Eastman et al. 1992). Combined these results suggest C3 provides a surface for plasminogen cleavage; however fibrinogen was more efficient for plasminogen cleavage over the 24 hours, with C3 not affecting plasminogen cleavage when incubated with fibrinogen.

To determine whether C3 influences plasminogen cleavage in the presence of fibrin, fibrin clots were formed in the presence and absence of C3 for 1 hour. Fibrin clots were overlayed with t-PA and plasminogen over a time course and plasminogen cleavage products were monitored by Western blot. As shown in Figure 35, Panel E, when t-PA and plasminogen were incubated with non-cross-linked fibrin clots in the absence of C3, plasminogen was converted to plasmin with the plasminogen band decreasing over the 24 period however a small amount of plasminogen remained at 24 hours, whilst the plasmin band increased over the 24 hours, similar to the observations in purified fibrinogen. There was no difference in plasminogen cleavage in the presence of fibrinogen or fibrin, possibly as both have been shown to be cofactors for plasminogen activation by t-PA (Fredenburgh et al. 1992;Horrevoets et al. 1997;Hoylaerts et al. 1982). In fibrin clots in the absence of C3, lower molecular weight band a appeared at 2 hours and increased in intensity over time. Similar to the observations in fibrin clots in the absence of C3, when t-PA and plasminogen were incubated with non-cross-linked fibrin clots in the presence of C3 (Figure 35, Panel F), plasminogen decreased over time with a small amount of plasminogen remaining at 24 hours, whilst the plasmin band increased in intensity over time. This result suggests there was no difference in plasminogen cleavage and plasmin generation between non-cross-linked fibrin clots in the presence and absence of C3. Whereas in non-cross-linked fibrin clot in the presence of C3 (Figure 35, Panel F), three lower molecular weight bands were observed, the first (band a) appeared at 1 hour and increased in intensity over time, whilst bands b and c were observed at 24 hours only. This suggests that plasmin autodigestion was enhanced in the presence of C3 in non-cross-linked fibrin clots.

To determine whether C3 influences plasminogen cleavage in the presence of cross-linked fibrin, cross-linked fibrin clots were formed in the presence and absence of C3 for 1 hour. Cross-linked fibrin clots were overlayed with t-PA and plasminogen over a time course and plasminogen cleavage products were monitored by Western blot. As shown in Figure 35, Panel G, when t-PA and plasminogen were incubated with cross-linked fibrin clots in the absence of C3, the plasminogen band remained constant over the first 4 hours and only decreased slightly after 24 hours with the majority remaining after 24 hours, this finding was in contrast to non-cross-linked fibrin clots in the absence of C3 where lower levels of plasminogen remained

after 24 hours (Panel E). In cross-linked fibrin clots (Panel G), the plasmin band increased over the 24 hour period, similar to the increase in the plasmin band in the presence of non-cross-linked fibrin (Panel E). In cross-linked fibrin clots (Panel G), only one lower molecular weight band (band a) was observed at 4 hours and increased in intensity after 24 hours, this was a similar observation to non-cross-linked fibrin (Panel E). As shown in Figure 35, Panel H, when t-PA and plasminogen were incubated with cross-linked fibrin in the presence of C3, the plasminogen band remained constant over the first 4 hours and only decreased after 24 hours with the majority remaining after 24 hours. This finding was similar to the observation in cross-linked fibrin in the absence of C3 (Panel G). This finding in cross-linked fibrin in the presence of C3 contrasts with the observation in non-cross-linked fibrin containing C3 (Panel F), where plasminogen cleavage steadily declined over the 24 hours. In the cross-linked fibrin clots containing C3 the plasmin band increased in intensity for the first 2 hours before decreasing slightly at 24 hours. This observation was in direct contrast to cross-linked fibrin in the absence of C3 (Panel G) and non-cross-linked fibrin in the presence of C3 (Panel F), where the plasmin band steadily increased over the 24 hours. In cross-linked fibrin clots containing C3, three low molecular weight bands were observed, bands a and b appearing at 30 minutes and both increase in intensity over the 24 hours whereas band c was present at 24 hours only. In cross-linked fibrin in the presence of C3, more low molecular weight bands appeared than in cross-linked fibrin in the absence of C3 (Panel G) and appeared sooner than the low molecular weight bands in non-cross-linked fibrin in the presence of C3 (Panel F). These findings show, less plasminogen cleavage in the presence of cross-linked fibrin clots compared to non-cross-linked fibrin clots, suggesting FXIII plays a role in plasmin generation. It has previously been shown that cross-linking hides the high affinity binding sites for t-PA, preventing t-PA binding and plasminogen cleavage (Husain et al. 1989) and therefore reducing plasmin generation. Less plasmin was also generated in cross-linked fibrin clots containing C3 compared to in the absence of C3 and the plasmin generated in cross-linked fibrin clots containing C3 underwent autodigestion to form more angiostatins which appeared sooner compared to cross-linked fibrin in the absence of C3.

As a small amount of plasmin was observed at time point 0 in the Western blots, to more clearly evaluate plasmin generation the amount of plasmin generation

over time was compared to time point 0 using densitometry. As shown in Figure 36, in the presence of fibrin the intensity of the plasmin band increased over time with a large increase at 24 hours, however in the presence of fibrin clots containing C3 the intensity of the plasmin band increased at a faster rate until 2 hours before plateauing for the remainder of the time course. In the presence of cross-linked fibrin the intensity of the plasmin band matched that of fibrin clots containing C3, except for an increase in band intensity at 24 hours. In the presence of cross-linked fibrin clots containing C3 the plasmin band was the least intense, with only a slight increase in band intensity over the first 4 hours before a decrease at 24 hours. The differences between the amount of plasmin generated in fibrin clots in the presence and absence of C3 and FXIII did not reach statistical significance at any time point.

When plasminogen was converted to plasmin in these Western blots a number of additional fragmentation bands were also observed. These fragmentation products were the same as those observed when plasminogen and t-PA were incubated with non-lysine analogues which promote plasminogen activation and autodigestion (Ohyama et al. 2004). In all experiments which led to plasmin generation, the fragmentation product at ~60 KDa was observed. This fragment most likely represents A61, an angiostatin made up of chain A (kringle domains 1-4 and part of kringle domain 5), which was produced by plasmin autodigestion (Kassam et al. 2001). The alternative fragments also likely represent minor angiostatins, angiostatin4.5 (52 KDa) and angiostatin (38 KDa) (Soff 2000). In cross-linked and non-cross-linked fibrin clots containing C3, more angiostatins were identified compared to in the absence of C3, angiostatins were also identified sooner in cross-linked fibrin clots containing C3 compared to non-cross-linked fibrin clots containing C3. This suggests that plasmin generated as a result of t-PA mediated plasminogen cleavage was broken down to more angiostatins and sooner following plasminogen cleavage, which would reduce the amount of plasmin available for fibrin clot lysis. Angiostatins are responsible for preventing angiogenesis and cell proliferation. However recently angiostatins have also been found to prolong fibrinolysis times by binding to fibrin and preventing t-PA mediated plasminogen activation (Ahn et al. 2011). Stack et al (1999) also found that angiostatin bound to t-PA preventing t-PA mediated plasminogen activation (Stack et al. 1999). These findings are consistent with the turbidimetric data which shows prolonged

fibrinolysis in cross-linked fibrin clots containing C3 compared to in the absence of C3 and FXIII, and highlights the possibility that angiostatin production may be a mechanism for prolonging fibrinolysis in the presence of C3. Alternatively angiostatins play a role in preventing excessive inflammation by preventing monocyte and neutrophil chemotaxis (Benelli et al. 2001). Angiostatin binds leukocyte β1 and β2 integrins thus preventing leukocyte attachment to the endothelium and extracellular matrix proteins and angiostatins bind Mac-1 a ligand responsible for leukocyte-fibrinogen binding (Chavakis et al. 2005). In this study, angiostatins also prevented the expression of tissue factor. These findings suggest that C3 promotes production of angiostatins required for preventing excessive inflammation and preventing further thrombus formation, whilst maintaining the current fibrin clot from fibrinolysis by plasmin.

In summary, these results suggest that in cross-linked fibrin clots, plasminogen cleavage remained similar in the presence and absence of C3, however less plasmin and increased numbers of lower molecular weight products were observed in the presence of C3, suggesting the plasmin generated underwent autodigestion to form A61, angiostatin4.5 and angiostatin. When comparing C3 containing fibrin clots in the presence and absence of FXIII, less plasminogen was cleaved and less plasmin was generated in the presence of FXIII. These findings support the results of the turbidity overlay experiment which showed that C3 prolongs fibrinolysis and prolonged fibrinolysis was dependent on FXIII-dependent cross-linking.

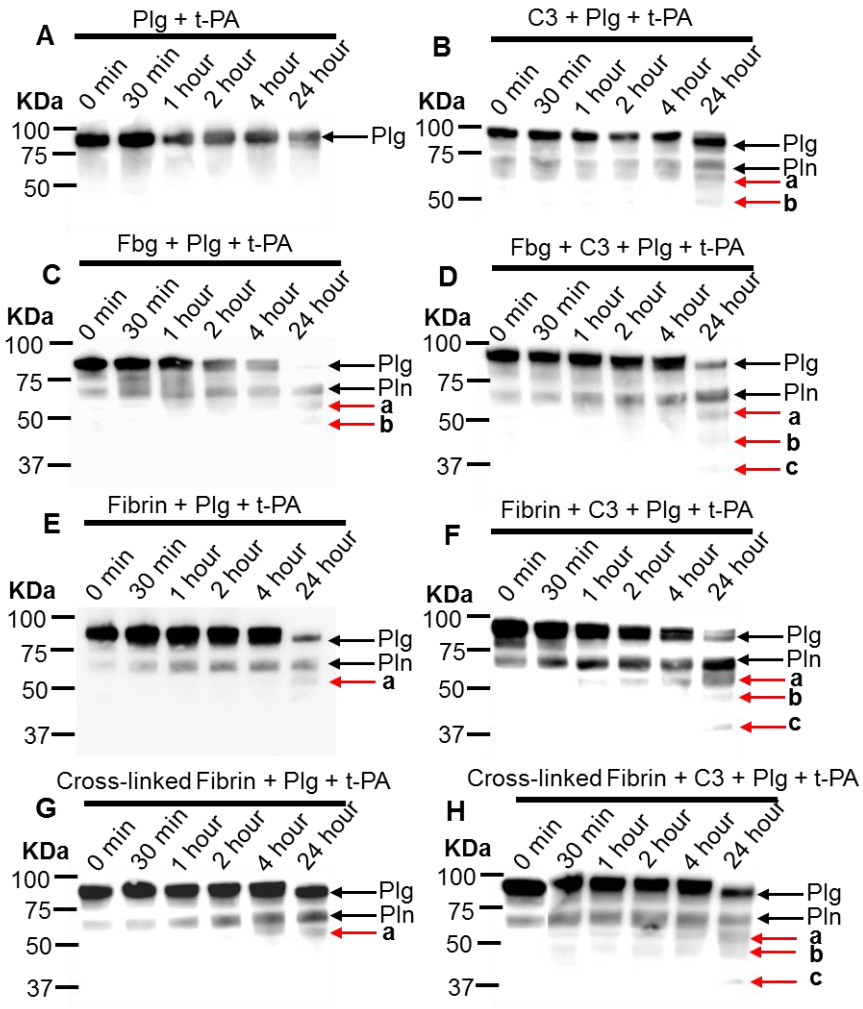

Figure 35: Time dependent t-PA mediated generation of plasmin in the presence and absence of C3, fibrin and FXIII.

Analysis of plasminogen cleavage by t-PA over a time course analysed by Western blot with an anti-plasminogen antibody. No plasminogen was cleaved by t-PA alone (**Panel A**). In the presence of C3 a small amount of plasminogen was cleaved to form plasmin (**Panel B**). In the presence of fibrinogen (**Panel C**) and C3 with fibrinogen (**Panel D**) the majority of plasminogen was cleaved to form plasmin at 24 hours. In the presence of non-cross-linked fibrin in the presence and absence of C3 the majority plasminogen was cleaved to plasmin at 24 hours (**Panel E and F**). In the presence of cross-linked fibrin (**Panel G**) and cross-linked fibrin containing C3 (**Panel H**) plasminogen cleavage remained constant over 4 hours with only a small amount cleaved at 24 hours. Red arrows (bands a-c) represent angiostatin production in all Western blots that have produced plasmin. Western blots representative of two independent experiments.

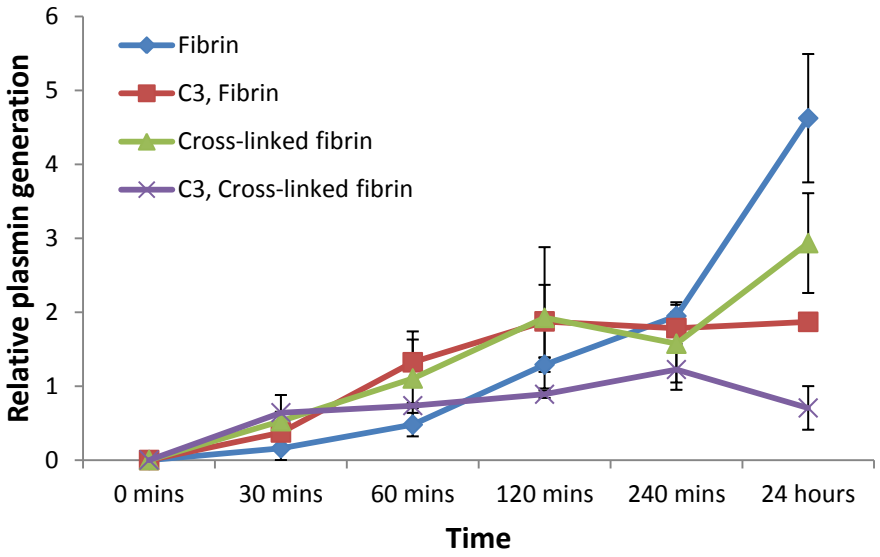

Figure 36: Densitometry analysis of plasmin generation over a 24 hour time course

Band intensity of plasmin measured from anti-plasminogen Western blot over a 24 hour period. Relative plasmin generation was calculated as difference in band intensity from time point 0 mins. Relative plasmin generation increasing over time in the presence of fibrin, but plateauing at 2 hours in the presence of fibrin clots containing C3. In the presence of cross-linked fibrin relative plasmin generation steadily increased over the 24 hours, however in the presence of cross-linked fibrin clots containing C3 relative plasmin generation increased slowly until 4 hours before decreasing at 24 hours. Mean of two independent experiments.

4.3.5.2. The influence of C3 on fibrin structure and fibrinolysis dependent on t-PA and plasminogen concentration

In the previous concurrent turbidimetric assay C3 was found to prolong fibrinolysis but not affect fibrin structure, suggesting C3 may interact with t-PA and/or plasminogen to prolong fibrinolysis. A concurrent turbidimetric assay was therefore performed to determine whether t-PA and plasminogen concentration influenced fibrin structure and fibrinolysis in the absence of C3 and to determine whether C3 had an additive affect on fibrin structure and fibrinolysis at reduced t-PA and plasminogen concentrations, which might suggest C3 interacts with t-PA and plasminogen. A concurrent turbidimetric assay was performed in the presence and absence of C3 with t-PA (Figure 37) and plasminogen (Figure 38) dose responses. As shown in Figure 37, in the absence of C3, maximum absorbance (Panel A) was significantly higher (P=0.002) and lysis time (Panel B) was significantly prolonged (P=0.001) with a decreased concentration of t-PA. As shown in Figure 37, panel C, in the presence of C3, maximum absorbance was significantly higher than in the absence of C3 at 5 and 10 ng/ml of t-PA but not at 50 ng/ml t-PA. Lysis time (Figure 37, Panel D) was significantly prolonged in the presence of C3 at all t-PA concentrations.

As shown in Figure 38, in the absence of C3, maximum absorbance (Figure 38, Panel A) was significantly higher (P=0.0001) and lysis time (Figure 38, Panel B) was significantly prolonged (P=0.0001) with a decrease in plasminogen concentration. As shown in Figure 38, Panel C, maximum absorbance was significantly higher in the presence of C3 compared to the absence of C3 at both 0.2 and 2 μg/ml of plasminogen but not at 20 μg/ml. Lysis time (Figure 38, Panel D) was significantly prolonged in the presence of C3 compared to the absence of C3 at all plasminogen concentrations, with lysis times prolonged by up to 1.5 fold at the lowest plasminogen concentration. Combined these findings suggest, that altering the t-PA and plasminogen concentration affects the overall clot structure in the absence of C3, resulting in a fibrin clots composed of thicker fibres and larger pores that takes longer to lyse. The increase in maximum absorbance observed with reduced t-PA and plasminogen was the opposite result previously observed in the C3 concentration-dependent turbidimetric assays. This finding may reflect the lower concentrations of t-PA and plasminogen used in this series of experiments (5-50

ng/ml t-PA incubated with 0.2-20 µg/ml plasminogen) compared to the previous turbidimetric experiments (100 ng/ml t-PA with 20 µg/ml plasminogen). Hess et al (2011) previously found that maximum absorbance was increased in the presence of C3 when using a lower plasminogen concentration (6 µg/ml). The increase in maximum absorbance observed in both the t-PA and plasminogen dose responses as a result of C3 could be accounted for by the temporal relationship between fibrin formation and fibrinolysis. Bauer et al (1994) found that with reduced t-PA concentrations, the time for protofibril formation and lateral aggregation of the fibrin fibres was increased, with plasmin generation occurring after lateral aggregation, whereas at higher concentrations plasmin generation occurs before lateral aggregation leading to fibrin breakdown and a reduced maximum absorbance (Bauer et al. 1994). Additionally, when clot formation and lysis occur concurrently adding another substrate in the form of C3 would decrease the effective plasmin concentration available for cleavage of fibrin thus increasing the maximum absorbance. These data suggest that in reduced t-PA and plasminogen concentrations, C3 further prevents early plasmin generation, allowing effective protofibril and lateral aggregation of the fibrin fibres to increase the maximum absorbance and in turn prolong fibrinolysis.

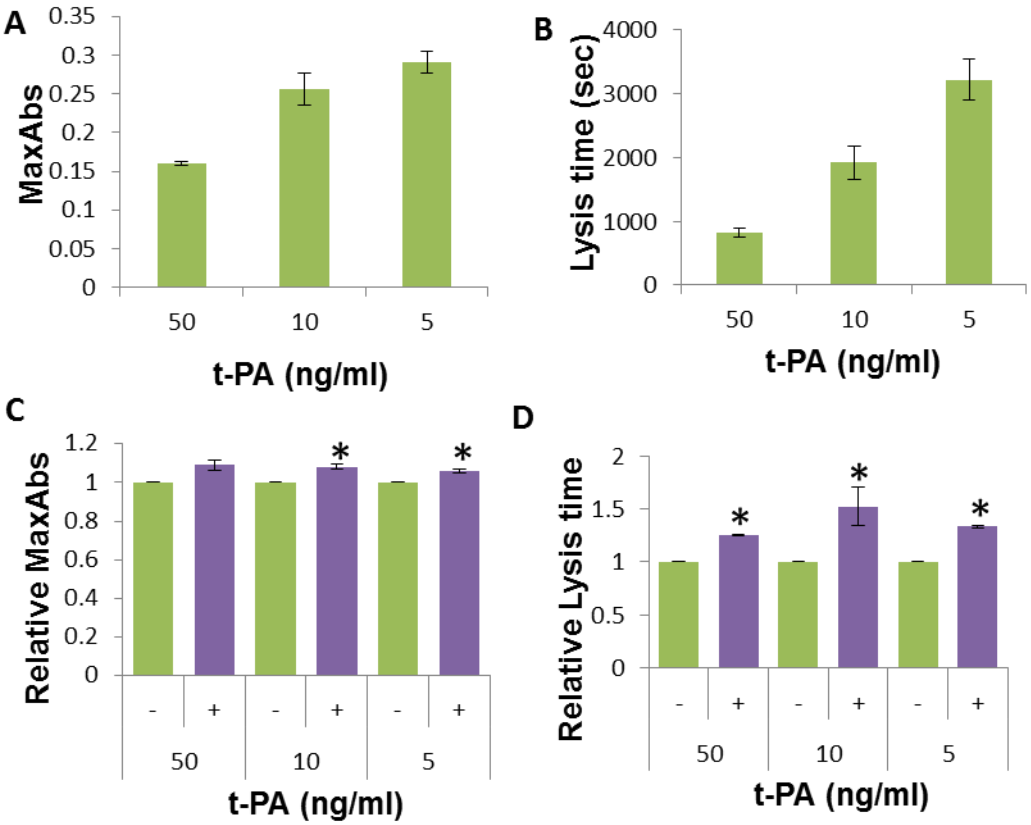

Figure 37: The effect of t-PA dose response on the influence of C3 in fibrin structure and lysis

Turbidimetric measurements at 340 nm were made using the concurrent assay in the presence of decreasing t-PA concentration in the presence and absence of C3. Relative maximum absorbance (MaxAbs) and lysis time was calculated as the difference between +/- C3 at each individual t-PA concentration. Maximum absorbance was significantly increased in the absence of C3 with decreasing t-PA (P=0.002) (**Panel A**), whereas maxiumum absorbance in fibrin clots containing C3 was significantly higher (t-PA 10 and 5 ng/ml) compared to fibrin clots in the absence of C3 (**Panel C**). Lysis time was significantly increased in the absence of C3 with decreasing t-PA (P=0.001) (**Panel B**), whereas lysis time was significantly longer in fibrin clots containing C3 at all t-PA concentrations (**Panel D**). Mean of three independent experiments. * indicates P = <0.05 after bonferroni adjustment when comparing +/- C3 at each individual t-PA concentration.

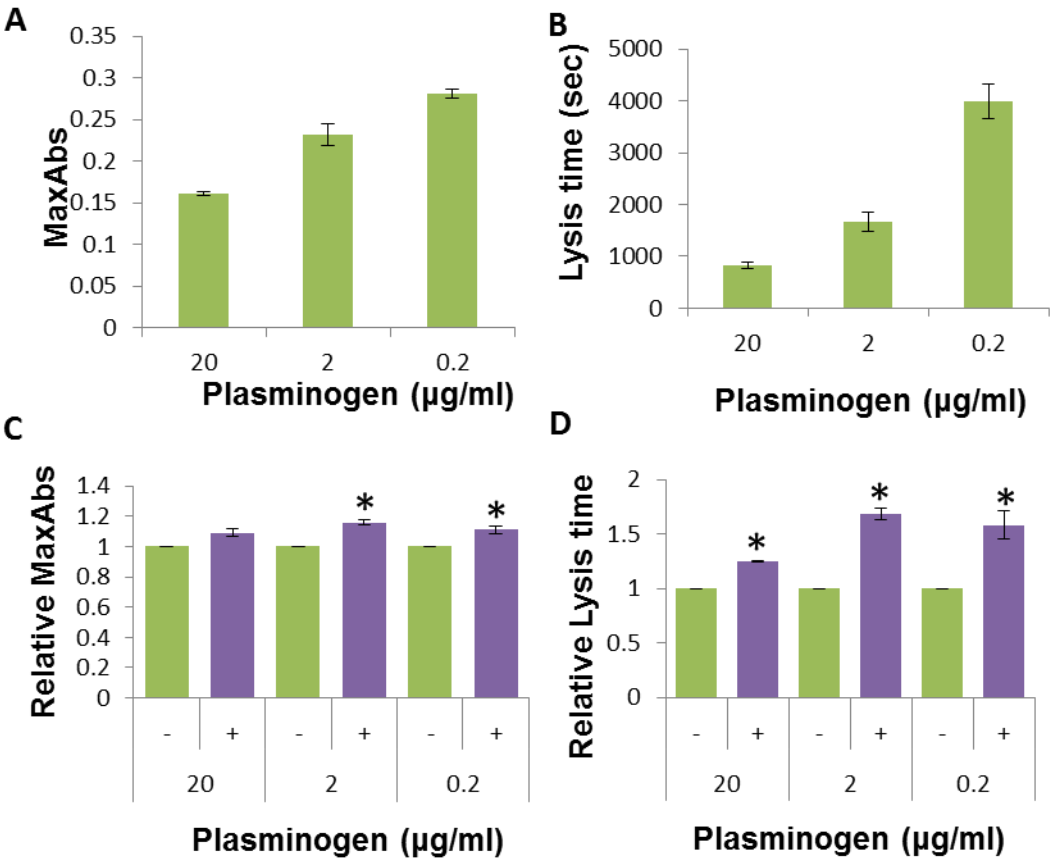

Figure 38: The effect of a plasminogen dose response on the influence of C3 in fibrin structure and lysis

Turbidimetric measurements at 340 nm were made using the concurrent assay in the presence of decreasing plasminogen concentration in the presence and absence of C3. Relative maximum absorbance (MaxAbs) and lysis time was calculated as the difference between +/- C3 at each individual plasminogen concentration. Maximum absorbance was significantly increased in the absence of C3 with decreasing plasminogen (P=0.0001) (**Panel A**), whereas maxiumum absorbance in fibrin clots containing C3 was significantly higher (plasminogen 2 and 0.2 µg/ml) compared to fibrin clots in the absence of C3 (**Panel C**). Lysis time was significantly increased in the absence of C3 with decreasing plasminogen (P=0.0001) (**Panel B**), whereas lysis time was significantly longer in fibrin clots containing C3 at all plasminogen concentrations (**Panel D**). Mean of three independent experiments. * indicates P = <0.05 after bonferroni adjustment when comparing +/- C3 at each individual plasminogen concentration.

4.3.6. The influence of C3 on the interactions between t-PA and plasminogen on a fibrin surface involved in plasmin generation

In the concurrent turbidimetric assay, C3 prolonged fibrinolysis in the presence and absence of FXIII. Furthermore, in the t-PA and plasminogen dose response experiments, C3 further enhanced the effect of t-PA and/or plasminogen on their own to prevent early plasmin generation and therefore prolong fibrinolysis, suggesting C3 may influence the interactions between t-PA, plasminogen and fibrin required for effective plasmin generation. In the absence of C3, fibrin acts as both a surface for plasmin generation and a substrate for generated plasmin. Plasmin generation requires interactions between fibrin, t-PA and plasminogen. Plasminogen and t-PA bind to fibrin within the D regions Aα140-160 and γ312-324 respectively (Bosma et al. 1988;Schielen et al. 1991) and also within the αC domain Aα221-610 (Tsurupa et al. 2001). Binding within the αC domain was high affinity (Kd Plasminogen 32 nM and tPA 33 nM) (Tsurupa et al. 2001) compared to the D regions (Kd ~ 1 μM for both tPA and plasminogen) (Yakovlev et al. 2000), suggesting the αC domain was essential in the initiation of fibrinolysis.

To determine whether C3 affects plasmin generation on a fibrin surface, fibrin was immobilised onto a 96 well plate and incubated with different orientations of t-PA, plasminogen and C3, each for 90 minutes. Plasmin generation was monitored by the cleavage of a chromogenic substrate S-2251. When t-PA was pre-incubated on the fibrin surface prior to plasminogen and S-2251, the maximum plasmin generation occurred after ~ 2½ hours (Figure 39, Panel A). Although the amount of plasmin generated at 4 hours between the different orientations did not significantly differ (P= NS) (Figure 39, Panel C), the rate of plasmin generation was reduced when C3 was incubated following a pre-incubation with t-PA and prior to plasminogen and S-2251 compared with t-PA pre-incubated on the fibrin surface prior to plasminogen and S-2251 although not significant (P=NS) (Figure 39, Panel D). This result suggests that C3 and t-PA bind to fibrin on separate sites, since C3 incubated first on the fibrin surface did not prevent t-PA mediated plasmin generation (Figure 40, Panel C). In unpublished data produced by Dr Kerrie Smith (section 9.4) using surface plasmon resonance a recombinant fragment of the αC domain (Aα233-425) bound to immobilised C3 with high affinity (Kd ~2.4 nM), which suggests that C3 binds within the region of t-PA and plasminogen binding,

however as no affect of C3 on plasmin generation was observed it suggests there was sufficient space on the αC domain to prevent steric hindrance when all three proteins were bound. Whereas when t-PA was incubated on the fibrin surface first, C3 prevented t-PA mediated plasmin generation. One possibility for this finding could be that conformational changes occur in t-PA which exposes a binding site for C3. This binding then prevents the formation of the ternary complex between t-PA, plasminogen and fibrin (Figure 40, Panel B). A recent study found that t-PA binds to fibrin first by the finger domain, resulting in exposure of a cryptic domain that was able to bind plasminogen (Kim et al. 2011), resulting in plasmin generation, if C3 were able to bind this cryptic site in t-PA it would prevent plasmin generation by preventing plasminogen binding to the t-PA. Whereas no decrease in the rate of plasmin generation was observed when t-PA and C3 were pre-incubated together on the fibrin surface suggesting that t-PA and C3 bind to fibrin at their separate sites with equal affinity, thus preventing the time required for conformational changes to occur in t-PA that exposes the C3 binding site (Figure 40, Panel D). To fully determine whether the effect of C3 on plasmin generation was dependent on binding to t-PA following fibrin binding, further experiments need to be performed using u-PA which does not have a fibrin-dependent effect.

When plasminogen was pre-incubated on the fibrin surface prior to t-PA and S-2251, the maximum plasmin generation was not reached within the 4 hour assay period, suggesting the reaction was significantly slower than that of t-PA when pre-incubated on the fibrin surface prior to the addition of plasminogen and S-2251, even though the concentration of proteins used in this experiment were the same as those used when t-PA was incubated on the fibrin surface (Figure 39, Panel B). The amount of plasmin generated (Figure 39, Panel E) and the rate of plasmin generation (Figure 39, Panel F) was reduced/slower when C3 was incubated on the fibrin surface following a pre-incubation with plasminogen and prior to the addition of t-PA and S-2251 or when C3 and plasminogen were incubated together on the fibrin surface prior to the addition of t-PA and S-2251 compared to plasminogen incubated on the fibrin surface prior to the addition of t-PA and S-2251, although not significant (P=NS). These findings suggest the amount and rate of plasmin generation was dependent on the orientation of when C3 was pre-incubated on the fibrin coated plates. Previous studies have shown that plasminogen can bind to two

fibrin monomers, suggesting plasminogen has two binding sites (Fears 1989), therefore plasminogen might bind both C3 and fibrin, with binding of C3 to plasminogen preventing the appropriate t-PA mediated interactions with plasminogen and fibrin required for plasmin generation (Figure 41, Panel B). When C3 was incubated on the fibrin surface first, C3 binds to a separate site on fibrin compared to t-PA and plasminogen, thus allowing t-PA and plasminogen to form the tertiary complex (Figure 41, Panel C), with no affect on plasmin generation, as suggested previously this result suggests C3, t-PA and plasminogen are able to bind to fibrin without steric hindrance. When plasminogen and C3 were incubated on the fibrin surface together a decrease in plasmin generation was observed suggesting C3 and plasminogen form a complex in solution which prevents plasminogen binding to fibrin and therefore decreases t-PA mediated cleavage of plasminogen at the fibrin surface (Figure 41, Panel D). In a recent study by Barthel et al (2012a) C3 and C3 cleavage products C3b, C3c and C3d were found to bind to plasminogen in solution and this binding interaction was found to be lysine dependent as a lysine analogue (εACA) inhibited binding of C3d by 75% (Barthel et al. 2012a). Since binding of plasminogen to fibrin was also dependent on lysine residues (Fears 1989) it suggests that C3 competes with fibrin for plasminogen binding via lysine residues and thus decreases plasmin generation. Barthel et al (2012a) did not suggest whether C3 bound to plasminogen will prevent plasminogen cleavage by t-PA. However if this was to occur it would suggest that C3 and C3 activation products produced by plasmin cleavage would form a positive feedback loop to regulate plasminogen activation. Since plasminogen binds to C3 in solution it was also possible that C3 may have been lost during the C3 purification at the lysine sepharose stage, however this must only account for a small amount of C3 due to the significantly lower plasminogen concentration in plasma compared to C3.

Combined these results suggest C3 binds to fibrin bound t-PA, C3 binds to fibrin bound plasminogen and C3 binds plasminogen in solution to prevent plasmin generation. These findings confirm the previous observations in the concurrent turbidimetric assay that show C3 prolongs fibrinolysis in the absence of FXIII and suggests a possible mechanism might be reduced plasmin generation as a result of interactions between C3 and plasminogen and between C3 and t-PA.

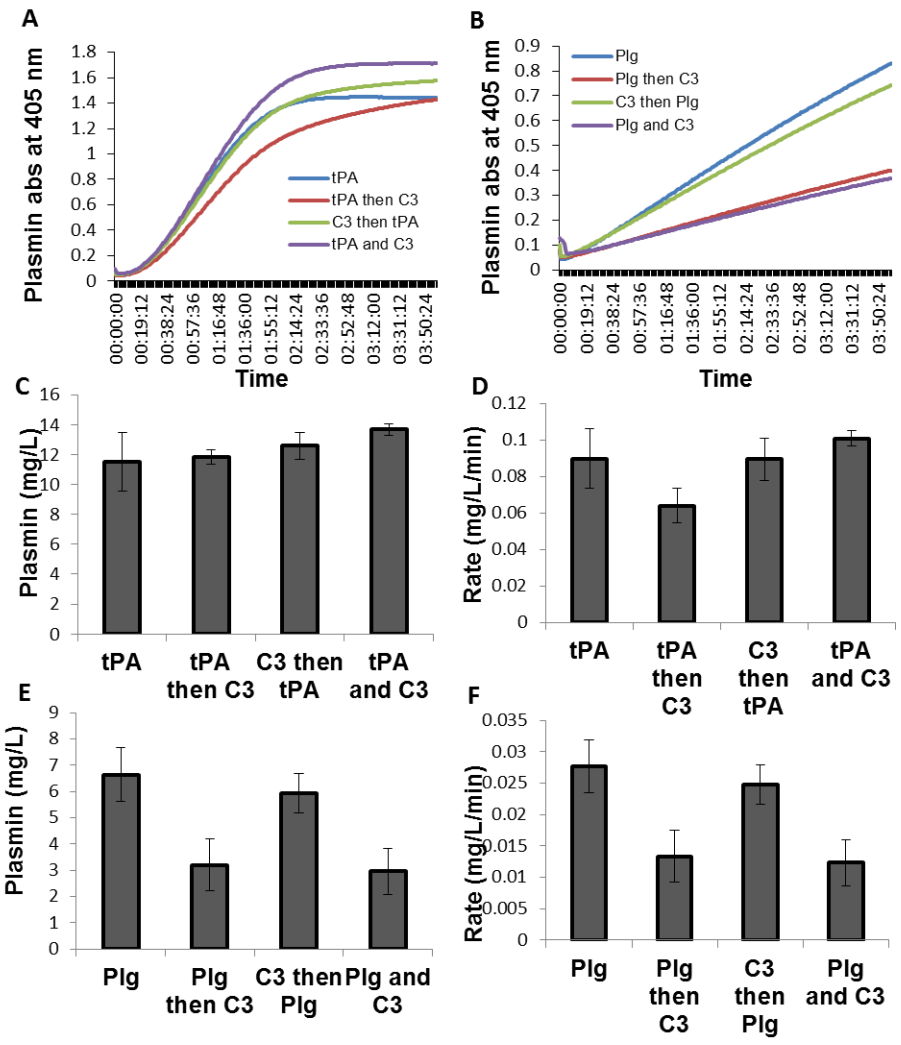

Figure 39: Plasmin generation in a plate based assay.

S-2251 cleavage monitored by changes in absorbance at 405 nm when t-PA (**Panel A**) or plasminogen (**Panel B**) were pre-incubated on an immobilised fibrin surface before, after or at the same time as C3. **Panel C**, the amount of plasmin generated at 4 hours, when t-PA was pre-incubated on an immobilised fibrin surface, showed no significant difference in the amount of plasmin between the orientations. **Panel D**, the rate of plasmin generation between 0-2 hours when t-PA was pre-incubated on an immobilised fibrin surface, showed a reduced rate when C3 was incubated following a pre-incubation with t-PA. **Panel E and D**, the amount of plasmin generated and the rate of plasmin generation at 4 hours, when plasminogen was pre-incubated on an immobilised fibrin surface, was reduced when C3 was incubated following a pre-incubation with plasminogen and when plasminogen and C3 were incubated together. Mean of three independent experiments (SEM).

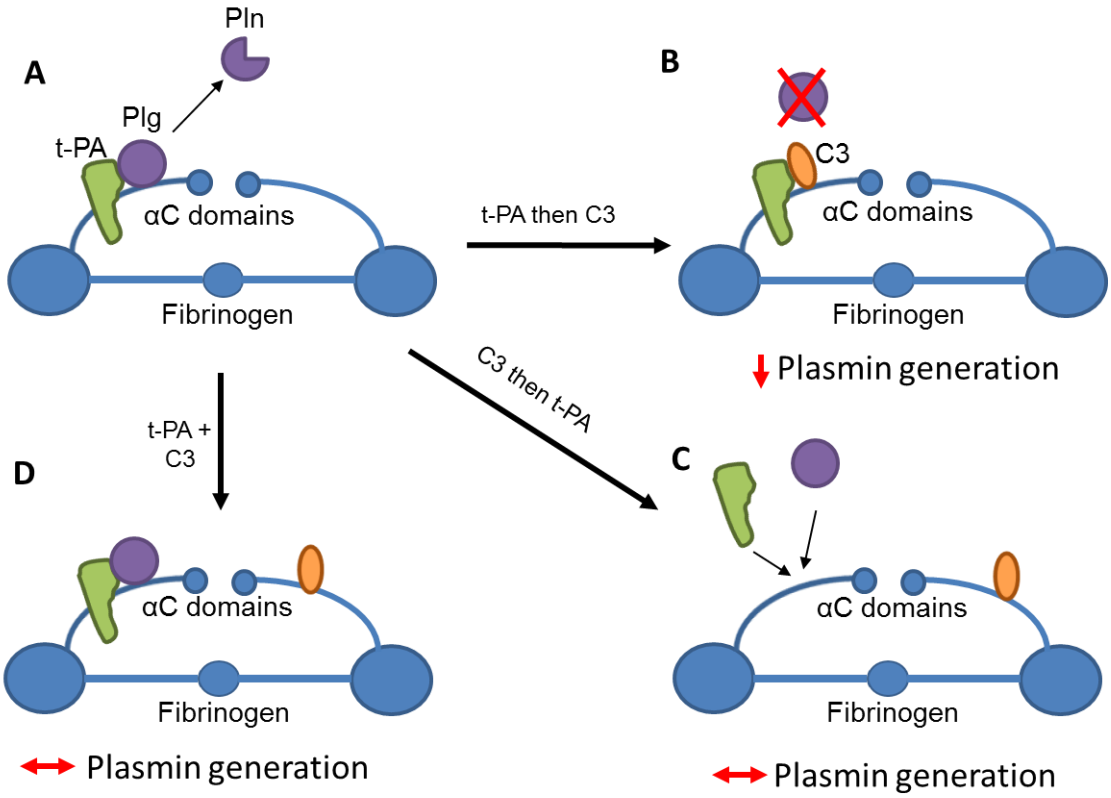

Figure 40: The interactions between C3 and t-PA that influence plasmin generation in the plate based assay

Panel A. t-PA and plasminogen (Plg) bind to fibrin in the formation of a tertiary complex, responsible for activating plasminogen to form plasmin (Pln). **Panel B**. When t-PA was incubated on the fibrin surface first this allows conformational changes to occur which exposes a binding site for C3. C3 once bound to t-PA prevents plasminogen binding to form the tertiary complex, thus plasmin generation was decreased. **Panel C**. When C3 was incubated on the fibrin surface first, C3 binds to a separate site on fibrin compared to t-PA and plasminogen, thus allowing t-PA and plasminogen to form the tertiary complex, with no affect on plasmin generation. **Panel D**. When t-PA and C3 were incubated on the fibrin surface together no affect on plasmin generation was observed suggesting C3 and t-PA bind to fibrin at separate sites and that C3 bound to fibrin before conformational changes occurred in t-PA which exposed a binding site for C3.

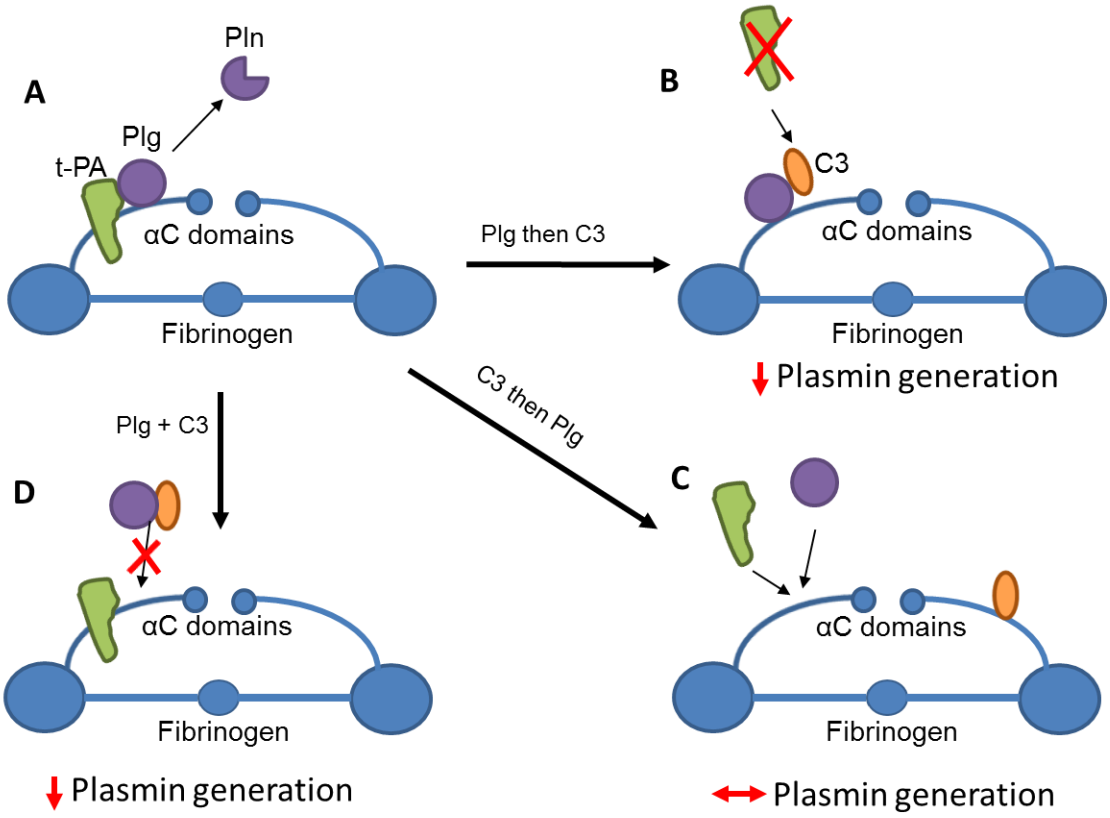

Figure 41: The interactions between C3 and plasminogen that influence plasmin generation in the plate based assay

Panel A. t-PA and plasminogen (Plg) bind to fibrin in the formation of a tertiary complex, responsible for activating plasminogen to form plasmin (Pln). **Panel B**. Plasminogen has two binding sites, therefore plasminogen can bind both C3 and fibrin, however binding of C3 to plasminogen prevents t-PA mediated interactions with plasminogen and fibrin resulting in reduced plasmin generation. **Panel C**. When C3 was incubated on the fibrin surface first, C3 binds to a separate site on fibrin compared to t-PA and plasminogen, thus allowing t-PA and plasminogen to form the tertiary complex, with no affect on plasmin generation. **Panel D**. When plasminogen and C3 were incubated on the fibrin surface together a decrease in plasmin generation was observed suggesting C3 and plasminogen form a complex in solution which prevents plasminogen binding to fibrin and therefore decreases t-PA mediated cleavage of plasminogen at the fibrin surface.

4.3.6.1. The influence of C3 on plasmin generation on the surface of fibrin clots incubated with t-PA and plasminogen

In section 4.2, C3 was shown to cross-link to fibrin and C3 within cross-linked fibrin clots was found to influence fibrinolysis rates in turbidimetric assays (Section 4.1.1), suggesting that C3 cross-linking may influence plasmin generation. To examine this possibility, plasmin generation was analysed by the cleavage of the chromogenic substrate S-2251 on the surface of fibrin clots containing +/- C3 and +/- FXIII, when either t-PA or plasminogen was incubated within the fibrin clot by monitoring the absorbance changes at 405 nm upon the addition of either t-PA or plasminogen to initite lysis. Previous optimisation experiments found there was no difference in plasmin generation between fibrin clots in the presence and absence of FXIII (Figure 13), suggesting plasmin generation was not FXIII-dependent.

When t-PA was incubated in the solid fibrin clots (Figure 42, Panels A-C), there was no significant difference in plasmin maximum absorbance, plasmin generation and rate of plasmin generation between cross-linked fibrin clots and non-cross-linked fibrin clots whether in the presence or absence of C3 (P = NS). There was a lower plasmin maximum absorbance, less plasmin generated and reduced rate of plasmin generation (Figure 42, Panels A-C) in cross-linked fibrin clots containing C3 compared in the absence of C3 although not significant (P=NS) There was a lower plasmin maximum absorbance, less plasmin generated and reduced rate of plasmin generation (Figure 42, Panels A-C) between non-cross-linked fibrin clots containing C3 compared to in the absence of C3 (Plasmin generation P=0.003, Rate of plasmin generation P=NS). These results confirm the optimisation experiment which shows that FXIII does not influence plasmin generation and further shows that C3 prevents plasmin generation on the surface of a fibrin clot. As outlined in Figure 40, Panel B, plasmin generation was reduced due to C3 binding conformationally changed t-PA, which prevents plasminogen binding, plasminogen cleavage and reduced plasmin generation. As C3 was incubated with t-PA and fibrin for an hour before plasminogen was added it suggests that this mechanism could have occurred, thus preventing the added plasminogen from binding t-PA and generating plasmin. This mechanism accounts for the affect of C3 in both the presence and absence of FXIII.

In the concurrent turbidimetric assay, C3 was found to prolong fibrinolysis in the presence of C3 and FXIII when plasminogen was incubated in the fibrin clot. Therefore the solid fibrin clot based plasmin generation assay was performed in fibrin clots incubated with plasminogen in the presence and absence of C3 and FXIII. As shown in Figure 43, Panels A & B there was a lower plasmin maximum absorbance and plasmin generation in cross-linked fibrin clots containing C3 and non-cross-linked fibrin clots containing C3 compared to cross-linked and non-cross-linked fibrin clots in the absence of C3. (P=0.02, P=NS respectively). There was no significant difference in plasmin maximum absorbance and plasmin generation between cross-linked fibrin clots containing C3 compared to non-cross-linked fibrin clots containing C3 or between cross-linked fibrin and non-cross-linked fibrin clots in the absence of C3 (P=NS). As shown in Figure 43, Panel C, the rate of plasmin generation reflected the final plasmin generation with lower rates of plasmin generation observed in cross-linked fibrin clots containing C3 and non-cross-linked fibrin clots containing C3 compared to cross-linked and non-cross-linked fibrin clots in the absence of C3 altough not significant (P=NS). There was no significant difference in rate of plasmin generation between cross-linked clots in cross-linked fibrin clots containing C3 compared to non-cross-linked fibrin clots containing C3 or between cross-linked fibrin and non-cross-linked fibrin clots in the absence of C3 (P=NS). These results confirm the optimisation of the solid clot based plasmin generation assay which found that FXIII does not affect plasmin generation (Figure 13) and further supports a role for C3 in the prevention of plasmin generation on the surface of a fibrin clot. As outlined in Figure 41, Panel B, plasmin generation was reduced when plasminogen bound to fibrin and C3 preventing t-PA mediated cleavage, whereas in Figure 41, Panel D, plasmin generation was reduced by plasminogen binding C3 in solution preventing plasmin attachment to fibrin and t-PA mediated cleavage of plasminogen. Both of these mechanisms may account for the reduced plasmin generation observed in the presence and absence of FXIII. These findings support the observations in the concurrent turbidimetric assay which suggest that C3 prolongs fibrinolysis in the presence and absence of FXIII. As the solid clots in these experiments contained either t-PA or plasminogen they don't reflect the differences observed in the turbidity overlay experiment which contained no t-PA and plasminogen during clot formation, which may account for why there

was not a larger difference in plasmin generation between cross-linked and non-cross-linked fibrin clots containing C3.

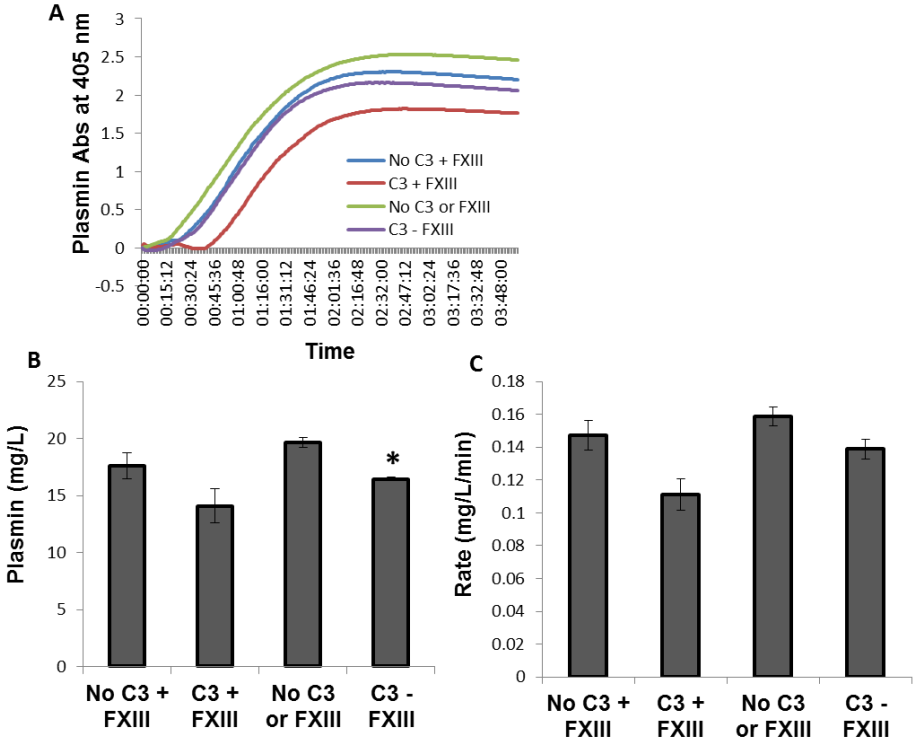

Figure 42: Plasmin generation in the presence of C3 and FXIII within a solid fibrin clot containing t-PA.

Fibrin clots containing t-PA in the presence and absence of C3 and FXIII were formed. Plasmin generation was monitored at 405 nm (change in absorbance) when plasminogen and S-2251 were overlayed on the fibrin clot (**Panel A). Panel B**: The amount of plasmin generated at 4 hours was reduced in cross-linked fibrin clots containing C3 and in non-cross-linked fibrin clots containing C3. No difference was observed between cross-linked fibrin clots compared to non-cross-linked fibrin clots containing C3 and between cross-linked fibrin clots and non-cross-linked fibrin clots in the absence of C3. **Panel C.** The rate of plasmin generation between 0-2 hours was reduced in cross-linked fibrin clots containing C3 although not significant. Mean of three independent experiments (SEM). ***** P=<0.05 between +/- C3 in the absence of FXIII.

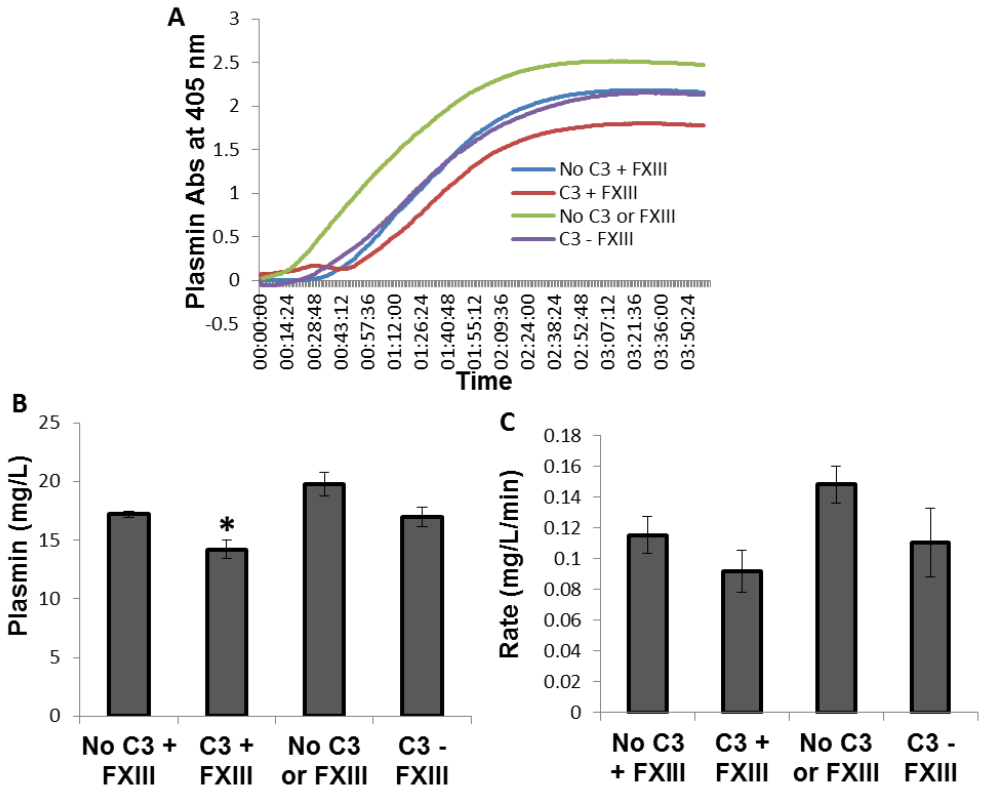

Figure 43: Plasmin generation in the presence of C3 and FXIII within a solid clot containing plasminogen.

Fibrin clots containing plasminogen in the presence and absence of C3 and FXIII were formed. Plasmin generation was monitored at 405 nm (change in absorbance) when t-PA and S-2251 were overlayed on the fibrin clot (**Panel A)**. **Panel B**. The amount of plasmin generated at 4 hours was reduced in cross-linked fibrin clots containing C3 and non-cross-linked fibrin clots containing C3. No difference in plasmin generation was observed in cross-linked and non-cross-linked fibrin clots in either the presence or absence of C3. **Panel C**. The rate of plasmin generation between 0-2 hours was reduced in cross-linked fibrin clots containing C3 and non-cross-linked fibrin clots containing C3. No difference in the rate of plasmin generation was observed in cross-linked and non-cross-linked fibrin clots in either the presence or absence of C3. Mean of three independent experiments (SEM). ***** P=<0.05 between +/- C3 in the presence of FXIII.

In summary, the present study has confirmed previous clinical study findings that suggest C3 prolongs fibrinolysis in both a purified and plasma based system. Although fibrin structure was unaffected by the presence of C3, C3 was found to cross-link the small primary amine of BP and C3 was cross-linked to fibrin in both purified and plasma clots. In addition, C3b was able to form non-enzymatic cross-links via its internal thioester with fibrin during the cross-linking process. C3 was also found to bind to fibrin in plasma clots. This study has also investigated potential mechanisms for prolonged fibrinolysis. C3 was found to be a substrate for plasmin with the generation of complement activation products of the C3 α chain and an unknown cleavage product of the C3 β chain, however as cleavage of C3 occured at a similar rate to fibrin cleavage it suggests C3 was not a competitive substrate for plasmin in fibrin clots. Alternatively C3 was found to influence plasminogen cleavage, plasmin generation and angiostatin production in cross-linked and non-cross-linked fibrin clots. This study has highlighted possible mechanisms for prolonging fibrinolysis which include binding of C3 to conformationally changed t-PA which prevents plasminogen binding and binding of C3 to plasminogen on the fibrin surface or in solution to prevent t-PA mediated plasminogen cleavage. These findings suggest C3 was an active component of fibrin clots and was localised to fibrin via cross-linking and binding and C3 prolongs fibrinolysis via several mechanisms most likely to prevent pathogen invasion.

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Chapter 5 Evaluating the influence of factor H on CVD risk and fibrin structure and function

Complement factor H was previously identified as a clot component in perfused solubilised plasma clots (Howes et al. 2012) and as shown by the previous chapter, incorporation of C3 into fibrin clots had a functional effect on fibrinolysis. Therefore the functional effects of factor H on fibrin structure and function were investigated. A factor H ELISA was therefore established to evaluate the percentage incorporation of factor H into plasma clots. In a study examining CVD risk in South Asian relatives of patients with T2DM compared with healthy South Asian controls, increased complement activation (formation of sC5b-9) was observed (Somani et al. 2012), suggesting the down regulation of an inhibitor in complement activation, therefore factor H was proposed as a potential CVD risk factor. In this study group factor H was measured to evaluate the relationship between factor H and CVD risk factors, complement activation and fibrin structure and function in South Asian relatives and controls.

5.1. Incorporation of factor H into plasma clots

Factor H has previously been identified as a plasma clot component (Howes et al. 2012). To establish the quantity of plasma factor H incorporated into plasma clots, factor H concentrations were compared in paired plasma and plasma-derived serum samples (n=78). As shown in Figure 44 the concentration of factor H within the clot increased with increasing plasma factor H concentration; however, the proportion of factor H incorporated into plasma clots varied between individuals from 0% to 34%, with a mean incorporation of 11.4% (SD 8.01). Since the concentration of factor H within plasma clots varied widely it suggests factor H may play a role in plasma clot structure and function.

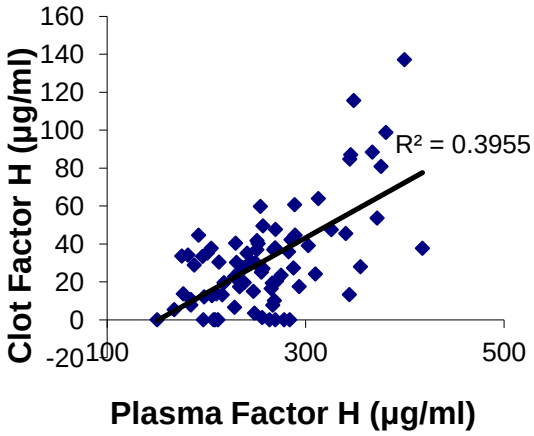

Figure 44: Incorporation of complement factor H into *ex vivo* plasma clots.

Factor H was measured by ELISA in paired plasma and plasma derived serum samples (n=78). Clot factor H was calculated as the difference between the serum concentration of factor H and the plasma concentration of factor H. **Panel A**: The concentration of factor H within plasma clots increased with increasing plasma factor H concentration.

5.2. Relationship between factor H and family history of T2DM in South Asians

5.2.1. Relationship between factor H and conventional risk factors for T2DM and CVD in South Asian relatives and controls

Factor H is a regulator of complement activation and as such alterations in the level of factor H may play a role in the inflammatory aspects of T2DM and CVD, therefore the relationship between factor H and conventional risk factors of T2DM and CVD were investigated. In South Asian relatives and controls (Table 12), plasma factor H correlated with the following T2DM and CVD risk factors: BMI, insulin, CRP and fibrinogen. Additionally in South Asian controls plasma factor H correlated with triglycerides, glucose and HOMA-IR. In linear regression analyses, BMI (1.8% variance) was an independent predictor of factor H in South Asian controls and CRP (3.6% variance) was an independent predictor of factor H in South Asian relatives.

Table 12 : Age and sex adjusted correlation coefficients between factor H and conventional risk factors for T2DM and CVD in South Asian controls and relatives

Factor H was significantly correlated with BMI, Insulin, CRP and fibrinogen in South Asian controls and relatives. Additionally factor H was significantly correlated with triglycerides, glucose and homeostatic model assessment of insulin resistance (HOMA-IR) in South Asian controls. HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

	South Asian Controls	South Asian Relatives
T2DM and CVD risk factors		
BMI	0.516†	0.368†
Cholesterol	0.153	0.040
HDL	-0.100	0.096
LDL	0.155	0.024
Triglycerides	0.284*	0.005
Glucose	0.203*	-0.069
Insulin	0.401†	0.221*
HOMA-IR	0.415†	0.189
CRP	0.588†	0.522†
Fibrinogen	0.474†	0.584†

Significant correlations: *$P<0.05$, †$P<0.001$

5.2.2. Relationship between factor H and complement components in South Asian relatives and controls

In previous work by Dr Somani, sC5b-9 was found to be significantly (P= <0.0001) higher in South Asian relatives compared to South Asian controls (Somani et al. 2012) and this was not entirely explained by other complement components, therefore factor H was analysed to determine if this explained the increase in

complement activation. There was no significant difference in factor H levels (*P*= 0.46) between South Asian relatives and the South Asian controls (Figure 45).

Plasma levels of factor H correlated with zymogen complement components (Table 13) C3, factor B and properdin (*P*= <0.001) in both South Asian relatives and controls, with no correlation observed between factor H and activated complement components fragment Bb, C3a-desArg and sC5b-9 in South Asian relatives; however, sC5b-9 was negatively correlated in South Asian controls (*P*= <0.05). In linear regression analyses, C3 and factor B were independent predictors of factor H in both South Asian controls (44.1% and 10.9% variance respectively) and South Asian relatives (25.5% and 9.2% variance respectively).

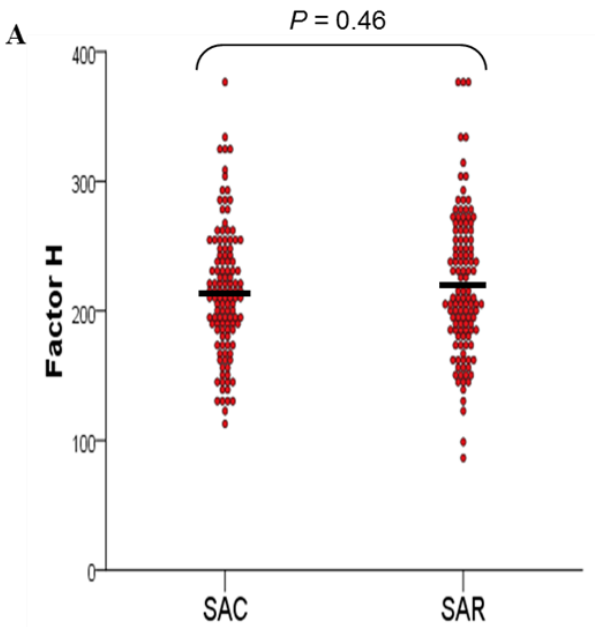

Figure 45: Complement factor H in South Asian relatives of subjects with T2DM compared with South Asian controls with no family history.

Factor H measured by ELISA was not significantly different between 119 South Asian relatives (SAR) and 119 South Asian controls (SAC).

Table 13: Age and sex adjusted correlation coefficients between factor H and complement components in South Asian controls and relatives.

Factor H was significantly correlated with C3, properdin and factor B in South Asian controls and relatives and was negatively correlated with sC5b-9 in South Asian controls.

	South Asian Controls	South Asian Relatives
Complement Components		
C3	0.686†	0.533†
sC5b-9	-0.230*	0.157
C3a-desArg	0.041	0.020
Properdin	0.370†	0.349†
Factor B	0.638†	0.509†
Bb	-0.098	-0.129

Significant correlations: *P<0.05, †P<0.001

5.2.3. Relationship between factor H and fibrin structure and function in South Asian relatives and controls

Factor H has been found to be plasma clot component and incorporation of factor H within plasma clots varied between individuals suggesting factor H may influence plasma clot structure and function. In the South Asian study group, plasma clot formation and lysis parameters were previously analysed by Dr Somani (Somani et al. 2012, unpublished data), who identified increased maximum absorbance (turbidity analysis and concurrent analysis) and reduced fibrinolysis times in South Asian relatives compared with controls (see appendix Table 18). Therefore the associations between factor H and ex vivo clot parameters were evaluated in this study. Initially, the combined study group was split into quartiles according to their plasma concentration of factor H. As shown in Figure 46, Panels A-D with increasing quartiles of factor H, lag times were significantly reduced and maximum absorbance was significantly increased. There was no significant difference in lysis times between the highest and lowest quartiles of factor H (Panel E); however, the area under the lysis curve was significantly increased with increasing factor H quartiles (Panel F).

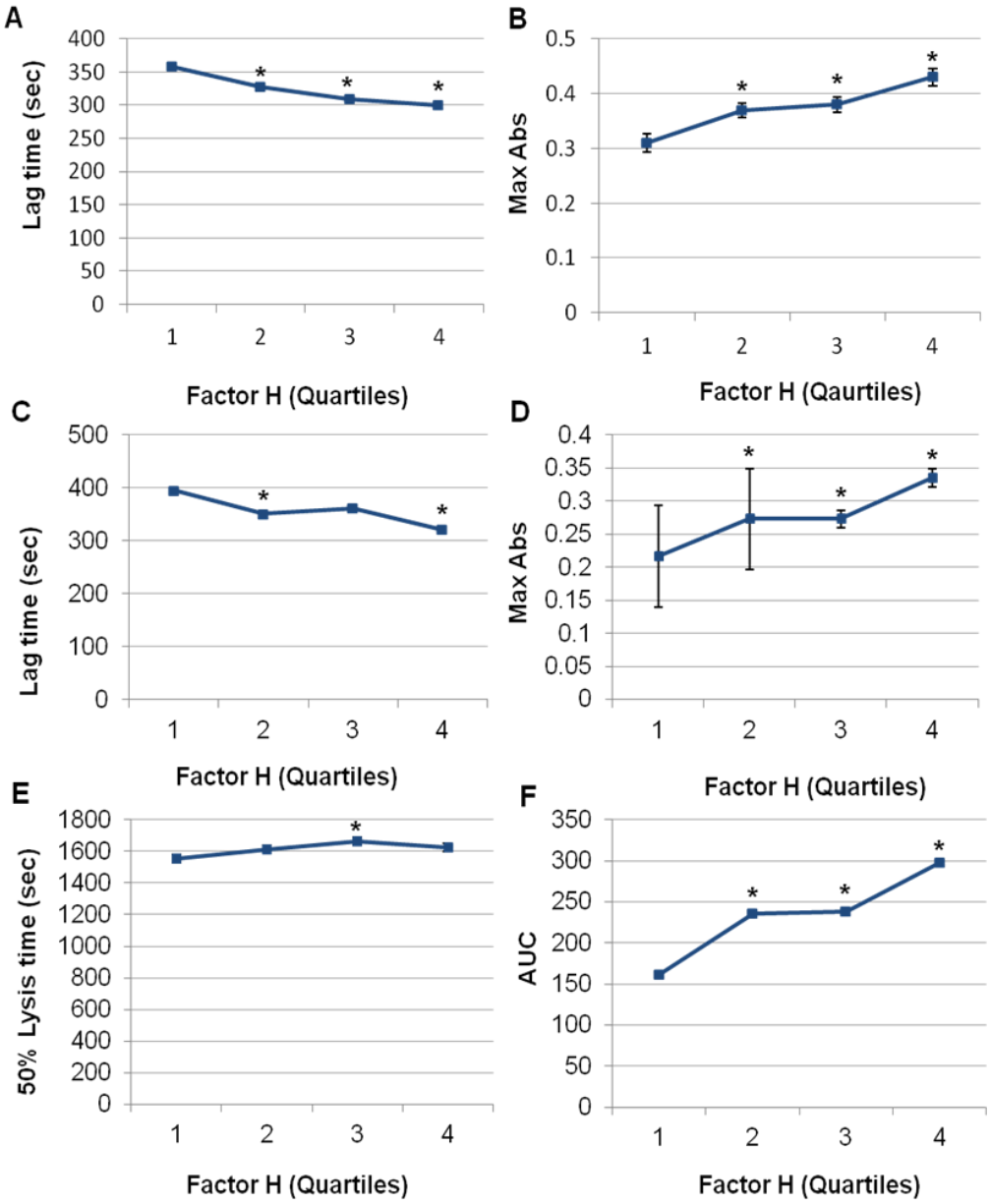

Figure 46: Role of factor H in plasma fibrin formation and lysis.

The combined South Asian relative and control study group was split into quartiles based on plasma factor H concentration and were analysed for turbidimetric clotting assay parameters (**Panels A & B**) and turbidimetric fibrinolysis assay parameters (**Panels C-F**). **Panels A & C**: Lag time decreased with increasing quartiles of factor H. **Panels B & D**: Maximum absorbance (MaxAbs) increased with increasing quartiles of factor H. **Panel E**: Time to 50% lysis was unaffected by factor H quartiles. **Panel F**: Area under the lysis curve (AUC) increased with increasing quartiles of factor H. Error bars are SEM. **P* = <0.05. when compared to quartile 1.

The age and sex adjusted correlations between factor H and turbidimetric parameters in South Asian relatives and controls are presented in Table 14. Factor H was significantly correlated with maximum absorbance (turbidity analysis and concurrent lysis analysis) and area under the lysis curve in both South Asian relatives and South Asian controls and with lysis time in South Asian controls. Factor H was significantly inversely correlated with lag time (concurrent analysis) in South Asian relatives and South Asian controls and lag time (turbidity analysis) in South Asian relatives only. In linear regression analyses, including adjustment for age, sex, fibrinogen, and PAI-1, factor H was independently associated with maximum absorbance (turbidity analysis) (P=0.005), 50% lysis time (P=0.006) and area under the lysis curve (P=0.041) in South Asian controls however, factor H was not independently associated with any fibrin parameter in South Asian relatives. The independent associations between factor H and fibrin parameters in healthy controls suggested that factor H may influence fibrin clot density and fibrinolysis. Therefore additional in vitro experiments were carried out to evaluate the role of complement factor H on fibrin formation and fibrinolysis, cross-linking and the interaction with the coagulation proteases thrombin and plasmin.

Table 14: Age and sex adjusted correlation coeffcients between factor H and turbidimetric parameters in South Asian relatives and controls

In South Asian controls, factor H was significantly correlated with maximum absorbance (T), maximum absorbance (F), lysis time, area under the lysis curve and was negatively correlated with lag time (F). In South Asian relatives, factor H was correlated with maximum absorbance (T), maximum absorbance (F), area under the lysis curve and was negatively correlated with lag time (F).

	South Asian Controls	South Asian Relatives
Turbidity clotting analysis		
Lag time (T)	-0.118	-0.278*
Maximum Absorbance (T)	0.546†	0.408†
Concurrent fibrinolysis analysis		
Lag time (F)	-0.238*	-0.306†
Maximum Absorbance (F)	0.477†	0.479†
Lysis time	0.425†	0.021
Area under the lysis curve	0.566†	0.445†

Significant correlations *P = <0.05, † P = <0.001

5.3. Evaluating the influence of factor H on fibrin formation and lysis

In the South Asian controls, factor H was independently associated with maximum absorbance, 50% lysis time and area under the lysis curve, suggesting factor H may influence fibrin clot structure and function in healthy individuals. To further clarify the clinical observations a turbidimetric assay was performed using purified proteins, to assess whether incorporated factor H influences fibrin formation and fibrinolysis. There was no significant (P= NS) difference in lag times (Figure 47, Panel A), maximum absorbance (Figure 47, Panel B) or 50% lysis times (Figure 47, Panel C) between fibrin clots containing no factor H and those containing increasing concentrations of factor H up to 100 μg/ml. At supraphysiological concentrations of

factor H (500 µg/ml) lag time was significantly longer (*P*= 0.001); however, maximum absorbance and lysis time were not affected by factor H (*P*= NS). These findings in a purified system suggest factor H plays little or no role in fibrin structure and function, except at supraphysiological concentrations of factor H, when lag time was prolonged, suggesting factor H may compete with fibrinogen and FXIII for thrombin cleavage. Since the observations in a purified system suggest factor H plays little or no role in fibrin formation and lysis it suggests the clinical observation in the South Asian study group may be due to other haemostatic determinants such as FXIII.

5.4. Evaluation of factor H cross-linking to fibrin

5.4.1. Cross-linking of factor H to fibrin in a purified system

As shown in appendix A, factor H was present in FXIII-depleted plasma clots but was nearly absent in normal pool plasma clots containing FXIII, suggesting factor H may be cross-linked to form higher molecular weigh products (Howes et al. 2012), therefore to determine whether factor H (procured from Quidel) was cross-linked to itself or to fibrin during clot formation, cross-linking assays were set up using purified proteins. To determine whether factor H forms cross-links with itself, factor H bands were monitored by SDS-PAGE and Western blotting with factor H antibody after an incubation of 4 hours in the presence and absence of FXIIIa. As shown in Figure 48, panel B there was no difference between the high molecular weight smearing/bands between purified factor H alone and purified factor H incubated with FXIIIa. As a positive control fibrinogen was analysed under the same conditions. As shown in Figure 48, panels A and C that in the presence of FXIIIa, fibrinogen was cross-linked to form γ-dimers and numerous α-polymers, with high molecular weight products present in the loading wells. Fibrin and factor H incubated together in the presence of FXIIIa for 4 hours produced the same factor H high molecular weight bands/smearing compared to in the absence of fibrinogen (Figure 48, Panels B). Figure 48, Panel B also shows that in the presence of thrombin a number of factor H containing lower molecular weight bands were produced, suggesting either that factor H was cleaved by thrombin or factor H was degraded during storage.

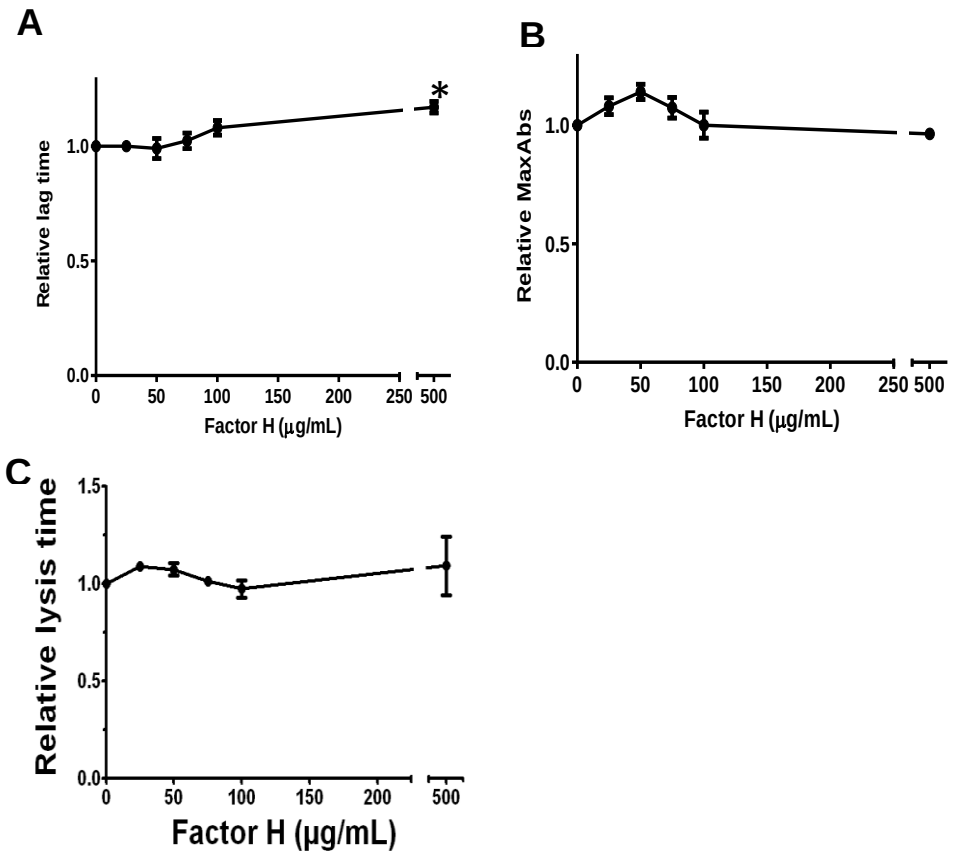

Figure 47: Evaluating the influence of factor H on fibrin formation and lysis.

Using purified proteins concurrent clot formation and lysis was measured in a turbidimetric assay at 340 nm. Relative lag time, maximum absorbance (MaxAbs) and lysis time were calculated as the normalised data to no factor H. **Panel A**: Lag time was increased at supraphysiological concentrations of factor H. **Panels B & C**: Maximum absorbance and lysis time were unaffected by factor H. Three independent experiments were performed (SEM). **P* = <0.05 after Bonferroni adjustment when comparing +/- factor H.

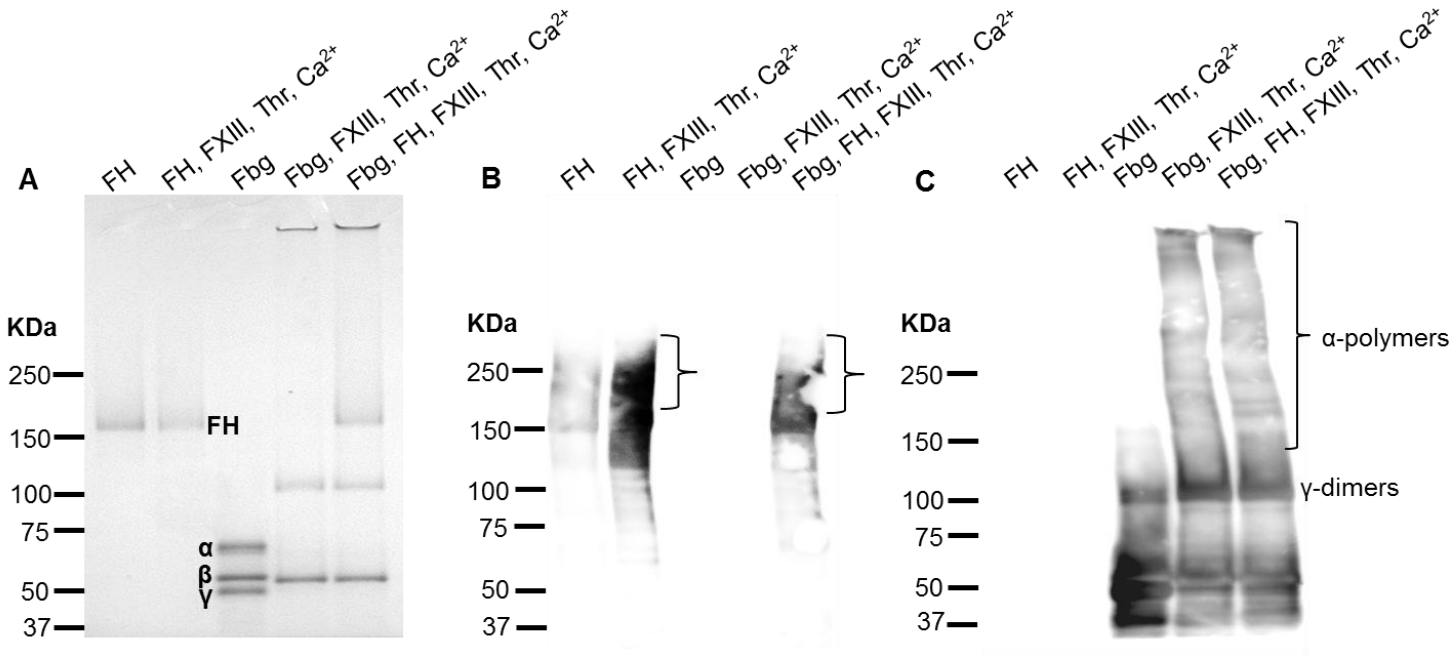

Figure 48: Analysis of factor H cross-linking to fibrin in a purified system.

Factor H and fibrinogen (Fbg) were incubated for 4 hours with thrombin (Thr), calcium (Ca^{2+}) and FXIII and analysed by Coomassie stained SDS-PAGE (**Panel A**) and Western blot with anti-factor H (**Panel B**) and anti-fibrinogen (**Panel C**) antibodies. Mean of two independent experiments. Parentheses indicate high molecular weight factor H products which appear to be present in the purified factor H and are unlikely to be FXIII-dependent cross-linked products.Numerous lower molecular weight cleavage products were also observed when factor H was incubated with thrombin.

5.4.2. Cross-linking of factor H to plasma clot components

Using purified proteins factor H was not cross-linked to fibrin, therefore to determine whether factor H was cross-linked to plasma proteins, plasma clots perfused with either TBS to remove non-bound proteins or with 2M NaCl to remove bound proteins were assessed by Western blot for factor H cross-linking (Figure 49). Factor H was observed in the TBS wash, and to a lesser degree in the NaCl wash, with no high molecular weight bands present in either the TBS or NaCl washed plasma clots (Figure 49, Panel A), suggesting that in plasma factor H was not cross-linked, however was capable of binding plasma clot components. As a positive control α2-AP was analysed in the same plasma clots. No bands were observed at the molecular weight of α2-AP; however, numerous high molecular weight bands were observed in both the TBS and NaCl wash (Figure 49, Panel B) suggesting α2-AP was cross-linked. Combined these results suggest factor H was cross-linked to form homodimers and factor H binds to plasma clot components.

5.5. Analysis of factor H cleavage by thrombin and plasmin

In a purified system factor H did not affect fibrin structure (maximum absorbance) or fibrinolysis, but did prolong lag time at supraphysiological concentrations of factor H, suggesting thrombin might cleave factor H. Furthermore in the cross-linking experiments low molecular weight bands were observed when factor H was incubated with thrombin and calcium. Clots formed and lysed in the concurrent turbidimetric analysis were analysed by SDS-PAGE and Western blotting to determine whether factor H was cleaved during fibrin clot formation/lysis. As shown in Figure 50 factor H was cleaved resulting in five cleavage products of ~ 140, 100, 70, 45 and 37 KDa, with all five cleavage products increasing in intensity with increasing factor H (25-100 µg/ml).

To determine whether thrombin was responsible for cleavage of factor H, purified factor H (obtained from Quidel) was incubated with increasing concentrations of thrombin for 4 hours and analysed by SDS-PAGE and Western blot. As shown in Figure 51 thrombin cleaves factor H into six products of ~140, 100, ~75, 60, 45 and ~32 KDa; however, the majority of factor H remained in its full

length form even at the highest thrombin concentration. Although these factor H cleavage products were observed in the cross-linking experiments in the presence of thrombin, several other products were also observed suggesting that factor H was also degraded. At the concentration of thrombin (0.025 U/ml) used in the concurrent turbidimetric assay only two of the cleavage products were observed, which suggests factor H was also cleaved by plasmin during fibrinolysis.

Since the lysed fibrin clots containing factor H analysed in Figure 50 also contained plasmin along with thrombin and a number of the bands were not accounted for by thrombin cleavage, purified factor H (obtained from Quidel) was incubated with increasing concentrations of plasmin for 4 hours and analysed for cleavage products by SDS-PAGE and Western blot. Plasmin cleaved factor H into numerous products of ~140, 100, ~75, 60, 45, 37, 24, 20 and 15 KDa. At the highest plasmin concentration the majority of full length factor H was cleaved to lower molecular weight products. This confirms that plasmin also cleaves factor H, however many of the cleavage products observed here were not observed in lysed cross-linked fibrin clots containing factor H, suggesting plasmin may have a higher affinity for cleaving fibrin rather than factor H, which may account for why no difference in fibrinolysis time was observed in the presence of increasing factor H concentrations.

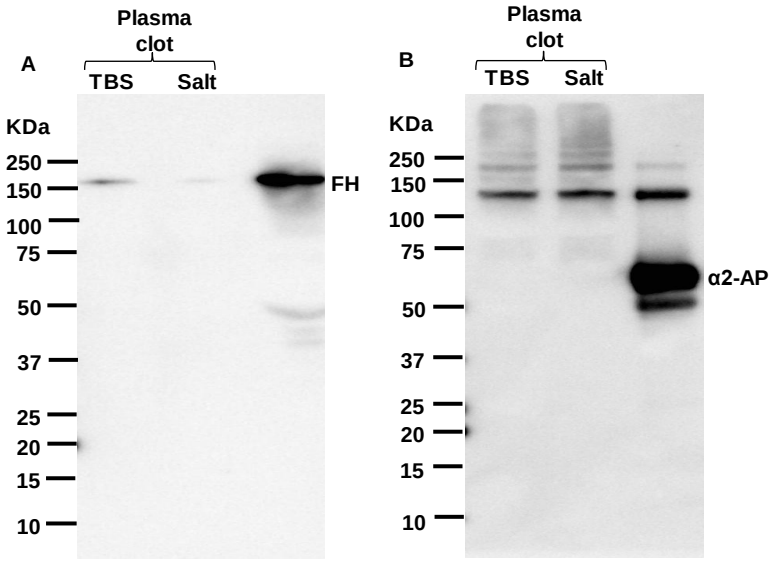

Figure 49: Analysis of factor H cross-linking in plasma clots.

Plasma clots formed in a humidified chamber were washed with either Tris buffered saline (TBS) or a 2 M NaCl wash for 4 hours. Plasma clots were solubilised and analysed by Western blot with anti-factor H (**Panel A**) and anti-α2-AP (**Panel B**) antibodies. Purified factor H (FH) and α2-antiplasmin (α2-AP) were included as controls. Western blots representative of two independent experiments. Western blots show that factor H was not cross-linked but does bind plasma clot components, whereas α2-AP was cross-linked forming high molecular weight products.

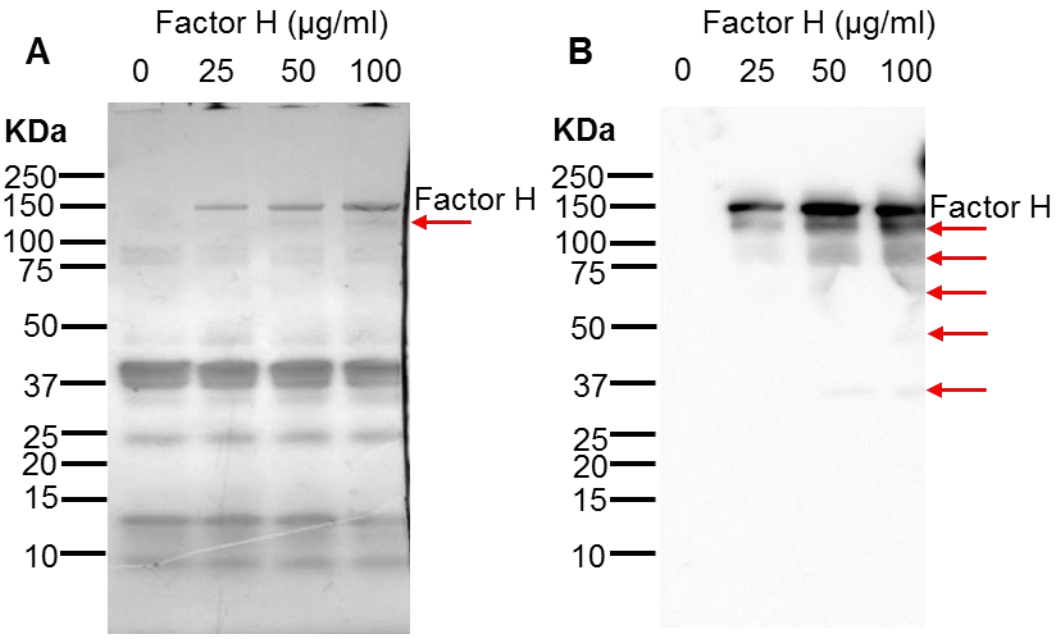

Figure 50: Concentration dependent cleavage of factor H within purified fibrin clots.

Purified clots produced during the concurrent turbidimetric and lysis analysis were analysed for factor H cleavage products by Coomassie stained SDS-PAGE (**Panel A**) and Western blot with an anti-factor H antibody (**Panel B**). Arrows indicate cleavage of factor H with products of ~ 140, 100, 70, 45 and 37 KDa.

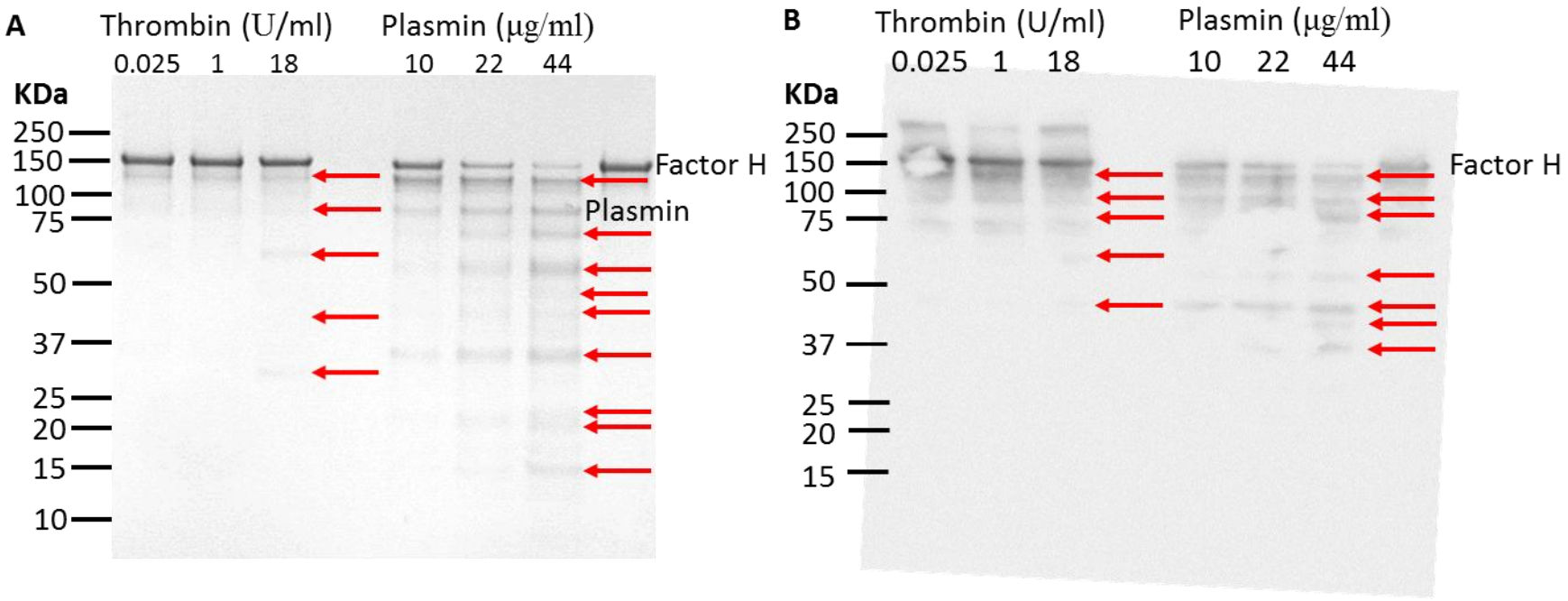

Figure 51: Cleavage of factor H by thrombin and plasmin.

Purified factor H was incubated with thrombin or plasmin for 4 hours at 37°C. The resulting cleavage products were analysed by Coomassie stained SDS-PAGE (**Panel A**) and Western blot with an anti-factor H antibody (**Panel B**). Arrows indicate cleavage products of factor H of ~140, 100, ~75, 60, 45, 37, ~32, 24, 20 and 15 KDa by thrombin and plasmin.

5.6. Discussion

Factor H was identified by mass spectrometry in perfused solubilised plasma clots by Dr Howes (Appendix A). To determine the amount of factor H incorporated into plasma clots during clot formation, paired plasma and plasma-derived serum were compared using ELISA. Eleven percent of plasma factor H was incorporated into a plasma clot, which was well below the 25% incorporation of C3 previously determined in the same plasma samples (Howes et al. 2012) and also below the level of α2-antiplasmin incorporation of 24-42% (Carter et al. 1999;Cederholm-Williams 1981;Sakata and Aoki 1980), which is known to be actively incorporated into fibrin clots by FXIII-dependent cross-linking (Fraser et al. 2011;Ichinose et al. 1983;Kimura et al. 1985a;Kimura and Aoki 1986). However as incorporation of factor H varied widely between individuals it may suggest that factor H was not actively being incorporated during clot formation but instead becomes trapped as a result of clot formation or it may play an active role in fibrin structure and function.

Several studies have investigated the role of the factor H polymorphism Tyr402His in CVD risk, with contradictory results (Kardys et al. 2006;Koeijvoets et al. 2009;Pai et al. 2007;Zee et al. 2006) Factor H can also be found in atherosclerotic lesions, with reduced levels present in areas containing C5b-9 (Seifert et al. 1989). In the superficial layer of early atherosclerotic plaques factor H binds to proteoglycans, thus limiting complement activation at the C3 convertase stage, whereas deeper in the intima factor H was absent enabling full activation of complement cascade and the generation of C5b-9 (Oksjoki et al. 2003), suggesting factor H plays a protective role in atherosclerosis. Correlation analyses were therefore performed between factor H and conventional risk factors for T2DM and CVD in the South Asian study group. In both the South Asian controls and South Asian relatives, factor H was positively correlated with BMI and insulin. Moreno-Navarrete et al (2010) also found that circulating levels of factor H expressed in omental fat deposits were associated with obesity, insulin resistance and metabolic parameters. Weight loss or improved insulin resistance caused a decrease in factor H levels (Moreno-Navarrete et al. 2010), which suggests that factor H was upregulated to help prevent uncontrolled alternative pathway activation observed in obesity and diabetes. Factor H was also secreted from pancreatic β cells and, as a result, inhibits insulin secretion when combined with

adrenomedullin, which could suggest that factor H acts as a chaperone protein for adrenomedullin in the regulation of insulin secretion (Martinez et al. 2001). Factor H was also positively correlated with the acute phase reactants CRP and fibrinogen in both South Asian controls and South Asian relatives, which might suggest underlying inflammation in these individuals.

Factor H is a regulator of the alternative complement pathway by acting as a cofactor for factor I in the cleavage of C3b to iC3b, aiding dissociation of the C3 convertase and competing with factor B for C3b binding (de Cordoba and de Jorge 2008). As a result, reduced levels of factor H may lead to increased levels of complement activation products. Therefore, factor H levels in South Asian relatives and controls were investigated to account for the increase in the complement activation product soluble C5b-9. However, no difference was observed between the South Asian controls and South Asian relatives, suggesting that inhibition of the alternative complement pathway by factor H was unaffected. This might suggest that reduced expression or function of other complement inhibitors contributes to the increased soluble C5b-9. For example the membrane-bound complement inhibitors CD55 and CD59 are responsible for preventing the assembly of the alternative pathway C3 convertase and the membrane attack complex respectively. In diabetic individuals CD55 and CD59 expression is down-regulated in peripheral blood leukocytes (Ma et al. 2009). Inactivation of CD59 by glycation leads to uncontrolled deposition of the membrane attack complex (Qin et al. 2004), suggesting that down-regulation of these inhibitors makes them likely candidates for the increase in soluble C5b-9 observed in the South Asian relatives. Factor H was positively correlated with zymogen complement components C3, properdin and factor B in both South Asian controls and South Asian relatives. This might reflect the general synthesis of acute phase reactants (complement C3 and factor B) by the liver as a result of TNFα stimulation of gene expression (Perlmutter et al. 1986). Factor H was negatively correlated with soluble C5b-9 in South Asian controls only; this was expected, as a change in the amount of inhibitor would reflect an opposite change in the amount of activation product and suggests normal control of complment activation in South Asian controls.

Fibrin formation and final fibrin structure has been found to be a major determinant in CVD risk. As patients with MI have altered fibrin architecture, with

stiffer fibrin clots and smaller pores, decreased permeability and slower lysis times (Collet et al. 2006;Fatah et al. 1996). Furthermore the fibrin structure of plasma clots significantly differs to purified fibrin clots (Carr 1988) suggesting plasma proteins contribute to the alterations in fibrin structure. To determine whether factor H alters fibrin formation and structure, turbidimetric parameters were compared across factor H quartiles in the South Asian study group. With increasing quartiles of factor H, the time for fibrin protofibril formation was shorter and the final fibrin structure was denser. In previous studies, increased fibrin density was associated with prolonged fibrinolysis (Collet et al. 2000;Collet et al. 2006), however in the present study there was no significant difference in lysis time between the lowest and highest quartiles of factor H, although in correlation analyses factor H correlated with inceased fibrin clot density and increased lysis times in the South Asian controls. Factor H was negatively correlated with time required for protofibril formation and positively correlated with fibrin density in South Asian controls and relatives, however after accounting for age, sex, fibrinogen and PAI-1, factor H was independently associated with maximum absorbance, 50% lysis time and area under the lysis curve in healthy individuals only, suggesting factor H may influence fibrin formation and lysis parameters. However these analyses can not exclude the affect of other haemostatic determinants which may affect fibrin formation and lysis.

To determine whether factor H plays a functional role in fibrin clot formation a turbidimetric assay was performed using purified proteins. Factor H was found not to influence the fibrin clot formation and fibrinolysis parameters investigated under physiologically relevant concentrations, suggesting that clot structure was unaffected by factor H. However, factor H present at supraphysiological concentrations significantly prolonged the time taken for protofibril formation (lag time), one possibility for this increase in lag time was the observation that factor H was cleaved by thrombin in the present study and by a previous study (Ohtsuka et al. 1993). As factor H was cleaved by thrombin, it might suggest that lag times should be affected by increased factor H over the physiological range; however, this was not observed. This could be due to the relatively low concentration of thrombin present in the reaction as well as the addition of fibrinogen and FXIII, which are major (and likely preferred) substrates for thrombin. These findings in the purified system suggest that factor H plays no functional role in fibrin formation and structure.

Previously incorporated proteins such as α2-AP have been found to be actively cross-linked to fibrin clots by FXIIIa (Fraser et al. 2011;Ichinose et al. 1983;Kimura et al. 1985a;Kimura et al. 1986). Therefore to determine whether factor H was actively incorporated into fibrin clots by FXIIIa-dependent cross-linking, cross-linking in purified and plasma based fibrin clots was investigated by SDS-PAGE and Western blot. Factor H incubated with FXIIIa in the absence of fibrin produced no additional high molecular weight products compared to purified factor H aloneThe same banding pattern was observed when factor H was incubated with fibrin in the presence of FXIIIa. No additional high molecular weight cross-linked bands were observed above 300 KDa, suggesting factor H was not cross-linked to high molecular weight fibrin α-chain products. Factor H has previously been identified to form non-covalent dimers and oligomers in plasma, which may account for the high molecular weight products observed in the purified factor H. Interestingly, recombinant fragments of factor H; SCR 6-8 and SCR 16-20 form dimers (Nan et al. 2008;Okemefuna et al. 2008) with the polymorphism His402 in SCR 6-8, promoting dimer formation (Fernando et al. 2007). Pangburn et al (2009) found that factor H dimer and oligomer formation was promoted in the presence of heparin and dextran sulphate. Factor H oligomer formation promoted binding to C3b, which resulted in increased cofactor activity and decay acceleration and therefore suppression of complement activation (Pangburn et al. 2009). These studies highlight the possibility that factor H and sections of the SCR which form dimers or oligomers, which function in complement regulation.

Similar to the purified cross-linking experiments, the perfused plasma clots were also incubated for 4 hours; however no factor H homodimers or cross-linking between cleavage products were observed, unlike the positive control α2-antiplasmin, which was actively cross-linked within plasma clots to provide protection against fibrinolysis (Kimura et al. 1985a;Kimura et al. 1985b;Sakata and Aoki 1982). This might suggest that in a complex mix of proteins such as plasma, factor H may have to compete with other substrates of FXIII for cross-linking. Alternatively since factor H was not cross-linked to fibrin, it might be lost from the plasma clots during perfusion. A small amount of factor H remained in the washed plasma clots, suggesting that although factor H was not actively cross-linked to fibrin, it was possible that factor H can bind to a plasma clot component. One

possibility may be that factor H may bind to fibrin as factor H contains structural homology with the coagulation protein FXIII B chain (Reycampos et al. 1990). FXIII B chain was found to bind fibrinogen via the γ' chain (Siebenlist et al. 1996) and the αC region (Smith et al. 2011), allowing the transport of FXIII to the site of active fibrin clot formation. If binding of factor H to fibrin were to occur then this would localise factor H to the site of coagulation and therefore the site of complement activation however binding studies are required in the future to confirm factor H binding to fibrin.

As a number of low molecular weight products were present in the cross-linking analyses, cleavage of factor H within fibrin clots was investigated. Analysis of lysed fibrin clots by SDS-PAGE showed that a small amount of factor H was cleaved under these reaction conditions. To determine whether this was due to thrombin or plasmin purified factor H was incubated with increasing concentrations of these enzymes. Thrombin cleaved factor H to form six products of ~140, 100, ~75, 60, 45 and ~32 KDa; with factor H cleavage occurring at a thrombin concentration as low as 0.025 µg/ml, suggesting that prolongation of lag time at supraphysiological concentrations of factor H could be due to thrombin cleavage. However, the majority of the factor H remained intact after 4 hours even at the highest factor H concentration of 18 µg/ml. Ohtsuka et al (1993) found that factor H was cleaved by thrombin forming one product of similar molecular weight to intact factor H after 5 minutes with the majority cleaved after 2 hours by high-performance liquid chromatography. As the amino terminus was the same as intact factor H, Ohtsuka et al (1993) suggested that the carboxyl terminus was the site of cleavage, in the formation of a hydrophobic factor H molecule with monocyte chemotactic activity (Ohtsuka et al. 1993). As the molecular weight of this hydrophobic factor H molecule and intact factor H appear the same it may account for why the majority of factor H appeared to be uncleaved after 4 hours by SDS-PAGE and Western blot in the present study. The additional cleavage products of factor H may be due to the high thrombin concentration (18 µg/ml compared with 5 µg/ml used by Ohtsuka et al. 1993) and longer incubation times used in the cleavage experiment.

Factor H was also cleaved in the present study by plasmin producing numerous products (~140, ~80, 60, 37, 24, 20 and 15 KDa). A similar cleavage pattern was observed with trypsin cleavage of factor H by Sim et al (1982) who

demonstrated within 25 minutes trypsin cleaved factor H to products of 141 and 34 KDa, whereas after 90 minutes trypsin further cleaved the 141 KDa chain to form products of 88, 26 and 24 KDa (Sim and Discipio 1982). Fontaine et al (1989) found that plasmin cleaved plasma factor H to form truncated factor H products on reduced gels of 43 and 40 KDa (Fontaine et al. 1989). In the presence of fibrin clots five factor H cleavage products were observed in the present study; three of the same cleavage products were observed after thrombin (0.025 μg/ml) cleavage of purified factor H, whereas all five cleavage products were observed after plasmin cleavage of purified factor H, suggesting that cleavage was the result of plasmin and thrombin proteolysis.

In the case of factor H, cleavage by thrombin results in a cleavage product which has monocyte chemotactic activity, whilst maintaining the inhibitory activity of intact factor H for C3bBb (C3 convertase) and C3b in the production of iC3b (Ohtsuka et al. 1993). Ohtsuka et al (1993) did not show the same product after plasmin cleavage of factor H; however, factor H fragmentation resulting from plasmin cleavage of factor H was previously identified in plasma and these fragments also retained cofactor activity for C3b (Fontaine et al. 1989;Sim et al. 1982). Factor H cleavage either as the result of thrombin and/or plasmin occurred in fibrin clots; however, this did not affect lag or lysis times over the physiological range, suggesting incorporated factor H may play an important role in controlling complement activation and the immune response within the clot rather than modulating clot structure or function. The function of factor H in the inhibition of complement activation within whole blood clots was highlighted by the ability of factor H to bind to activated platelets via receptors GPIIb/IIIa or thrombospondin-1 (Vaziri-Sani et al. 2005), and preventing C3 activation by aiding factor I mediated cleavage of C3 (Hamad et al. 2010). Without this control in complement activation by factor H on the surface of platelets, full complement activation and the formation of the membrane attack complex would lead to stimulation of α-granule release of FV and promote a pro-thrombotic state (Wiedmer et al. 1986). In patients with aHUS, platelet surface levels of factor H are significantly decreased; resulting in complement component deposition and platelet microparticle release (Stahl et al. 2008). As platelet microparticles are known to be pro-thrombotic (Keuren et al. 2006), this study suggests a protective role of factor H in thrombosis. The importance

of factor H in controlling complement within a clot was highlighted by the ability of thrombin and plasmin cleaved factor H to still retain cofactor activity for cleavage of C3b by factor I (Fontaine et al. 1989;Ohtsuka et al. 1993;Sim et al. 1982). Thrombin cleaved factor H may further aid the immune response by chemotaxis of monocytes for pathogen removal by phagocytosis (Ohtsuka et al. 1993). Since the present study has shown that factor H does not affect fibrin structure or function, it suggests factor H plays a regulatory role within the clot in regards to complement activation.

In summary, the present study confirmed that factor H was a plasma clot component, and showed that only small amounts were incorporated into plasma clots. The present study has shown that levels of factor H are not significantly different between individuals with a family history of T2DM and healthy controls suggesting complement activation in these individuals was the result of down-regulation of alternative complement inhibitors; however the correlation between factor H and acute phase reactants suggest factor H was a marker of ongoing inflammation in these individuals. In plasma clots factor H was associated with increased clot density and prolonged lysis in healthy individuals, however the in vitro data suggests factor H plays minor roles in fibrin formation and overall fibrin structure and lysis even though factor H was found to be cross-linked and cleaved by thrombin and plasmin. Previous studies have shown that factor H oligomer formation, and cleavage products of factor H remain functionally active, suggesting factor H may play a protective role in fibrin clots by preventing uncontrolled complement activation rather than modulating fibrin clot structure and function.

Chapter 6 Identification of novel FXIII substrates using a functional proteomics approach

In several recent studies the cellular substrates of tissue transglutaminase have been identified using BP, the biotin labelled lysine analogue commonly utilised to identify reactive glutamine residues (Fleckenstein et al. 2004;Orru et al. 2003). In this study, the method was adapted to identify novel substrates of FXIII in plasma as summarized in Figure 52. Initially, BP was cross-linked to proteins in fibrinogen depleted plasma by FXIIIa. The biotin labelled plasma proteins were purified from non-biotin labelled plasma proteins by avidin affinity chromatography prior to MS identification of the parent protein and reactive glutamine residues.

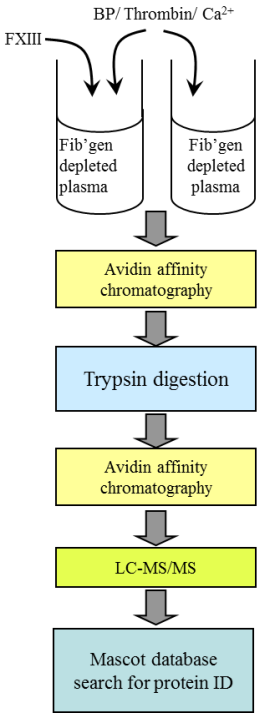

Figure 52: Proposed method for the identification of novel FXIII substrates in plasma

BP was incubated in fibrinogen depleted plasma in the presence and absence of FXIII. BP labelled proteins were separated from non-BP labelled proteins by avidin affinity chromatography prior to MS identification of the parent protein. Reactive glutamine residues were identified by MS following an additional avidin affinity chromatography of the BP containing peptides.

6.1. FXIII-dependent cross-linking of BP into plasma proteins

6.1.1. Optimisation of the BP cross-linking reaction in normal pool plasma

Cross-linking of BP into plasma proteins by FXIIIa was assessed by Western blotting with streptavidin HRP in the following sections. Initial optimisation experiments were performed to determine: (1) the optimum concentration of BP and (2) the optimum concentration of FXIII, to maximise incorporation of BP and to minimise the number of protein-protein cross-links. Normal pooled plasma was initially used for these optimisation steps to enable monitoring of FXIII-dependent cross-linking of fibrinogen, therefore allowing the assessment of protein-protein cross-link formation.

Cross-linking of BP into plasma proteins was found to be concentration dependent (Figure 53, Panel A) with increased band intensity with increasing BP. At 10 mM and 25 mM BP a number of high molecular weight bands were observed, suggesting protein-protein cross-linking was still taking place. These protein-protein cross-links were confirmed to be fibrin cross-linking bands as shown in Figure 53, panel B. To determine whether FXIII concentration was a rate-limiting factor in the cross-linking reactions, 100-fold excess and 1000-fold excess FXIII (plasma concentration of FXIII, 0.022 mg/ml) relative to the concentration of other plasma proteins in the reaction were compared. Although the banding pattern for both FXIII concentrations were similar, there appeared to be a greater amount of protein-protein cross-links formed especially in the presence of either 10 mM or 25 mM BP (Figure 53, Panel A), which were confirmed as protein-protein cross-links in the fibrinogen Western blot (Figure 53, Panel B).

To enable maximal cross-linking in reactions, samples were required to contain excess BP; therefore BP was removed from the samples by centrifugation using a 3 KDa molecular weight cut off spin column. The flow-through was incubated with FXIII, thrombin and calcium on fibrinogen coated plates and cross-linking of residual BP was assessed by streptavidin alkaline phosphatase. As shown in Figure 53, Panel C in all of the reactions, BP was present in excess; with residual BP cross-linking onto the fibrinogen coated plates in a concentration dependent manner. The amount of excess BP was independent of FXIII concentration (Figure

53, Panel C). Final concentrations of 100 mM BP and 0.022 mg/ml FXIII were chosen for subsequent analyses.

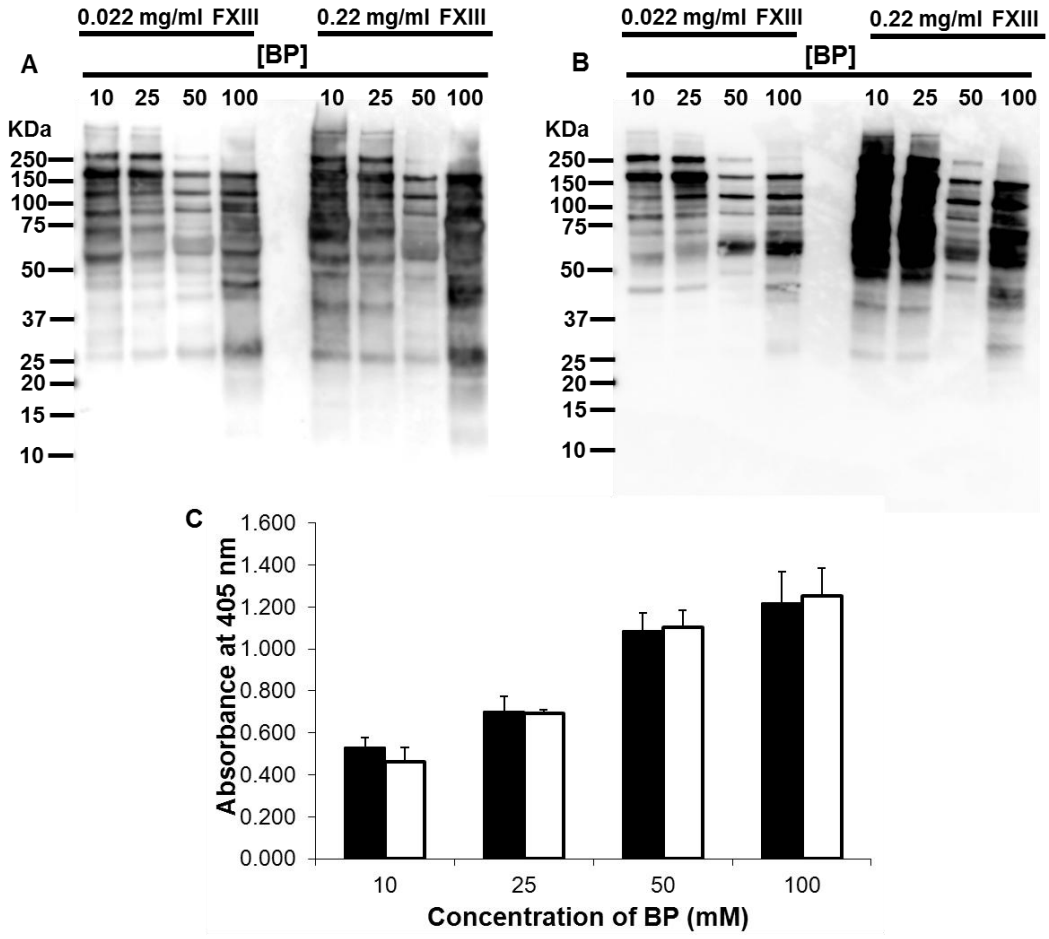

Figure 53: Concentration dependent cross-linking of BP to plasma proteins

BP (10-100 mM) was incorporated into normal pooled plasma in the presence of 0.022 mg/ml or 0.22 mg/ml FXIII as analysed by streptavidin HRP Western blot (**Panel A**). High molecular weight fibrin cross-linked products were confirmed by anti-fibrinogen (**Panel B**) Western blot. **Panel C.** Analysis of excess BP by FXIII activity assay. Excess BP from the cross-linking reaction with 0.022 mg/ml FXIII (black) and 0.22 mg/ml FXIII (white) was removed by de-salting spin column (Millipore) and analysed by FXIII activity assay, with concentration-dependent cross-linking of BP to fibrinogen coated plates. Data presented as mean of triplicate experiments (SEM).

6.1.2. Optimisation of the incubation time for the BP cross-linking reaction in fibrinogen depleted plasma

The optimum concentration of BP and FXIII were determined in normal pooled plasma; however, as fibrinogen is a major substrate of FXIII and is relatively abundant in plasma (~ 2 mg/ml), fibrinogen depleted plasma was used to facilitate the identification of less abundant FXIII substrates. Fibrinogen depleted plasma was confirmed to be fibrinogen free by the Clauss fibrinogen assay, which found no clotting after 30 minutes. Diluted normal pooled plasma and fibrinogen depleted plasma (1 in 100 dilution) were also examined by Western blot for the presence of fibrinogen. Although a small amount of fibrinogen was present in the fibrinogen depleted plasma, this was significantly less than that observed in normal pooled plasma (Figure 54). These experiments confirm that fibrinogen was almost completely depleted and residual fibrinogen was insufficient for clot formation.

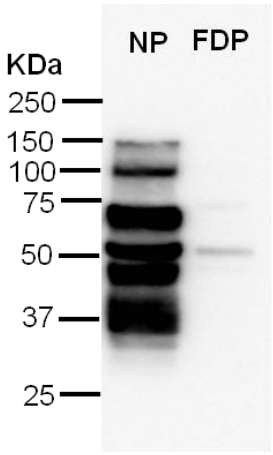

Figure 54: Depletion of fibrinogen in fibrinogen depleted plasma

Western blot (anti-fibrinogen antibody) of normal pooled plasma (NP) and fibrinogen depleted plasma (FDP) show that FDP contains a small amount of fibrinogen compared to NP.

In the previous optimisation steps described in section 6.1.1 BP cross-linking reactions were incubated for two hours only. Incubation time was optimised in fibrinogen depleted plasma (Figure 55). Although the banding patterns observed between the three time points were similar, there was a greater intensity of

biotinylated proteins after three hours as observed by streptavidin HRP, with no additional increase in band intensity after four hours. This experiment also confirmed the use of 100 mM BP in fibrinogen depleted plasma with the greatest intensity of biotinylated proteins (Figure 55, Panel A). Excess BP was also assessed by FXIII activity assay. At all three time points BP was in excess (Figure 55, Panel B); however, with increasing time a reduced amount of BP at 50 mM and 75 mM BP was observed. At 100 mM BP the levels remained similar over the three incubation time points, thus confirming the use of 100 mM BP in fibrinogen depleted plasma. The optimum incubation time of three hours was chosen for subsequent experiments.

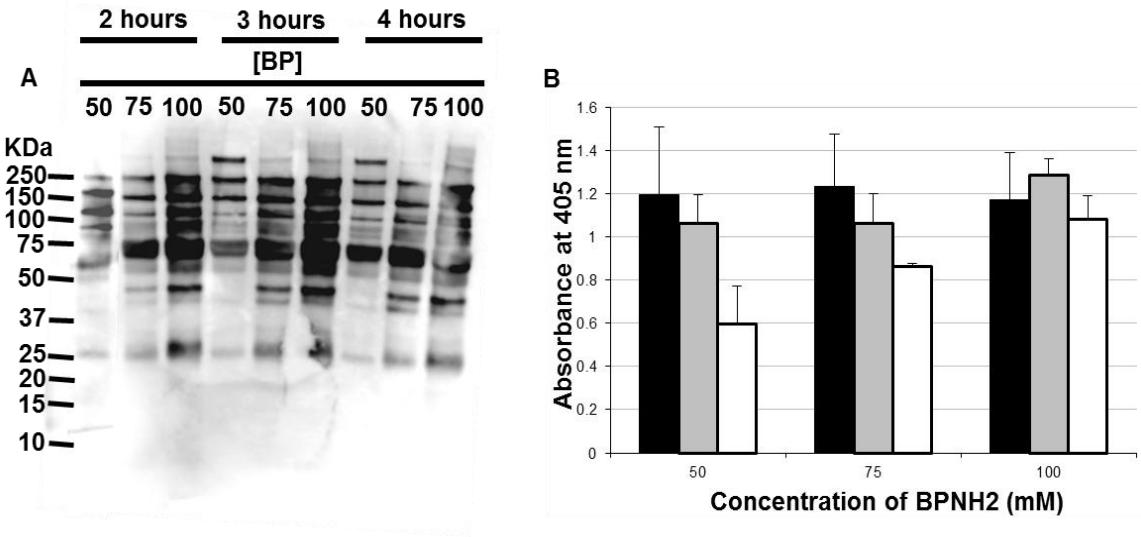

Figure 55: Time dependent cross-linking of BP to FXIII substrates in fibrinogen depleted plasma.

Panel A. Streptavidin HRP Western blot of BP (50-100 mM) incorporation into plasma proteins by FXIIIa over time. Three hours was considered the optimum incubation time. **Panel B**. Excess BP from the cross-linking reaction at 2 hours (black) 3 hours (grey) 4 hours (white) was removed by de-salting spin column (Millipore) and analysed by FXIII activity assay. Reduced excess BP was observed with increasing incubation times in the presence of 50-75 mM BP with no difference in excess BP in the presence of 100 mM. Data presented as mean of triplicate experiments (SEM).

6.1.3. Establishment of the positive and negative controls for mass spectrometry analysis.

Once the optimal conditions were established, the negative and positive controls were assessed by streptavidin HRP for BP incorporation. FXIII-B subunit binds to the alternatively spliced variant of γ-fibrinogen, γ' (Siebenlist et al. 1996), which represents up to ~15% of plasma fibrinogen (Moaddel et al. 2000). FXIII-A2B2 also binds to the αC region of fibrinogen specifically between residues 371-425 (Smith et al. 2011). These findings confirm a proposal that FXIII might circulate in plasma bound to fibrinogen (Greenberg and Shuman 1982), suggesting a possibility that fibrinogen depleted plasma would also be depleted of FXIII. To test this theory BP incorporation into plasma proteins was compared in the presence and absence of exogenous FXIII. Biotinylated plasma proteins were observed under both conditions (Figure 56, Panel A). Western blotting therefore confirmed that fibrinogen depleted plasma was not free of FXIII or other transglutaminases such as tissue trasglutaminase (TG2) and was therefore not a suitable negative control for MS analyses. As a negative control for the non-specific interactions with the avidin affinity chromatography column, fibrinogen depleted plasma incubated in the absence of BP was assessed. As expected, no bands in either the normal pooled plasma, fibrinogen depleted plasma or purified fibrinogen were observed in the absence of BP (Figure 56, Panels A and B).

As a positive control for the proteomic investigation, purified fibrinogen was used, as the cross-linking sites have been well characterised by previous investigators. Cross-linking of BP occurred in the fibrinogen α and γ chains, with a small amount of biotinylation in the fibrinogen β chain (Figure 56, Panel B). BP was also cross-linked to a small number of high molecular weight proteins (Figure 56, Panel B), which corresponded to BP incorporation into high molecular weight protein contamination of either purified fibrinogen or purified FXIII (Figure 56, Panel C, arrows). The positive control of purified fibrinogen and the negative control of fibrinogen depleted plasma in the absence of BP were chosen for subsequent experiments.

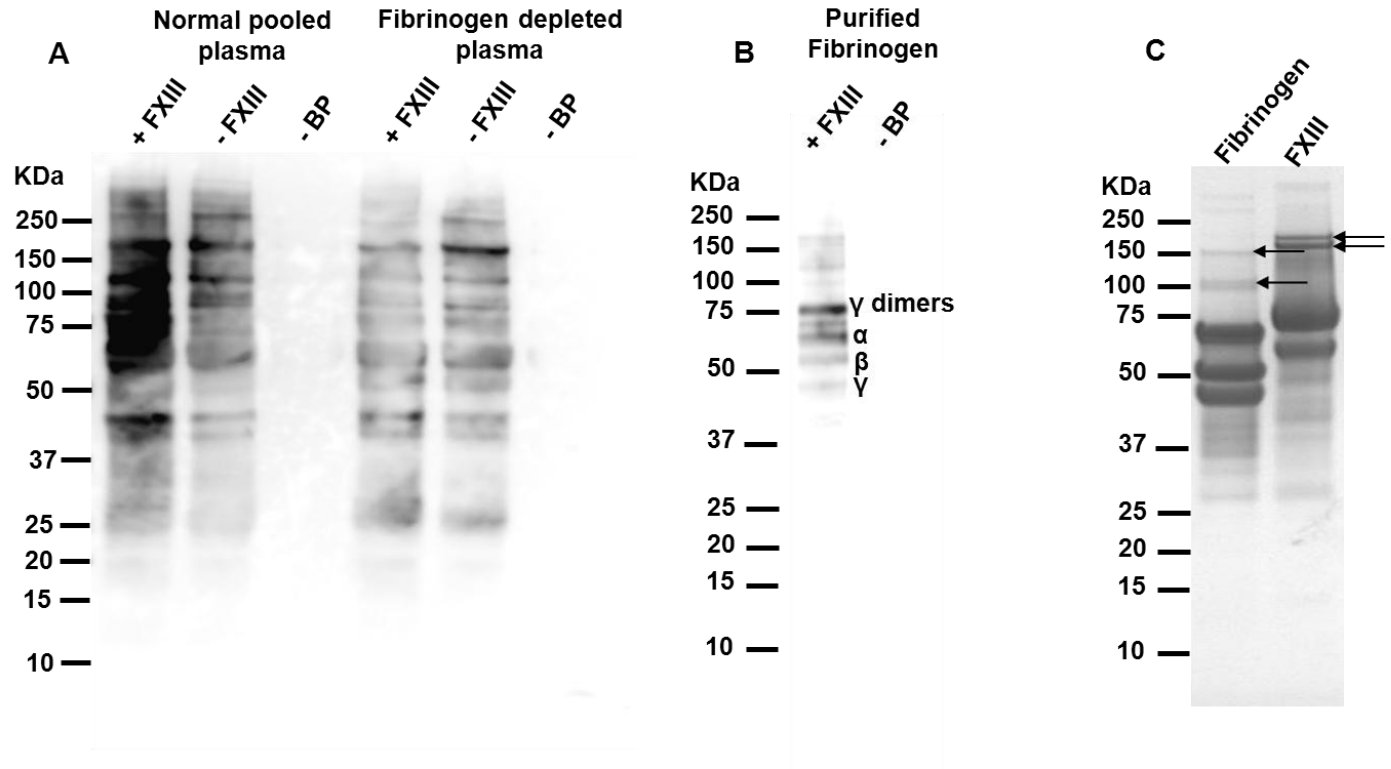

Figure 56: Evaluation of the positive and negative controls for mass spectrometry.

Streptavidin HRP Western blot of BP incorporation into plasma proteins (**Panel A**) and purified fibrinogen (**Panel B**) in the presence and absence of FXIII, with no BP incorporation in the absence of BP. **Panel C**. Coomassie stained gel of purified fibrinogen and purified FXIII. Arrows indicate possible high molecular weight contamination in the fibrinogen and FXIII preparations that could be cross-linked in panel B.

6.2. Purification of BP labelled plasma proteins by monomeric avdin affinity chromatography

To remove BP labelled plasma proteins from non-labelled plasma proteins, monomeric avidin was used due to its ability to bind biotin within the BP. Monomeric avidin was chosen over other avidin techniques due to its high binding capacity, low non-specific binding and reusability.

6.2.1. Removal of free BP by dialysis

Non-incorporated BP can interact with the avidin column, preventing binding of BP labelled proteins to the column and therefore reducing the maximum protein binding capacity of the column. Several methods for removal of free BP were evaluated. Two different dialysis conditions were explored; firstly BP labelled protein samples were dialysed three times for 30 minutes in a total of 3 litres of TBS, secondly for two times 1 hour and overnight in a total of 6 litres of TBS using a 3 KDa molecular weight cut off membrane, and then applied to the avidin column. The protein concentration was monitored at 280 nm. Protein containing fractions were concentrated by acetone precipitation and analysed for the presence of biotinylated proteins by Western blotting with streptavidin HRP. As shown in Figure 57, panels A & C, the majority of the protein was washed from the column in the first few fractions, and these fractions did contain biotinylated plasma proteins (Figure 57, Panels B & D), suggesting insufficient removal of free BP. A small amount of biotinylated protein was eluted from the column during the elution step, fractions 17-26 (Figure 57, Panels C & D), suggesting that a proportion of BP labelled proteins bound to the avidin column, but sufficient free BP prevented the majority of BP labelled protein from binding to the avidin column. These experiments suggest that the samples would require long periods of dialysis and large volumes of buffer for the effective removal of BP and that dialysis was an inefficient method for the removal of free BP from BP labelled proteins.

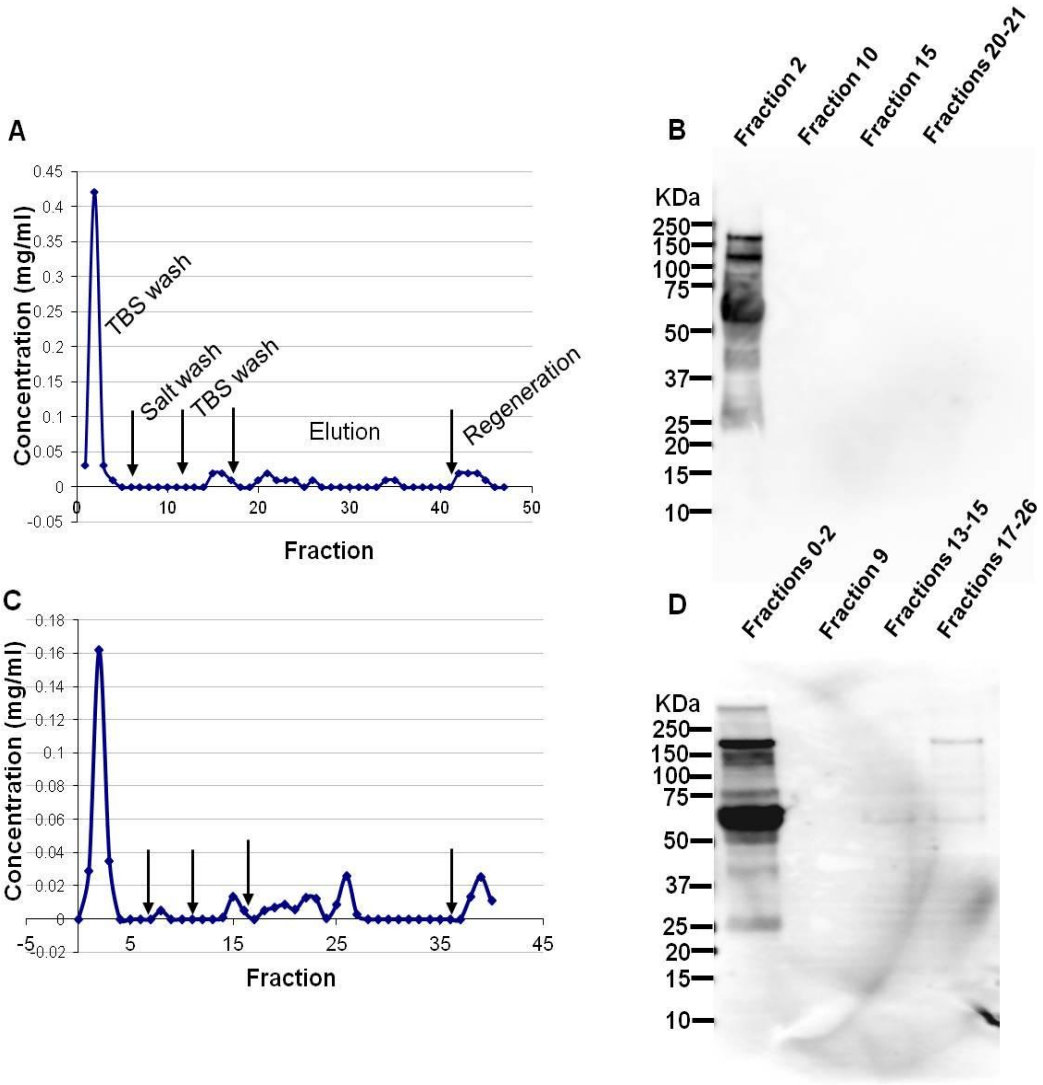

Figure 57: Removal of free BP by dialysis.

Panels A & B. Dialysis in 3 litres of TBS for a total of 90 minutes. **Panels C & D**. Dialysis in 6 litres of TBS for a total of 14 hours. **Panels A & C.** Chromatogram of the monomeric avidin affinity purification shows that the majority of the protein eluted during the first few fractions (arrows represent a change in buffer). **Panels B & D.** Acetone precipitation and analysis of fractions by streptavidin HRP Western blot, confirms that BP labelled proteins were washed from the column in the first few fractions, with a small amount in the elution factions (Panel D) only.

6.2.2. Removal of free BP by gel filtration

Gel filtration separates components via size exclusion, therefore, as BP was relatively small at 328.47 Da compared to plasma proteins, it was expected that this method would separate free BP from the BP labelled proteins. Two different Sephadex G25 columns were evaluated, one of 7 cm by 0.5 cm and one of 7 cm by 2.5 cm as shown in Figure 58. Protein content of the fractions was monitored at 280 nm and BP content of the fractions was monitored by FXIII activity assay. In the first column the elution profiles of BP and protein overlap, with fibrinogen and plasma proteins eluting between fractions 3-13, whereas BP elutes between fractions 6-30 (Figure 58, Panels A & B). In the second column peak separation was improved, with fibrinogen and plasma proteins eluting between fractions 10-20 and BP eluting between fractions 19-45 (Figure 58, Panel C & D). This suggested that the 7 cm by 2.5 cm Sephadex G25 column gave adequate separation for subsequent experiments.

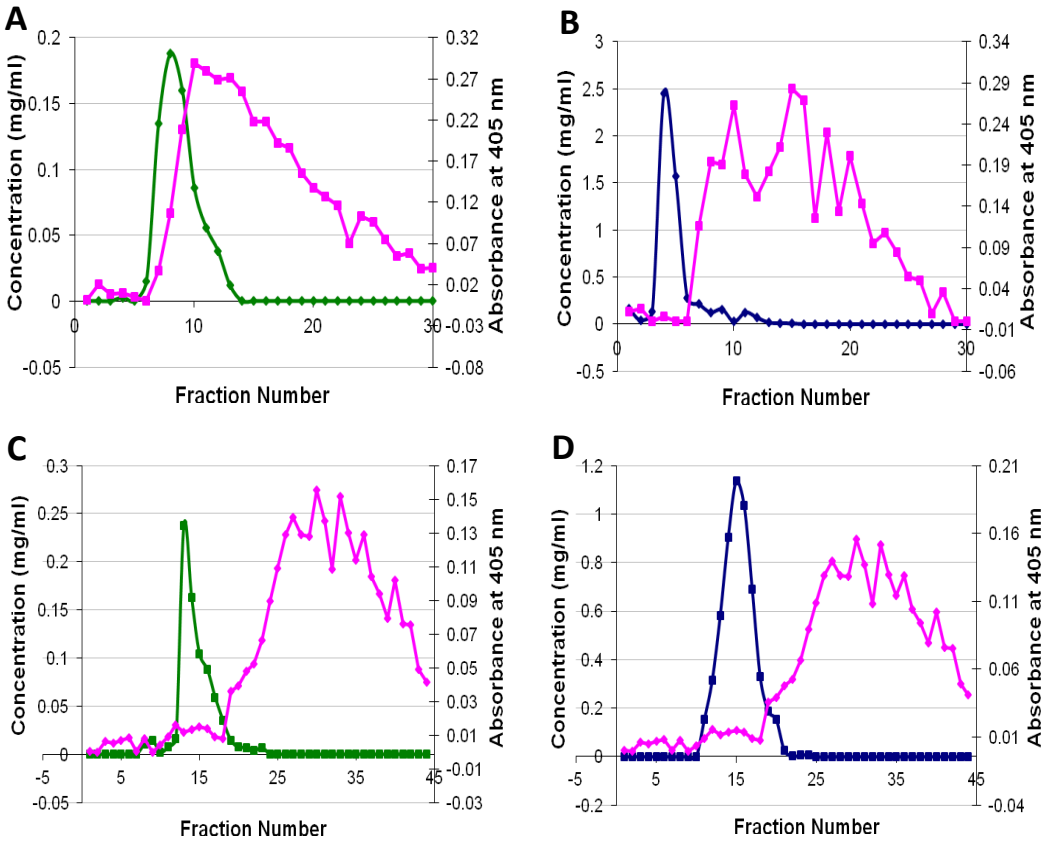

Figure 58: Removal of free BP by gel filtration.

Using a sephadex G25 column of height 7 cm by width 0.5 cm (**Panels A & B**) plasma proteins (blue), purified fibrinogen (green) eluted between fractions 3-13 as monitored at 280 nm, whereas BP (pink) eluted between fractions 6-30 as monitored by FXIII activity assay. Using a sephadex G25 column of height 7 cm by width 2.5 cm (**Panels C & D**) plasma proteins (blue) and purified fibrinogen (green) eluted between fractions 10-20, whereas BP (pink) eluted between fractions 19-45. The sephadex G25 column height 7 cm by width 2.5 cm was used for subsequent experiments.

6.2.3. Purification of BP labelled proteins and negative control by gel filtration and monomeric avidin affinity chromatography

The negative control (fibrinogen depleted plasma incubated with FXIIIa in the absence of BP), positive control (fibrinogen incubated with FXIIIa and BP) and BP labelled fibrinogen depleted plasma samples were purified by gel filtration to remove excess BP label. Although the negative control did not contain BP, it underwent gel filtration to replicate the conditions under which the BP labelled plasma sample were treated. Protein was eluted from the gel filtration column between fractions 9 and 22 (Figure 59, Panel A, C & E) and BP eluted from the gel filtration column between fractions 16 and 45. Fractions 9 to 19 were pooled, concentrated in a 3 KDa molecular weight spin column and applied to the monomeric avidin affinity column. Following an incubation of one hour on the monomeric avidin column, the protein was eluted from the column using the optimised in-house method of purification (Section 3.17.2). In the negative control, the majority of the protein was washed from the column between fractions 1 and 5 with a small amount of protein observed in the salt wash and additional TBS wash step (Figure 59, Panel B). As expected, no protein eluted from the column during the elution or regeneration steps. In the positive control, a small amount of protein was washed from the column between fractions 1 to 15, with the majority eluting during the elution step between fractions 16 and 28 (Figure 59, Panel D). In the BP labelled plasma protein sample, the majority of the protein eluted during the initial wash step between fractions 1 and 8. A smaller amount of protein was eluted from the avidin column during the elution step between fractions 16 and 22 and also during the regeneration step between fractions 23 to 26 (Panel F). The elution fractions from all three samples underwent acetone precipitation and were stored for MS analysis. A small amount of the BP labelled plasma sample was also analysed for BP labelled proteins by Western blot with streptavidin HRP. As shown in Figure 59, panel G the majority of the protein washed from the column during the initial TBS wash was non-biotinylated, with only a small presence of biotinylated proteins. On the other hand, proteins present in the elution fractions were biotinylated, with the greatest amount of biotinylated proteins present in fractions 18-19.

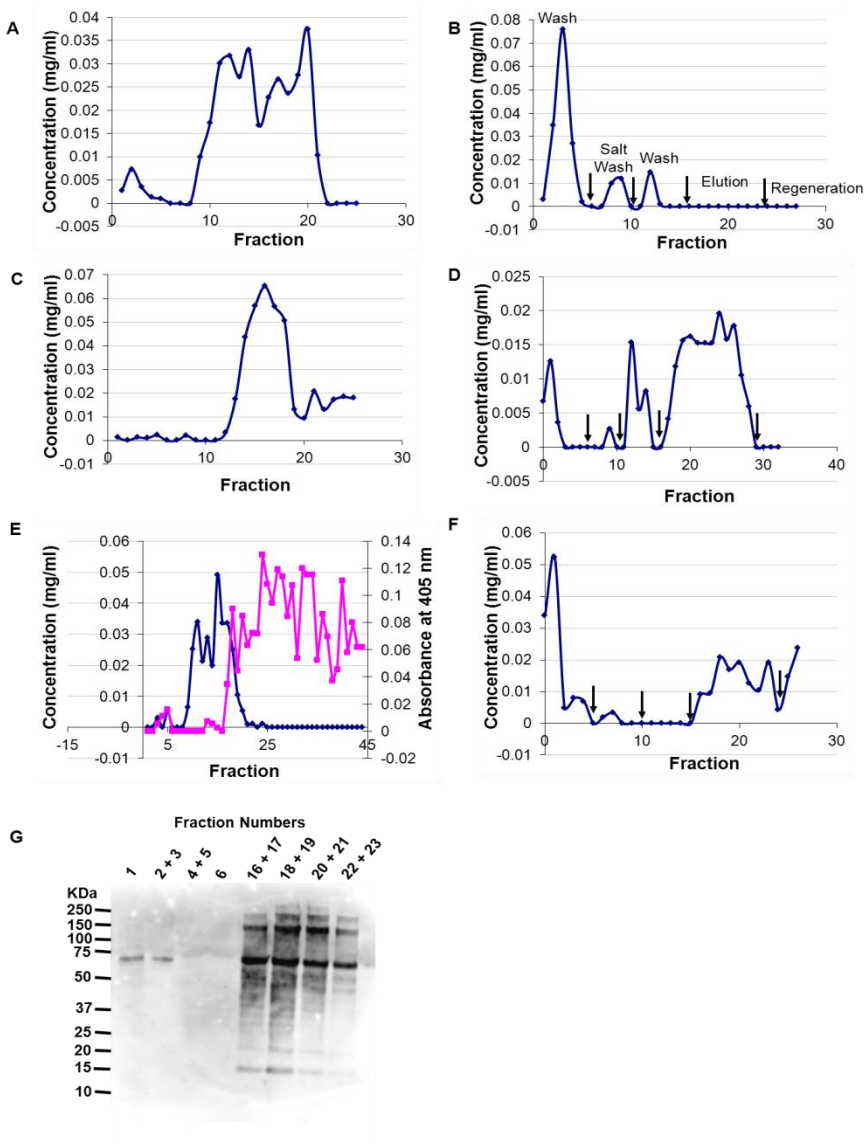

Figure 59: Purification of BP labelled plasma proteins, positive and negative controls by monomeric avidin affinity chromatography

Panels A, C & E: Protein eluted from the gel filtration column monitored at 280 nm (blue) between fractions 9 and 22 and BP eluted from the gel filtration column monitored by FXIII activity assay (pink) between fractions 16 to 45. **Panels B, D & F**: Chromatogram of the avidin affinity column purifications monitored at 280 nm with non-BP labelled proteins removed during the wash steps and BP labelled proteins eluted during the elution fractions. Arrows represent the buffer changes specified in Panel B. **Panels A & B**: Negative control. **Panels C & D**: Positive control. **Panels E & F**: BP labelled plasma proteins. **Panel G**: Streptavidin HRP Western blot of concentrated fractions from Panel F confirm purification of BP labelled proteins

6.3. Identification of BP labelled proteins by mass spectrometry

The acetone precipitated BP-labelled plasma proteins obtained from the avidin affinity column were trypsin digested and analysed by LC-MS/MS to identify both the parent protein and the glutamine site of BP incorporation. The resulting peptide matches were directly analysed in MASCOT to help identify the parent proteins. The negative control was analysed first and, as expected, no proteins were identified. The positive control was then analysed with no detection of fibrinogen, as there was insufficient protein for identification. The biotinylated plasma protein sample was analysed last, with the identification of human serum albumin only (Table 15), however no BP labelled peptides were identified within the human serum albumin. Failure to identify the positive control and any known plasma substrates of FXIII resulted from the strong presence of human serum albumin in the sample and insufficient protein concentration; therefore, the following modifications to the method were evaluated (a) reduce the monomeric avidin column size to help prevent sample dilution (b) increase the protein concentration (c) reduce BP to prevent non-specific incorporation into albumin (d) performing the acetone precipitation at York University immediately prior to MS analysis.

Table 15: Characterisation of BP labelled plasma proteins analysed by LC-MS/MS peptide mass fingerprinting

Protein Identified	Accession number	MW (KDa)	MOWSE Score	Expected value	Number of peptide matches
Human Serum albumin	gi|6013427	70.7	184	3.9e-05	4

*MASCOT MOWSE search score is -10 log (*p*), where *p* is the probability that the observed match is a random event. Search scores >56 indicate identity at the *p*< 0.05 level.

6.4. Further optimisation of the monomeric avidin purification method

To increase the protein yield obtained during monomeric avidin affinity chromatography, several modifications to the previous method were performed including reducing the monomeric avidin column size to prevent sample dilution, increasing the initial protein concentration of the sample and reducing the BP concentration to reduce non-specific binding to albumin.

6.4.1. Establishment of the column size for the purification of BP labelled proteins

To help prevent sample dilution, the column volume of the avidin column was reduced from 2 mls to 0.5 mls in the first instance. To check whether the binding capacity of the avidin column was affected by column volume, biotinylated BSA was applied to the column and protein concentration was monitored at 280 nm. As shown in Figure 60, panel A the majority of the biotinylated BSA was washed from the column between fractions 0 to 5 with a maximum concentration of 0.12 mg/ml in fraction 2. A smaller amount of biotinylated BSA eluted between fractions 7 and 10 during the elution step, suggesting the binding capacity of the column was significantly impaired. The column volume was therefore increased to 1 ml and once again the biotinylated BSA elution was monitored at 280 nm. As shown in Figure 60, panel B the majority of the biotinylated BSA eluted during the elution step between fractions 7 and 13 with a maximum concentration of 0.09 mg/ml in fraction 9. A small amount of biotinylated BSA was washed from the column between fractions 1 and 3; however, these levels were significantly improved compared to the 0.5 ml column volume. A column volume of 1 ml was therefore considered to be the minimum for future purification of biotinylated plasma proteins.

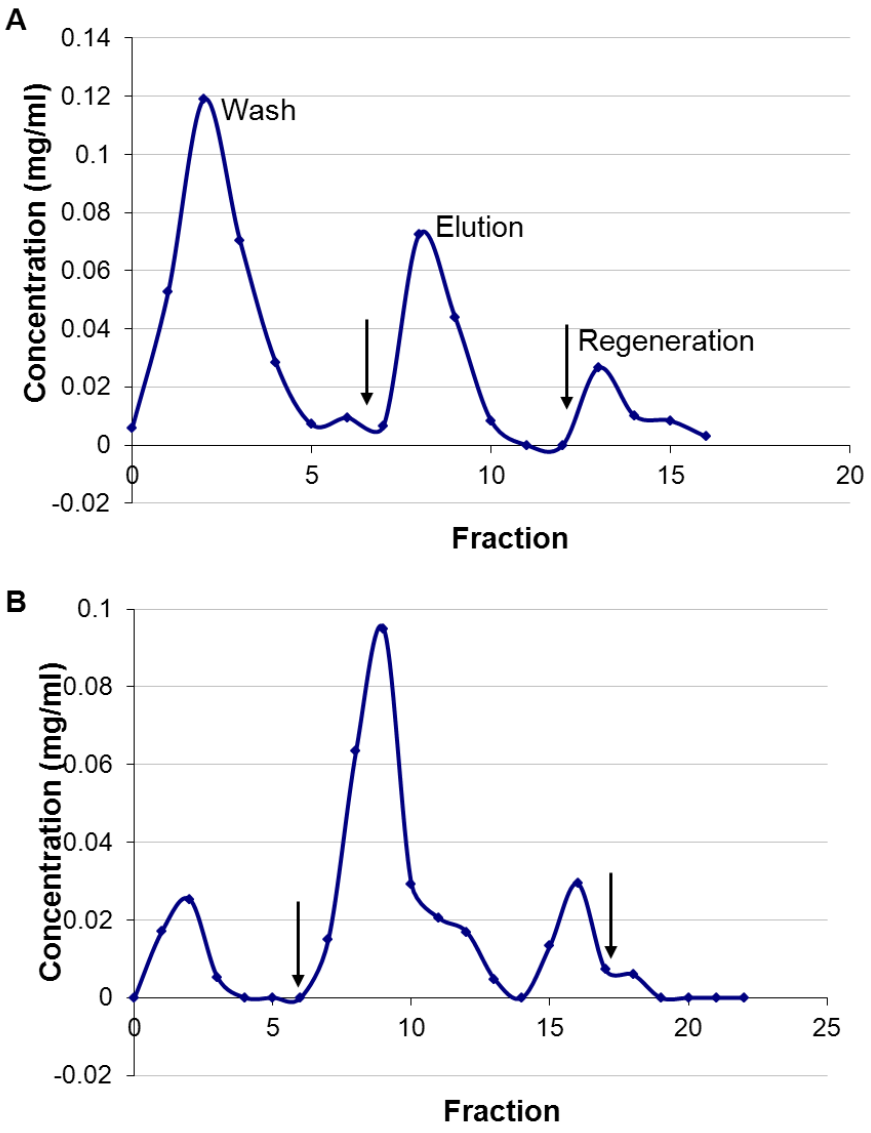

Figure 60: Evaluation of binding capacity of the monomeric avidin column after a reduction in column volume.

Biotinylated BSA elution chromatogram from the avidin affinity column monitored at 280 nm. A column volume of 0.5 ml (**Panel A**) had a binding capacity of 140 µg whereas a column colume of 1 ml (**Panel B**) had a binding capacity of 330 µg. Arrows represent changes in buffers.

6.4.2. Optimisation of the initial protein concentration

The initial protein concentration was low due to the initial dilution of the plasma, which may have prevented the capture of sufficient plasma proteins by avidin affinity chromatography; therefore resulting in insufficient protein for MS. A 10-fold (2.05 mg/ml) and 30-fold (9.38 mg/ml) increase in fibrinogen depleted plasma protein concentration was incubated in the presence and absence of BP for BP labelling as previously described in section 6.1. Fractions 10 to 19 from the gel filtration column were pooled, concentrated for each control and BP labelled sample and applied to the monomeric avidin column. For both negative controls, plasma proteins were washed from the avidin column between fractions 1 and 4 (Figure 61, Panels A & D). No protein was eluted during the elution step.

For the 10-fold BP labelled sample, plasma proteins were washed from the column between fractions 0 and 3 with a maximum concentration of 0.8 mg/ml in fraction 1. No protein was eluted during the elution step (Figure 61, Panel B). To determine whether biotinylated proteins were present in the initial wash fractions, fraction 1 was acetone precipitated and analysed by Western blot with streptavidin HRP. As shown in Figure 61, panel C the wash fractions did indeed contain biotinylated proteins that were not purified from the non-biotinylated plasma proteins. As a greater proportion of BP labelled proteins was expected in the 30-fold plasma sample, it was also purified by monomeric avidin. The plasma proteins were washed from the column between fractions 1 and 3, with a maximum concentration of 3.5 mg/ml in fraction 1. A small amount of protein was eluted during the regeneration step only (Figure 61, Panel E).

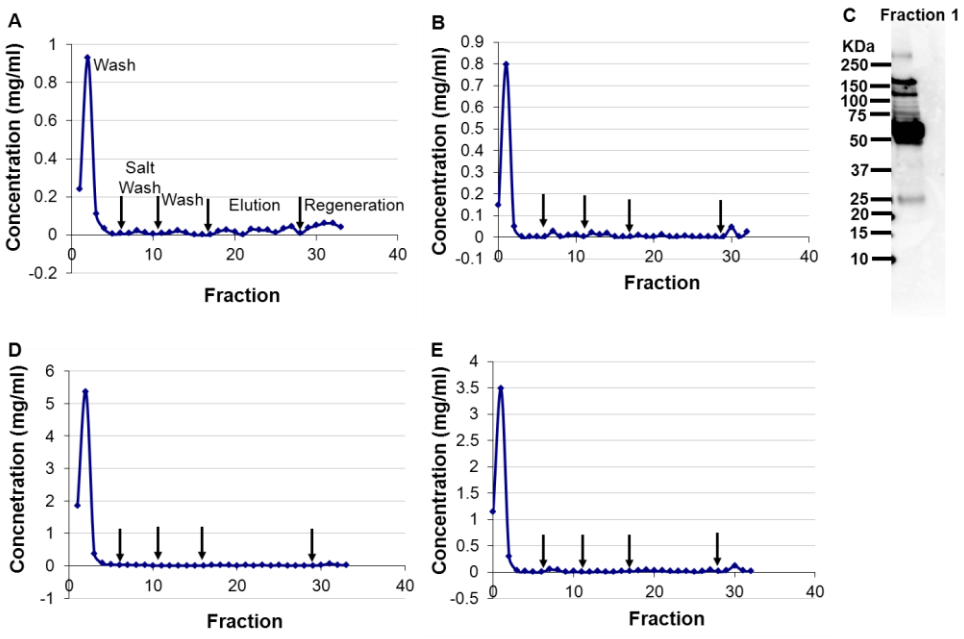

Figure 61: Purification of negative control and BP labelled plasma proteins by monomeric avidin affinity chromatography after an increase in the inital protein concentration.

Chromatogram of protein elutions monitored at 280 nm for the negative control (**Panels A & D**) and BP labelled plasma (**Panels B & E**) after an incubation with a 10-fold (**Panels A & B**) and 30-fold (**Panels D & E**) increase in initial plasma concentration. In all chromatograms protein elutes from the monomeric avidin column in the wash step. Arrows indicate the buffer changes outlined in panel A. **Panel C**. Fraction 1 of panel B was acetone precipitated and analysed by streptavidin HRP Western blot and was found to contain BP labelled plasma proteins.

As no purified BP labelled proteins were obtained after changing the column volume or increasing the initial plasma concentration using the previously optimised method, five reactions were combined and purified by monomeric avidin chromatography. Five negative control reactions containing 1 in 100 dilutions of fibrinogen depleted plasma (total protein content of 0.7 mg/ml) in the presence of FXIIIa and five test samples containing 1 in 100 dilutions of fibrinogen depleted plasma (total protein content of 0.7 mg/ml), 5 mM BP and FXIIIa were set up. Each reaction was individually gel-filtered as described previously. Fractions 10 to 19 from all five reactions were pooled, concentrated and applied to the monomeric avidin column, with the negative control purified first. As shown in Figure 62, Panel A, the majority of the plasma proteins were washed from the column between fractions 1 and 3 with a maximum concentration of 1.55 mg/ml in fraction 2. A small amount was also washed from the column during the salt wash. As shown in Figure 62, Panel B, the majority of BP labelled protein was washed from the column between fractions 1 and 4, with a maximum protein concentration of 0.75 mg/ml in fraction 2. A smaller amount of protein was washed from the column during the salt wash; however, no protein was eluted from the column during the elution step.

6.4.3. Establishment of BP concentration to reduce human serum albumin labelling

Human serum albumin was the only protein to be identified by MS, however no BP labelled peptides from human serum albumin were determined, suggesting BP may be non-specific at high concentrations, therefore BP incorporation into albumin was monitored over a range of BP concentrations by streptavidin HRP Western blot and compared with an anti-albumin Western blot. In reactions using 5 mM and 10 mM BP, the amount of biotinylated albumin was significantly reduced compared to the 50 mM and 100 mM BP reactions (Figure 63, Panel A), however BP containing high molecular weight bands were also found to contain albumin (Figure 63, Panel B), suggesting human serum albumin was cross-linked by FXIIIa to form multimers.

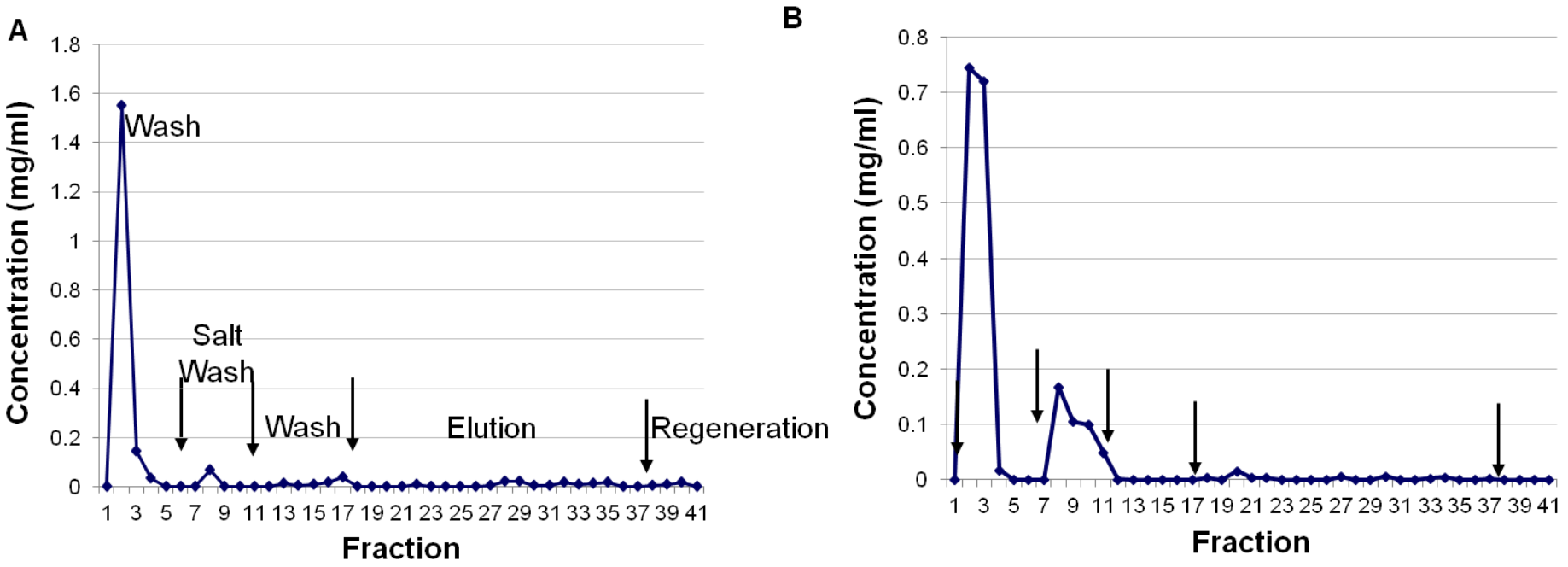

Figure 62: Purification of pooled BP labelled plasma reactions by monomeric avidin affinity chromatography.

Panel A. Purification of the negative control resulted in all the protein washing from the column during the wash steps. **Panel B**. Purification of the biotinylated plasma protein samples resulted in all the protein washing from the column during the wash steps. Protein elution was monitored at 280 nm. Arrows represent changes in buffer steps outlined in panel A.

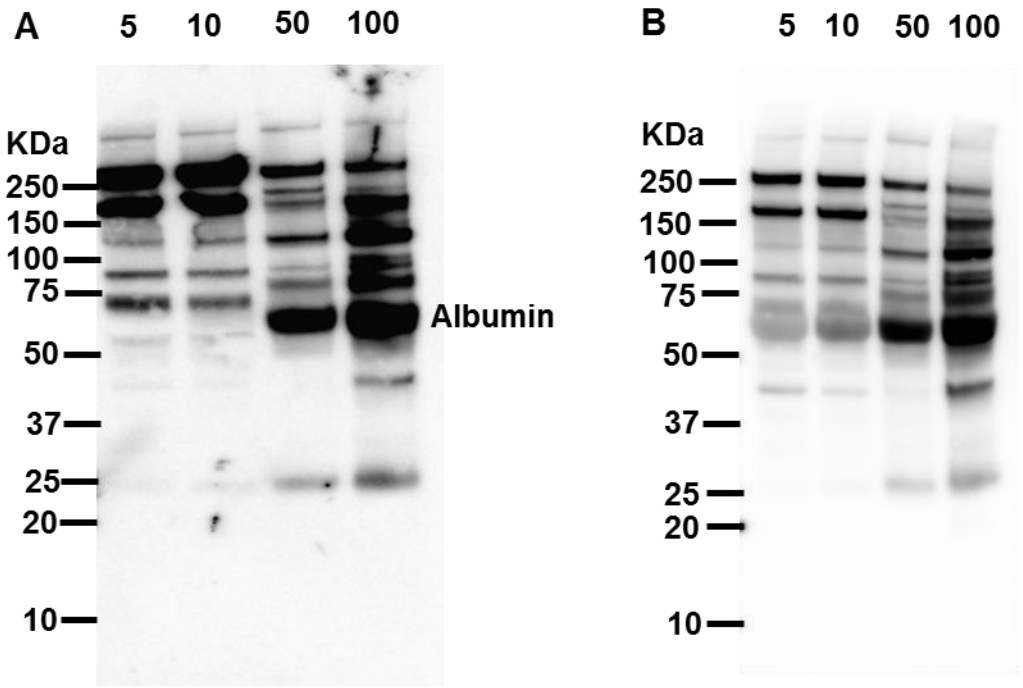

Figure 63: Identification of BP labelling of albumin at reduced BP concentrations

BP from 5 mM to 100 mM was incubated with fibrinogen depleted plasma in the presence of FXIIIa. BP labelled plasma proteins were observed by streptavidin HRP Western blot (**Panel A**) and compared to an anti-albumin Western blot (**Panel B**). At all concentrations of BP, BP was incorporated into albumin and albumin multimers.

This series of optimisation experiments show an inability to optimise the monomeric avidin method further as the binding capacity of the monomeric avidin column was checked and found to be unaltered from that specified in section 3.17.3.2 and no excess BP was observed in the BP labelled plasma samples by FXIII activity assay (data not shown). Furthermore, BP incorporates into human serum albumin at reduced BP concentrations forming albumin multimers, therefore any further series of experiments would require starting plasma depleted in both fibrinogen and human serum albumin. Therefore a gel based approach to identifying FXIII substrates in plasma clots was established.

6.5. Identification of plasma FXIII substrates by SDS-PAGE

As an alternative method for the identification of FXIII substrates, normal pooled plasma clots and FXIII deficient plasma clots formed in a humidified incubator and perfused with buffer were compared by SDS-PAGE. The resulting plasma clots were reduced in 100 mM DTT, separated by electrophoresis on 3-8 % tris-acetate gels and silver stained; the resulting bands in the normal pooled plasma were compared to the FXIII deficient plasma to determine possible FXIII substrates. The normal pooled plasma clots contained a large number of high molecular weight proteins that were poorly resolved under these conditions (Figure 64). Although the FXIII deficient plasma clots were better resolved, the band intensity was insufficient for MS identification of plasma proteins.

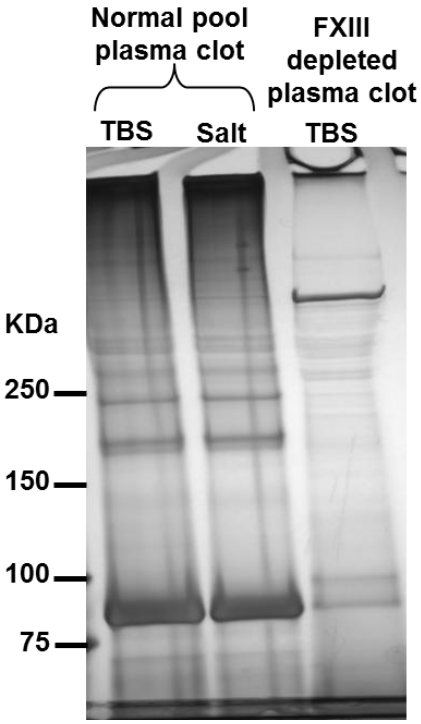

Figure 64: SDS-PAGE of a silver stained, perfused, solubilised plasma clots.

Normal pooled plasma and FXIII depleted plasma clots were perfused with either tris buffered saline (TBS) or a salt wash and analysed by SDS-PAGE on 3-8% tris-acetate gels (silver stained) show poor resolution in the high molecular weight range and insufficient protein in the FXIII depleted plasma clots. Gel representative of three independent experiments.

Following the poor resolution of the higher molecular weight proteins, different temperature and staining conditions were evaluated. Figure 65 panel A, shows that higher molecular weight proteins stained with silver were poorly resolved whether undergoing a 15 minute treatment at 95°C or one hour at 37°C. However, the lower molecular weight bands appeared to be better resolved at 37°C (1 hour) compared to the 95°C (15 minutes). Although silver staining was highly sensitive and allowed the identification of a large numbers of protein bands, the experiments described above suggest it was impossible to distinguish bands in the high molecular weight range by SDS-PAGE. As an alternative, reduced plasma clots were analysed by SDS-PAGE and stained with coomassie. As shown in Figure 65, panel B, there was insufficient protein bands observed from the plasma clots having undergone incubation at 95°C. Whereas at 37°C the protein bands were poorly resolved and showed protein smearing, possibly suggesting the protein was not reduced completely. Due to the time constraints of this project, further evaluation of this method was not established; however future progression of this method could include the evaluation of different reducing agents (Urea), the amount of protein loaded onto the gels or the amount of time the gels were run for.

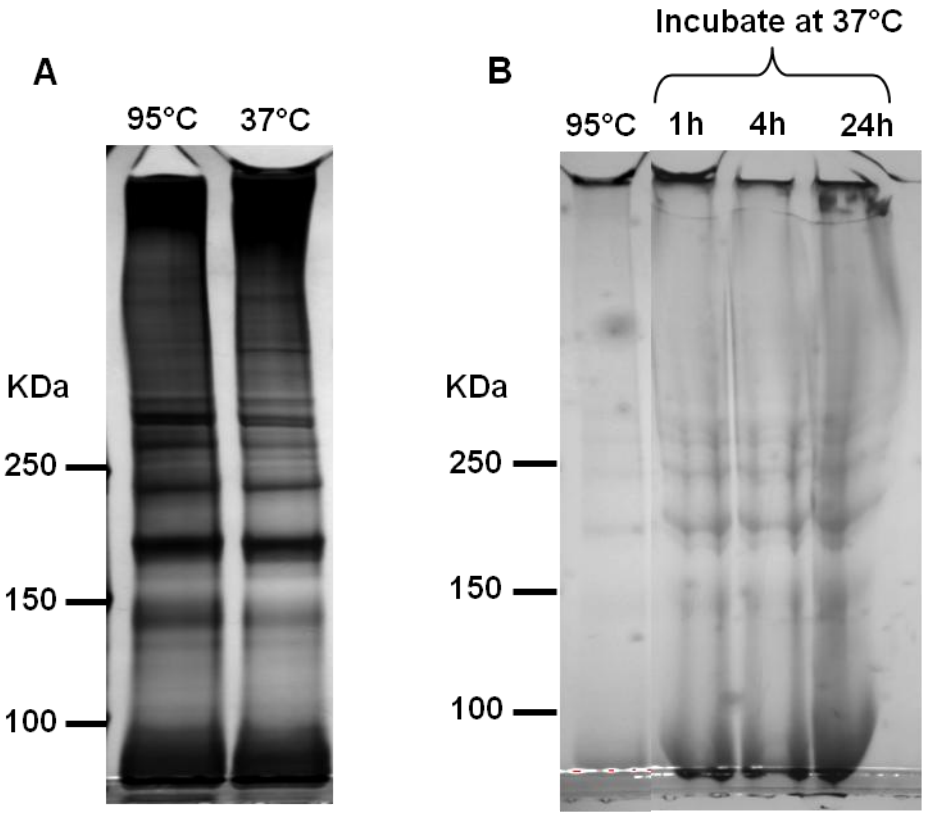

Figure 65: Temperature dependent reduction of solubilised plasma clots

Panels A & B. Normal pooled plasma clots solubilised by 100 mM DTT and loading buffer at 95°C for 15 minutes (**Panels A & B**) or at 37°C for 1 hour (**Panel A**) or 1-24 hours (**Panel B**). Plasma proteins seperated by SDS-PAGE on a 3-8% Tris Acetate gel and were either silver stained (**Panel A**) or Coomassie stained (**Panel B**). Panel A shows that the plasma proteins were insufficiently resolved within the high molecular weight range, whereas panel B shows insufficient protein and protein which was poorly reduced.

6.6. Discussion

The plasma proteome is dynamic and as such contains a large number (~106) and range of protein concentrations (55 000 000 000 pM, albumin to 1-5 pM, IL-6) (Hortin and Sviridov 2010). For example, the top 10 most concentrated proteins make up ~90% of the protein in plasma (Anderson et al. 2004). For this reason it was important to investigate subproteomes which perform specific functions within plasma, to maximise the yield and analyse the sample in depth. Several processing techniques including protein depletion, fractionation and selection for specific protein-protein interactions can be used to reduce protein complexity and allow the identification of greater numbers of proteins with lower protein concentrations. To facilitate identification of FXIII substrates, a combination of the above techniques were employed. Protein depletion of fibrinogen from plasma was essential in the identification of less abundant substrates. Fibrinogen is one of the ten most abundant plasma proteins at a concentration of around 2 mg/ml and is the main substrate for FXIIIa. Selection for specific protein-protein interactions was carried out using the biotinylated probe, BP. This is a lysine analogue that can be cross-linked by FXIIIa to reactive glutamine residues within substrates and, as a result, could form an effective probe for the isolation and evaluation of FXIII substrates by avidin affinity chromatography. BP was cross-linked into the reactive glutamine residues of a number of plasma proteins with protein molecular weights ranging from ~15 KDa through to >300 KDa. The optimum BP concentration was determined to be 100 mM, as it produced the largest proportion of biotinylated proteins whilst reducing protein-protein cross-linking interactions. The BP concentration used in the present study was significantly higher than the reported 10 mM BP used for cross-linking purified α2-antiplasmin (Lee et al. 2000); however, it was deemed appropriate to use higher concentration of BP to allow the maximum number of cross-linking reactions to occur within a complex mixture of proteins. The optimum FXIII concentration was examined to determine whether FXIII was a rate limiting factor in the cross-linking reaction. No obvious differences were observed for 100-fold and 1000-fold excess FXIII concentrations, possibly as the ratio of FXIII to other plasma proteins was already 100 fold greater. The cross-linking of BP into plasma substrates of FXIII was also examined over time. A three hour incubation time was considered optimum as the greatest intensity of bands was observed. Although FXIII substrates such as

fibrin (Standeven et al. 2007) and α2 antiplasmin (Lee et al. 2001) are known to cross-link more rapidly to fibrin, von Willebrand factor required an incubation time of three hours to cross-link to fibrin in plasma (Hada et al. 1986), suggesting for the purpose of identifying potential novel substrates longer incubation times were appropriate.

Fibrinogen depleted plasma with no exogenous FXIII was initially evaluated as a negative control, since FXIII can bind to fibrin(ogen) by several identified interactions it was possible that FXIII was also depleted from the fibrinogen depleted plasma. To test this proposal, FXIII depletion in fibrinogen depleted plasma was evaluated by incubating fibrinogen depleted plasma and BP in the absence of added FXIII. As cross-linking of BP to plasma proteins was observed, this suggests that plasma FXIII may not be bound exclusively to fibrinogen in plasma. Immunodepletion of FXIII from the fibrinogen depleted plasma was considered; however, this would require prolonged sample handling, possible sample dilution and no guarantee that all the FXIII would be removed. Alternatively incorporation of BP into fibrinogen depleted plasma proteins may be the result of other members of the transglutaminase family such as TG2. Erythrocytes are a known source of plasma TG2 and TG2 has previously been found to cross-link fibrin in thrombi (Mutch et al 2010). Therefore, an alternative negative control containing fibrinogen depleted plasma in the absence of BP was investigated. This negative control would allow the cross-linking reactions to take place; however, due to the absence of the biotinylated probe, the proteins would be eliminated during avidin affinity chromatography. This would therefore allow the analysis of non-specific binding of streptavidin HRP to plasma proteins and the non-specific binding of proteins to the monomeric avidin affinity column. No bands were observed in the absence of BP, suggesting streptavidin does not bind non-specifically to the plasma proteins.

FXIIIa cross-links fibrin to provide the strength and rigidity to a clot. FXIII initially cross-links the fibrin γ chain between Lys406 of one γ chain and either Gln398 or Gln399 on a neighbouring γ chain to form γ-dimers (Purves et al. 1987). FXIII also forms cross-links between fibrin α chains involving Gln221, Gln237, Gln328, Gln366 (Cottrell et al. 1979) and Lys508, Lys556, Lys539, Lys580, Lys583, Lys418, Lys448, Lys601, Lys606, Lys427, Lys446, Lys429, lys208, Lys224 and Lys219 to form α-polymers (Sobel et al. 1996). Therefore, as fibrinogen was a major

substrate of FXIII and the glutamine cross-linking sites have been previously characterised, fibrinogen was chosen as an appropriate positive control to determine the specificity of BP cross-linking. As expected BP, was cross-linked to both the α and γ chains of fibrinogen, which confirms previous findings that suggest the α and γ chains are the only chains to be cross-linked (Standeven et al. 2007). The intensity of the α chain band was greater than that of the γ chain, possibly because there are four reactive glutamine residues within the α chain, compared to only two known reactive glutamine residues within the γ chain. A small amount of cross-linking of BP was also observed in the fibrinogen β chain that has not been previously reported (Standeven et al. 2007). This either suggests that the small size of the biotin probe allows it to be bound non-specifically to the β chain or that the biotin probe has been cross-linked to a currently unknown glutamine site. However as the fibrinogen β chain remains at 55 KDa after fibrin cross-linking in previous studies (Standeven et al. 2007), it suggests that BP was most likely non-specifically incorporated into the β chain. BP was also cross-linked into a number of high molecular weight complexes by FXIIIa in purified fibrinogen. These bands represent the formation of γ-dimers, suggesting protein-protein cross-linking occurred, as the γ-chain contains more than one reactive glutamine residue, additionally higher molecular weight bands represent cross-linking of contaminating proteins that were not removed from the purified fibrinogen and purified FXIII or high molecular weight cross-linked fibrin α chains. Incorporation of BP into plasma proteins was optimised to maximise the number of protein identifications, therefore validation of any cross-linking sites identified by MS would be required to exclude proteins which are non-specifically identified.

Avidin affinity chromatography was employed to allow the separation of non-BP labelled plasma proteins from FXIII substrates which contained the BP label. Several avidin purification methods are summarised in Table 9. Monomeric avidin was chosen as the purification method of choice due to its high binding capacity, low non-specific binding and lower binding affinity, allowing the avidin-biotin interaction to be reversible with 2 mM free biotin. Commercial biotinylated BSA was used to check the purification method and the overall binding capacity. As expected, the biotinylated BSA bound to the monomeric avidin column and eluted during the elution step using both the manufacturers and alternative methods with a binding capacity of 1.3 mg of biotinylated BSA per 2 mls of monomeric avidin. The binding

capacity was significantly lower than the specified 2.4 mg of biotinylated BSA per 2 mls of monomeric avidin quoted by the manufacturer (Thermo fisher scientific Inc 2011). In the first series of experiments, the protein concentration applied to the column was significantly lower than the binding capacity of the column (summarised in Table 16), to allow the capture of all BP labelled proteins in the plasma sample.

As free BP contains the biotin tag, it is also able to attach to the monomeric avidin column, thus preventing the attachment of BP labelled plasma proteins. Also, due to its small size, attachment to the avidin column may be more accessible compared to large proteins containing only one or a few cross-linked BP molecules. To remove excess BP from the plasma sample, dialysis was evaluated using a 3.5 KDa molecular weight cut off membrane, which was significantly larger than the 328.47 Da of the BP. However, BP- labelled plasma proteins were observed in the wash fractions from the monomeric avidin column and were only observed in the elution fractions after significantly longer dialysis times with increasing levels of buffer, suggesting BP still remained in the sample. This could be due to the high starting concentration of BP or insufficient buffer exchange. Dialysis was therefore considered ineffective for the removal of BP, as large volumes of buffer would be required with increased sample handling time, which might lead to protein degradation. Gel filtration was considered as an alternative to the removal of BP. Gel filtration separates components via their size, with larger proteins unable to penetrate the matrix pores and therefore eluting first, whereas smaller proteins/contaminants penetrate the matrix pores and elute later (Hagel 2001). Therefore BP can be readily separated from much larger plasma proteins. This technique has been previously described for the removal of free BP from cross-linked tissue transglutaminase substrates (Fleckenstein et al. 2004;Orru et al. 2003). An additional benefit of using this technique was that BP elution from the column can be fully monitored using the FXIII activity assay, therefore ensuring the efficient removal of BP from the plasma sample. A Sephadex G25 column was selected to separate the plasma proteins from the BP, since the exclusion limit of 5000 Da (Hagel 2001) would prevent any protein over 5000 Da being retained on the column, with other lower molecular weight proteins and BP retained by interactions with the matrix pores of the column to elute later. Fleckenstein et al (2004) used a Sephadex G50 superfine column for the removal of free BP from biotinylated proteins. Sephadex G50 has an exclusion limit

of 30,000 Da (Hagel 2001) and was considered inappropriate for plasma samples, as low molecular weight plasma proteins would be excluded with the BP and would therefore minimise peak separation. Column dimensions were shown to have a large influence on the separation of protein from BP, with peak separation significantly improved with a wider column diameter. One explanation for this could be that the surface area of the column was increased allowing increased penetration of the BP into the matrix pores resulting in improved peak seperation. Gel filtration was therefore used in the separation of BP from all biotinylated samples.

Once the free BP had been successfully removed, biotinylated purified fibrinogen and biotinylated plasma samples were successfully separated from non-biotinylated proteins by monomeric avidin affinity chromatography. Elution fractions were acetone precipitated to remove salt from the samples and to concentrate the proteins for MS analysis. Protein samples were analysed by MS using a bottom-up approach, whereby the proteins were digested with trypsin before mass analysis using an HCTultra ETD II ion trap mass spectrometer. The bottom-up approach is known to be particularly effective in the analysis of complex samples and in the identification of chemical modifications within peptides (Yates et al. 2009) such as the cross-linking of BP to reactive glutamine residues of FXIII substrates. The HCTultra ETD II ion trap mass spectrometer used for the analysis of the BP labelled proteins was a tandem mass spectrometer that combines electrospray ionisation with either collision induced dissociation or electron transfer dissociation. Collision induced dissociation is known to be particularly effective for sequence information; however, fragmentation by this technique usually occurs at the weakest bond, usually at the site of a post-translational modification, such as the BP incorporation site, whereas electron transfer dissociation is known to be particularly effective at detecting post-translational modifications; however, it results in multiply charged fragments (Hartmer et al. 2008;Yates et al. 2009), making it harder to identify the parent protein and its sequence using protein databases (Sharma et al. 2010). This mass spectrometer was therefore used to combine the advantages of collision induced dissociation with those of electron transfer dissociation in the identification of BP labelled plasma proteins due to the posttranslational modification. Human serum albumin was the only plasma protein to be identified in the MS analysis. Human serum albumin has not been previously characterised as a FXIII substrate; however,

has been previously found to be cross-linked by tissue transglutaminase forming dimers and high molecular weight polymers (Thung et al. 1989). Gupta et al. (2007) further showed that BP could be cross-linked to human serum albumin by tissue transglutaminase in atherosclerotic plaque tissue (Gupta et al. 2007); however the reactive glutamine residues were not identified by this study. As FXIII and tissue transglutaminase come from the same enzymatic family, there is a possibility the FXIII may also cross-link human serum albumin; however, no BP labels were identified in the MS analyses, suggesting that either human serum albumin bound non-specifically to the monomeric avidin during the purification stages or the MS was unable to pick up the BP label. Subsequent experiments confirmed BP incorporation into human serum albumin monomers and polymers which cross-reacted with both an anti-albumin antibody and streptavidin-HRP, thus confirming the observation of human serum albumin polymer formation previously observed in the presence of tissue transglutaminase (Thung et al. 1989). Human serum albumin is highly abundant in plasma and therefore all the cross-linked bands containing BP were also found to contain albumin at both high and low BP levels. Depletion of albumin from the fibrinogen depleted plasma was considered, however although depletion of albumin from serum was good at over 90% (Bjorhall et al. 2005) depletion of albumin from plasma was lower at 72%, with significant loss of low abundance proteins (cytokines) observed in this study (Granger et al. 2005). Analysis of albumin depleted from serum, identified 67 proteins which were also depleted along with the albumin, 57 of these proteins were either of low molecular weight or low abundance (Zhou et al. 2004). These studies suggest albumin will most likely remain in the plasma sample after depletion, with possible depletion of unknown FXIII substrates during the depletion steps.

Several issues were identified from the MS analysis; firstly the protein concentration of the samples was too low, due to the loss of protein at each step as highlighted in Figure 66. Initially the starting plasma sample was depleted of fibrinogen. It was possible that during the immunodepletion procedure plasma substrates of FXIII were also depleted, due to binding to fibrinogen or non-specifically to the immunodepletion column. Another reason could be that the initial starting concentration of protein was not sufficient for BP labelling, preventing the capture of enough FXIII substrates by avidin affinity chromatography. Although the

avidin affinity column was able to purify BP labelled plasma proteins, these were in small quantities compared to non-BP labelled proteins. Alterations to the BP labelling method were evaluated to help increase the initial protein concentration however, this resulted in no BP labelled proteins eluting from the monomeric avidin column even though BP labelled proteins were present in the sample by streptavidin Western blot. One possibility for the absence of BP labelled proteins in the elution step could be that FXIII substrates are present in plasma at relatively low concentrations with possibly only one or two reactive glutamine sites available for BP labelling per protein, therefore making it harder to attach to the avidin sites from within a complex mixture of proteins such as plasma where the majority would not be labelled. This may account for why the BP labelled BSA attached to the avidin column readily, as the whole sample was BP labelled with eight moles of biotin per mole of BSA. This may account for why previous studies have used DTT to reduce protein disulphide bonds, during the BP incorporation method to identify substrates of tissue transglutaminase (Orru et al. 2003;Ruoppolo et al. 2003), however reducing proteins into their component chains may lead to the identification of BP labelled proteins by FXIII which would not be otherwise cross-linked in plasma. Finally the loss of protein could be due to the acetone precipitation method. Proteins only precipitate in ice cold acetone; therefore the absence of a cold centrifuge for centrifugation of samples from the freezer would allow the sample to warm during centrifugation and in turn cause the re-suspension of the proteins and overall loss of yield. Although numerous changes had been made to the reaction conditions, removal of free BP and the avidin column itself (changes highlighted in Table 16), BP labelled plasma proteins of sufficient quantity was not obtained to allow the identification of any proteins other than human serum albumin by MS

Alternatively, the low yield could be due to the need of a large interaction time between the biotinylated protein and the monomeric avidin. According to the manufacturer's instructions, an interaction time of one hour was advised between the monomeric avidin and BP labelled proteins (Thermo fisher scientific Inc 2011). Whereas a recent study found that even after an interaction time of three hours, biotinylated cytochrome C remained in the supernatant following monomeric avidin affinity chromatography (Spross and Sinz 2012). Chromatography techniques such as avidin affinity chromatography will evolve, allowing for greater sensitivity in the

future. For example, in a recent study monomeric avidin attached to a monolithic stationary phase column was found to have a higher binding capacity (7.1 mg of BSA/ml of column), better enrichment of biotinylated proteins and required a reduced interaction time between the biotinylated protein and the column of 132 seconds (Spross et al. 2012) compared to commercially sourced monomeric avidin (Thermo fisher scientific Inc 2011). Alternatively, avidin or streptavidin could be used more extensively in the future due to the design of cleavable linker regions within biotin probes (Landi et al. 2010).

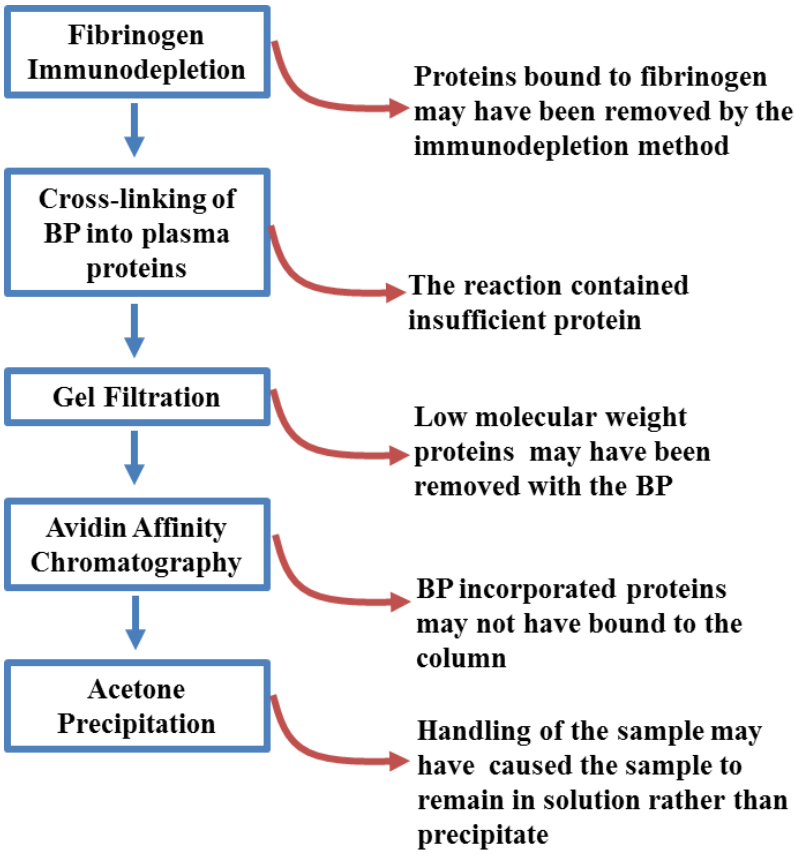

Figure 66: Stages representing the loss of protein in the proteomics approach

1. Protein loss during immunodepletion of fibrinogen from plasma could have occurred by the non-specific binding to the immunodepletion column or to fibrinogen. 2. The starting concentration of proteins in the BP cross-linking reaction was insufficient. 3. Due to the exclusion limit of the gel filtration column, proteins of <5000 KDa may have been lost during purification. 4. As observed in the following series of experiments, BP labelled proteins had difficulty binding to the monomeric avidin column. 5. Due to the absence of a cold centrifuge proteins may have remained in solution during the acetone precipitation method.

As an alternative method for the identification of FXIII substrates, perfused plasma clots and perfused FXIII-deficient clots were compared. The plasma clot proteome encompasses proteins which either bind and/or are cross-linked by FXIIIa during fibrin clot formation. By perfusing plasma clots with TBS all non-incorporated plasma proteins were removed, whereas perfusion with a salt wash

enabled the removal of bound proteins. Protein bands from perfused solubilised normal pooled plasma clots were compared with perfused solubilised FXIII deficient plasma clots by SDS-PAGE to determine possible FXIII substrates. Previously Dr JM Howes used this technique to identify complement C3, factor H and fibronectin as FXIIIa dependent cross-linked substrates (Howes et al. 2012). Therefore, to identify additional plasma proteins, 100 µl of plasma were used to form the clots compared to only 50 µl previously used (Howes et al. 2012). However, this resulted in poor resolution of the high molecular weight bands in the plasma clot. One possibility for this could be the accumulation of un-resolved fibrin α-polymers in the high molecular weight range, as fibrin polymer formation was also identified previously in 2-DE polyacrylamide gels (Howes et al. 2012). Secondly, there was insufficient protein within the bands to allow the effective identification by MS. Different staining techniques were investigated; silver staining was highly sensitive, allowing the identification of larger numbers of proteins; however, due to this sensitivity band intensity was insufficient for protein identification by MS. Coomassie blue stain was less sensitive than silver stain and as a result less abundant proteins were not picked up; however, due to its reduced sensitivity clear bands on a Coomassie stained gel usually indicate sufficient protein for MS analysis. In the future, alternative denaturing reagents could be used, to provide better separation of the higher molecular weight products within fibrin clots. In a recent study, plasma clots were washed with a mixture of urea, thiourea and CHAPS (3-[(3-cholamidopropyl)diethylammonio]-1-propanesulfonate) to remove all non-covalently bound proteins for analysis by MS (Talens et al. 2012). This study only identified bound proteins (proteins in the supernatant); however this technique could be used in the future to identify the cross-linked components of the clot.

The plasma clot subproteome has previously been investigated by comparing the differences in plasma and plasma derived serum samples. Proteins known to be involved in coagulation were lower in serum compared to plasma. Whereas complement proteins (complement C3 and factor H) were also found to be reduced in serum (Misek et al. 2005), suggesting they are incorporated into a plasma clot. Analysis of the low molecular weight peptidome of serum identified coagulation proteins (prothrombin and fibrinogen) and complement proteins (C3 and C4) with peptides most likely produced by cleavage by proteases during plasma clot formation

(Tammen et al. 2005). Niessen et al (2011) not only found that complement proteins were present in plasma and whole blood clots but also found that complement proteins C1q, C5, C6 and C9 are actively cleaved by proteases using proteomics by comparing the changes in molecular weight (Niessen et al. 2011). These studies highlight the importance of coagulation and complement proteins in ex vivo thrombus formation. The identification of complement proteins within plasma clots using proteomics shows that complement activation occurs during thrombus formation; however proteomics is unable to determine the functional role of complement in thrombus formation, thus supporting the need for in vitro studies to validate the proteomic observations.

In summary, the present study has established a functional proteomics based method for the identification of reactive glutamine containing substrates of FXIIIa using BP. Using this method, human serum albumin was identified as a potential substrate and was confirmed to form cross-links with itself in the formation of human serum albumin multimers. Although further alterations to the method were made, the purification of BP labelled proteins by monomeric avidin proved to be the problem, which prevented the present study identifying more FXIII substrates. Further advances in avidin purification and MS technology may make this technique possible in the future. As an alternative method for the identification of FXIII substrates, a gel based approach proved just as problematic with insufficient protein and poor resolution within the high molecular range; however future optimisation could include trying different reducing agents to improve high molecular weight bands and combining numerous clots to increase the protein content.

Table 16: Methods evaluated for the purification of BP labelled plasma proteins by monomeric avidin affinity chromatography.

Highlighted in yellow are the changes made.

Reaction Number	1	2	3	4	5	6
Plasma Concentration	0.81 mg/ml	0.72 mg/ml	0.73 mg/ml	2.05 mg/ml	8.64 mg/ml	~0.67 mg/ml per sample
BP	100 mM	100 mM	100 mM	100 mM	100 mM	5 mM
Reaction Volume in TBS	1 ml	1 ml	1 ml	500 µl	500 µl	5x 1 ml
Method of removal of excess BPNH2	3.5 KDa Dialysis 3x 30 min in 1 Litre of TBS	3.5 KDa dialysis 2x 1 hour and overnight each in 2 litres of TBS	7cm by 2.5cm sephadex G25 column followed by concentration in 3 KDa vivaspin column	7cm by 2.5cm sephadex G25 column followed by concentration in 3 KDa vivaspin column	7cm by 2.5cm sephadex G25 column followed by concentration in 3 KDa vivaspin column	7cm by 2.5cm sephadex G25 column followed by concentration in 3 KDa vivaspin column
Protein applied to avidin column	0.63 mg/ml	0.34 mg/ml	0.31 mg/ml	1.53 mg/ml	5.62 mg/ml	2.26 mg/ml
Avidin column volume	2 mls	2 mls	2 mls	1 ml	1 ml	2 mls

Chapter 7 Conclusions

Complement and coagulation cascades are derived from common ancestral genes (Krem et al. 2002) and are activated to prevent blood loss and pathogen invasion, and, as a result, are required to co-localise at the injury site. In previous work performed in DCDR, complement proteins C3 and factor H were identified as novel plasma clot components using a proteomics based technique (Howes et al. 2012). Therefore, these complement proteins were analysed to determine their role in fibrin formation and fibrinolysis.

Complement and coagulation/fibrinolysis proteins interact at numerous levels as highlighted in Figure 67. During coagulation thrombin cleaves fibrinogen and FXIII to initiate clot formation, whilst thrombin cleavage of C3 results in the generation of C3b and C3a at high concentrations, most probably during the thrombin burst, when the greatest thrombin concentration is generated (Brummel-Ziedins et al. 2005). C3a is an anaphylotoxin that attracts phagocytes for the targeted destruction (phagocytosis) of pathogens. To prevent excess inflammation as a result of anaphylatoxin (C3a and C5a) production, TAFIa, a fibrinolysis inhibitor, inactivates C3a and C5a by cleaving the carboxyl terminal lysine or arginine residues (Campbell et al. 2001;Leung et al. 2008). Complement activation may also result from thrombin generated C3b in the production of the alternative pathway C3 convertase in the presence of factor B and factor D, ultimately resulting in more C3a, C3b and the generation of the membrane attack complex (C5b-9). This could occur in fibrin clots as C3b can non-covalently attach to fibrin during cross-linked fibrin formation. Factor H may form a protective role in fibrin clots against excessive complement activation with thrombin and plasmin cleaved factor H still retaining cofactor activity for cleavage of C3b by factor I (Fontaine et al. 1989;Ohtsuka et al. 1993;Sim et al. 1982). Complement activation in whole blood thrombi was suggested by (Distelmaier et al. 2009) by comparing systemic plasma to coronary thrombi. Within coronary thrombi levels of C3c, C3a and C5a were significantly increased; alternatively, thrombi levels of factor B decreased compared to systemic plasma factor B levels, suggesting factor B is consumed in the formation of the alternative pathway C3 convertase within thrombi, which was confirmed by the localised deposition of C5b-9. The main role for the membrane attack complex within thrombi

is in the effective removal of pathogens by cell lysis as part of the innate immune response. The importance of complement activation within fibrin clots is highlighted by several recent studies which found that FXIIIa dependent entrapment of *E.coli* and *S.aureus* within fibrin clots helped to prevent systemic infection (Wang et al. 2010). Whereas Loof et al (2011) found that the intrinsic pathway of coagulation was activated on the surface of *S.pyogenes* bacteria, resulting in FXIIIa dependent immobilisation within fibrin clots and the prevention of bacterial release (Loof et al. 2011). The present study has also shown that complement C3 is incorporated into fibrin clots through binding and cross-linking reactions, which would allow C3 to localise to the site of active pathogen invasion, furthermore in the presence of C3 and FXIII fibrinolysis was significantly prolonged, suggesting complement C3 and FXIII play important roles in the prevention of pathogen invasion. Since systemic infection was prevented in these studies it suggests that bacterial killing must take place within the fibrin clot and therefore suggests a role for complement activation and FXIII in this process. Once such mechanism of pathogen removal is phagocytosis, both C3a and thrombin cleaved factor H provide chemotaxis of monocytes for pathogen removal by phagocytosis (Ohtsuka et al. 1993;Sarma et al. 2011). Phagocytes bind to the opsonins C3b and iC3b via CR1, 3 and 4 (Gros et al. 2008;Hatano et al. 2009) and as shown in the cross-linking experiments in the present study, C3b is active and able to bind to free hydroxyl and amine groups via its internal thioester within fibrin clots.

An alternative mechanism for the formation of the membrane attack complex may be in platelet activation. C3b has been shown to bind P-selectin on the surface of activated platelets, resulting in the generation of the C3 convertase following factor B recruitment and therefore the generation of C3a and C5b-9 (Del, I et al. 2005). C5b-9 produced as a result of complement activation on activated platelets promotes the pro-thrombotic state by aiding factor V release from α granules and the formation of the prothrombinase complex on the platelet surface (Wiedmer et al. 1986). The function of factor H in the inhibition of complement activation within whole blood clots is highlighted by the ability of factor H to bind to activated platelets via receptors GPIIb/IIIa or thrombospondin-1 (Vaziri-Sani et al. 2005), and preventing C3 activation by aiding factor I mediated cleavage of C3 (Hamad et al. 2010). In patients with aHUS, platelet surface levels of factor H are significantly

decreased; resulting in complement component deposition and platelet microparticle release (Stahl et al. 2008). As platelet microparticles are known to be pro-thrombotic (Keuren et al. 2006), this study suggests a protective role of factor H in thrombosis. Hamad et al (2010) found that C3(H_2O) bound to activated platelets, which in the presence of factor I was cleaved to form iC3(H_2O). The resulting iC3(H_2O) was able to bind complement receptor 1, suggesting a role for iC3(H_2O) in the attachment of monocytes and neutrophils to platelets and therefore the site of potential phagocytosis (Hamad et al. 2010). The formation and role of platelet-leukocyte (monocytes or neutrophils) complexes in thrombosis has previously been examined in an in vivo baboon model following an arteriovenus shunt. Whereby activated platelets expressed P-selectin on their surface which when bound to leukocytes promoted leukocyte accumulation within a thrombus and promoted the deposition of fibrin (Palabrica et al. 1992). Scholz et al (2002) has since shown that tissue factor bound to platelet microparticles is transferred to the monocyte surface in a CD62P dependent manner (Scholz et al. 2002), thus promoting the initiation of coagulation resulting in fibrin formation. Furthermore, platelet microparticles bound to neutrophils, promoted the expression of CD11b (which in complex with CD18 forms CR3) and the phogocytic activity of the neutrophils (Jy et al. 1995).

Alternatively, during fibrinolysis the process of complement activation may be controlled by the coagulation protease plasmin along with conventional complement inhibitors to help prevent host tissue damage. In the present study plasmin cleaved C3 into C3b and C3a and further cleaved C3b into C3c, C3d and cleavage products of the β chain, all of which prevent the formation of the C3 convertase and therefore complement activation in favour of phagocytosis by the attachment of C3b, iC3b and C3c to opsonise pathogens and promote the immediate clearance via complement receptor 1 on neutrophils and eosinophils (Gros et al. 2008;Hatano et al. 2009) or the secondary effect by promoting the adaptive immune response in the stimulation of the B cell response via complement receptor 2 attachment to C3dg or C3d (Sarrias et al. 2001). Amara et al (2010) recently confirmed plasmin cleavage of C3 in the generation of C3a and further showed C3a recruited neutrophils. Furthermore in a purified system plasmin at physiological levels has recently been shown to inhibit the alternative pathway C3 convertase by 40% and production of the membrane attack complex by 40% (Barthel et al. 2012a);

however, C3b degradation by plasmin was inhibited by α2-antiplasmin (Barthel et al. 2012a), suggesting α2-antiplasmin promotes complement activation and the formation of the C3 convertase, however it is unknown whether α2-antiplasmin plays a similar role in plasma clots. As shown in the present study C3 prolongs fibrinolysis rates via several possible mechanisms including substrate competition, plasmin generation and angiostatin production as described in Chapter 4, possibly to prevent systemic pathogen invasion. The importance of this is highlighted by the number of pathogens that exploit the fibrinolytic system to evade fibrin entrapment and the immune system (Summarised in Table 17), with mechanisms including recruitment of t-PA or plasminogen, cleavage of fibrinolysis inhibitors or by the production of proteins with similar effects to the human fibrinolytic proteins. Several of the pathogens summarised in Table 17, recruit/bind plasminogen on their surfaces to evade the immune system and fibrin entrapment. Interestingly, C3 also binds plasminogen, as discussed above, suggesting C3 may prevent pathogen binding/recruitment of plasminogen, thus protecting the fibrin clot from plasmin degradation and preventing systemic pathogen invasion.

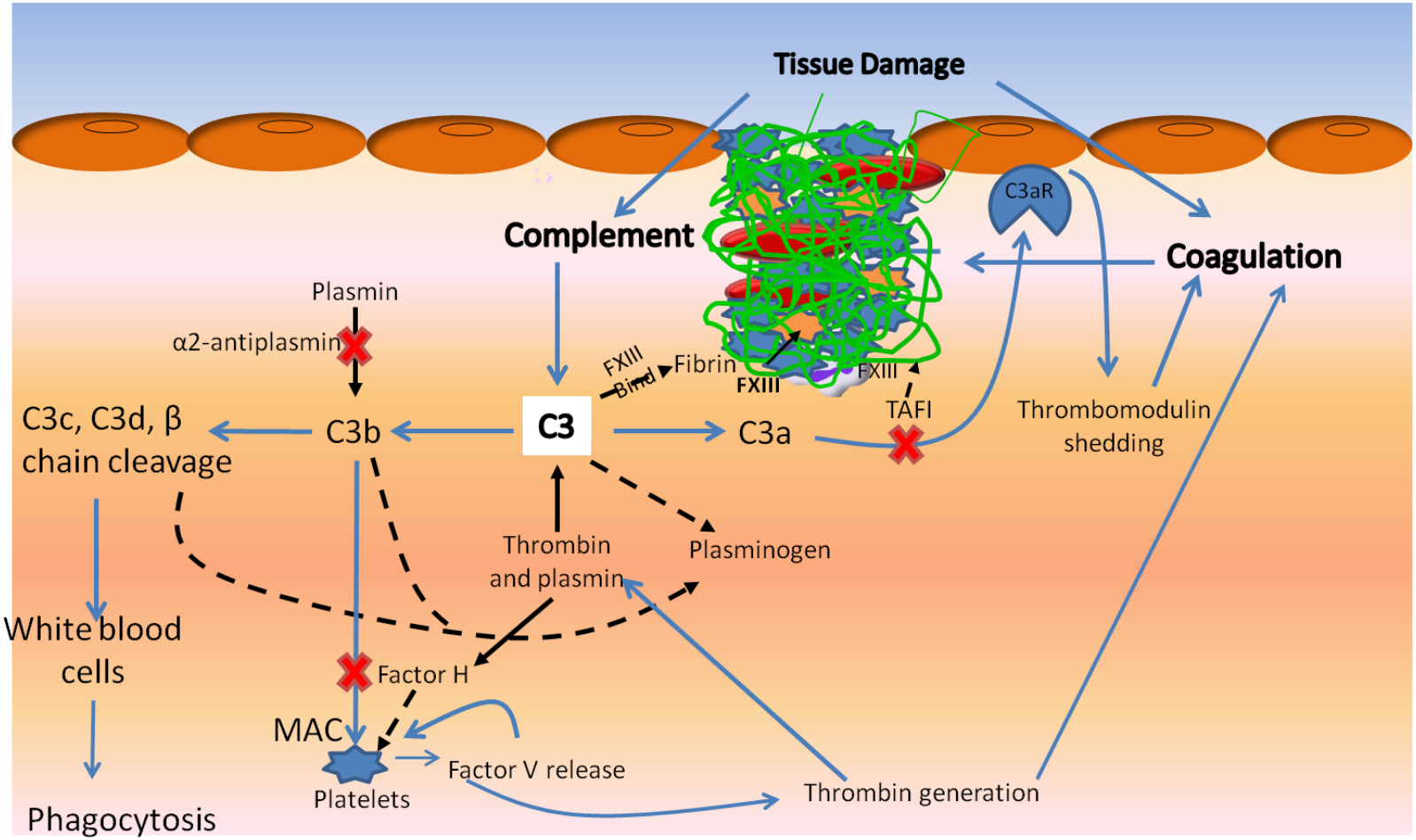
Tissue Damage
Complement
Coagulation
C3aR
Plasmin
α2-antiplasmin
FXIII
Bind
Fibrin
FXIII
FXIII
TAFI
Thrombomodulin
shedding
C3c, C3d, β
chain cleavage
C3b
C3
C3a
Thrombin
and plasmin
Plasminogen
White blood
cells
Factor H
MAC
Factor V release
Platelets
Thrombin generation
Phagocytosis

Figure 67: Interaction between C3, factor H and components of the coagulation/fibrinolytic cascades.

Tissue damage results in the initiation of coagulation, resulting in fibrin clot formation and complement activation. During fibrin formation complement C3 binds and is cross-linked to the forming fibrin and pathogens are trapped in the forming clot. C3 is cleaved within fibrin clots either by the C3 convertase or by thrombin/plasmin cleavage. C3 and its cleavage products also bind to plasminogen, possibly to prevent recruitment of plasminogen by pathogens. The C3 cleavage product C3a binds to the C3a receptor (C3aR), which initiates thrombomodulin shedding, responsible for increased fibrin formation and platelet clumping. Thrombin activatable fibrinolysis inhibitor (TAFI) inhibits this process by cleaving C3a into C3a-desArg. Cleavage products C3b recruit the remaining complement components in the formation of the membrane attack complex (MAC), which lyse both pathogens within the fibrin clot and platelets. Platelets release factor V from α granules, aiding the formation of the prothrombinase complex and therefore coagulation by the production of thrombin. Platelets are protected from this lysis by the complement inhibitor factor H. C3a promotes chemotaxis of white blood cells to the clot site, whilst opsonisation by C3b and iC3b of the clot may promote white blood cell phagocytosis of pathogens within fibrin clots.

Table 17: The mode of exploitation of the fibrinolytic system by pathogens

Pathogen	Mode of action	Reference
H.influenzae	Binds plasminogen via protein E on its surface, cleavage by u-PA resulted in cleavage of fibrin and C3b	(Barthel et al. 2012b)
B. burgdorferi	Binds plasminogen via lipoprotein A on its surface, activated by host plasminogen activators. Plasmin produced is protected from α2-antiplasmin	(Fuchs et al. 1994)
B. burgdorferi	Binds plasminogen via ErpP, ErpA, ErpC on its surface, activated to plasmin by u-PA	(Brissette et al. 2009)
Y. pestis and *S. enterica*	Membrane bound proteases, Omptins, inactivate PAI-1	(Haiko et al. 2010)
M. fermentans	Binds plasminogen via two sites on its surface, cleavage by u-PA results in host cell invasion	(Yavlovich et al. 2001)
Streptococci	Bind plasminogen via M proteins on its surface, activated by streptokinase produced by the streptococci	(Bennasr et al. 1994)
Staphylococci	Produce the plasminogen activator staphylokinase; however, a small amount of plasmin is required for full activation	(Grella and Castellino 1997)
Y. pestis	Pla is a plasminogen activator and inactivates α2-antiplasmin	(Kukkonen et al. 2001)

In the context of cardiovascular disease, increased fibrinolysis times and decreased plasmin generation lead to a pro-thrombotic state, eventually leading to the development of occlusive thrombi and ultimately MI or a stroke. Increased fibrinolysis times as a result of C3 is particularly important in CVD, as individuals with CVD and T2DM have higher plasma levels of C3 compared with healthy controls, with increased C3 levels pre-dating disease presentation. (Ajjan et al. 2005;Carter et al. 2009;Muscari et al. 2000;Onat et al. 2005;Somani et al. 2006). In

relatives with a family history of diabetes, levels of C3 were significantly higher than healthy controls, with a significant association of C3 with prolonged fibrinolysis times observed in these individuals (Schroeder et al. 2010). Hess et al (2011) also found that fibrinolysis times were prolonged in type 1 diabetic children compared with controls and this correlated with plasma levels of C3 in these individuals (Hess et al. 2011), suggesting C3 is pro-thrombotic and may contribute to increased CVD risk. A recent study demonstrated that C3b deposition on endothelial cells as a result of P-selectin binding was responsible for microvascular thrombosis (Morigi et al. 2011). Once bound to P-selectin C3b recruited factor B to form the C3 convertase and generated C3a. C3a once bound to C3aR on the endothelial cells, was responsible for thrombomodulin shedding from the endothelial cells and therefore resulting in increased fibrin deposition and platelet clumping. Thrombomodulin is an important cofactor in the thrombin mediated activation of the anticoagulant protein C and the fibrinolysis inhibitor TAFI (Van de Wouwer and Conway 2004), and therefore has anti-coagulant functions, however as highlighted by Morigi et al (2011) thrombomodulin shedding resulted in a pro-thrombotic state. The importance of this study in cardiovascular thrombosis is highlighted by the increased expression of P-selection on endothelial cells at the site of active atherosclerotic plaque formation (Johnson-Tidey et al. 1994) and increased deposition of complement activation products C3b on endothelial cells during atherosclerotic plaque development (Yin et al. 2007) suggesting these events might lead to a pro-thrombotic state as a result of thrombomodulin shedding. Alternatively, TAFI may be important in inhibiting this process, as TAFI can inactivate C3a and C5a by cleaving the carboxyl terminal lysine or arginine residues (Campbell et al. 2001;Leung et al. 2008) in the formation of C3a desArg. Unlike C3a, C3a desArg formed as a result of carboxypeptidase B cleavage was unable to bind C3aR (Wilken et al. 1999). This suggests that TAFI may prevent thrombomodulin shedding and the resulting fibrin formation and platelet clumping observed in the presence of intact C3a, however if thrombomodulin shedding were to occur then activation of TAFI would be prevented and the protective mechanisms of TAFI would also be lost.

In the context of CVD, proteomics is only starting to be fully appreciated in the discovery of biomarkers. By comparing plasma from patients with coronary artery stenosis with controls with normal arteries, 95 proteins were differentially

expressed, with up-regulation of proteins from the following protein groups: complement (C1, C3, C4, C5a and factor B), coagulation (fibrinogen γ chain, prothrombin) and fibrinolysis (α2 antiplasmin) (Donahue et al. 2006). A decrease in α-antitrypsin isoforms and increase in fibrinogen γ isoforms was observed in patients with acute coronary syndrome compared with healthy controls (Mateos-Caceres et al. 2004). A recent study comparing systemic plasma to plasma from an atherosclerotic plaque rupture site from MI patients, found that the latter contained 9 proteins which were differentially expressed including an increase in complement factor H related protein 2 and a decrease in complement factor B, C5, C7, carboxypeptidase N, plasminogen, protein S, pigment epithelial derived factor and matrix metalloprotease 9 (Distelmaier et al. 2012). This study has highlighted the local complement activation and potential regulation (complement factor H related protein 2) that occurs at coronary atherosclerotic plaque rupture sites. These studies have highlighted the role of complement as a marker of CVD and further showed that complement was involved in disease pathogenesis, which confirms the role of complement in disease pathogenesis previously observed in the in vitro data.

Proteomic studies have also highlighted the importance of complement and coagulation factors during plasma clot formation. In a proteomic study by Distelmaier et al (2009), complement activation was observed in the coronary thrombi from patients with acute MI, with increased levels of C3c, C3a and C5a and deposition of the activation products C3d and C5b-9. As a result of anaphylatoxin production, neutrophils were actively recruited to the thrombus site, suggesting complement activation either as a result of the conventional pathways or as a result of thrombin and plasmin generation potentiate the detrimental effects of complement in MI. Several studies have compared plasma to plasma derived serum, with components of the coagulation and complement cascades found to be depleted in serum compared to plasma. Interestingly, C3 and clusterin were found to have molecular weights matching cleavage products in serum with C3 cleavage resulting in C3b and C3dg (Misek et al. 2005). Analysis of the serum peptidome (low molecular weight peptides) found fibrinogen, prothrombin, albumin, apolipoprotein A1, collagen, C4, C3 and several cellular components (Tammen et al. 2005). These studies suggest C3 is cleaved and confirms the cleavage products of C3 observed in the present study following plasmin cleavage of C3. Initiation of coagulation in

plasma and whole blood by the addition of tissue factor and calcium, removed the fibrin clot, and then monitored cleavage products of coagulation and complement proteins (changes in molecular weight) in the supernatant compared to plasma controls. This method observed the majority of coagulation and complement proteins, however cleavage only occured in FX, FV, prothrombin, protein S, fibrinogen, FXIII, complement C1r, C5, C6 and C9(Niessen et al. 2011). Complement C3 cleavage in this study was not identified however this could be because they measured components in the supernatant and not the actual thrombi. Whereas the direct analysis of perfused solubilised plasma thrombi identified complement proteins C3 and factor H along with proteins involved in coagulation (fibrin α, β and γ chains, FXIII, α2-macroglobulin, kininogen) protein inhibition (inter-alpha-trypsin inhibitor) and binding proteins (fibronectin) (Howes et al. 2012). These studies have not only highlighted the importance of complement and coagulation proteins in plasma clot formation, but also highlighted the ability of complement proteins to be cleaved and potentially activated within plasma clots. In the future, proteomics could be used to profile protein production and protein cleavage resulting from CVD processes, allowing quick and cheap diagnostic tests to be developed for the identification of at risk patients. For example, in the case of MI, sera from MI patients compared with normal individuals were found to produce increased levels of C3f and FpA, both of which underwent additional amino terminal truncation in the formation of a distinct peptide pattern (Marshall et al. 2003). Alternatively, proteomics could be used to determine the changing protein profile of CVD patients following treatment, allowing practitioners to determine the best treatment profile for different stages of the disease.

In conclusion, this study has shown that complement C3 and factor H are not only substrates of coagulation specific enzymes (FXIII, thrombin and plasmin), but in the case of C3 are also involved in fibrin function by influencing the interactions and functions of fibrinolytic proteins. These effects may be important in preventing pathogen invasion whilst promoting the complement response within the fibrin clot required for pathogen removal. Whilst factor H did not have the same effects within fibrin clots as C3, its function within fibrin clots is likely protective, to prevent uncontrolled complement activation and tissue damage. This study has added to the

growing body of evidence which suggests complement and coagulation pathways interact for the purposes of blood loss and pathogen removal. This study has also highlighted the importance of identifying proteins involved in plasma clot formation by proteomics and validating their roles in fibrin formation. In the context of CVD, understanding the mechanisms involved in fibrin formation and lysis are important in determining at risk individuals.

Chapter 8 References

2011, *Global Atlas on cardiovascular disease prevention and control*, World Health Organisation, Geneva.

AbdAlla, S., Lother, H., Langer, A., el, F.Y., & Quitterer, U. 2004. Factor XIIIA transglutaminase crosslinks AT1 receptor dimers of monocytes at the onset of atherosclerosis. *Cell*, 119, (3) 343-354.

Aebersold, R. & Mann, M. 2003. Mass spectrometry-based proteomics. *Nature*, 422, (6928) 198-207.

Ahn, J.H., Lee, H.J., Lee, E.K., Yu, H.K., Lee, T.H., Yoon, Y., Kim, S.J., & Kim, J.S. 2011. Antiangiogenic kringles derived from human plasminogen and apolipoprotein(a) inhibit fibrinolysis through a mechanism that requires a functional lysine-binding site. *Biological Chemistry*, 392, (4) 347-356.

Ajjan, R., Grant, P.J., Futers, T.S., Brown, J.M., Cymbalista, C.M., Boothby, M., & Carter, A.M. 2005. Complement C3 and C-reactive protein levels in patients with stable coronary artery disease. *Thrombosis and Haemostasis*, 94, (5) 1048-1053.

Amara, U., Flierl, M.A., Rittirsch, D., Klos, A., Chen, H., Acker, B., Bruckner, U.B., Nilsson, B., Gebhard, F., Lambris, J.D., & Huber-Lang, M. 2010. Molecular Intercommunication between the Complement and Coagulation Systems. *Journal of Immunology*, 185, (9) 5628-5636.

Amento, E.P., Ehsani, N., Palmer, H., & Libby, P. 1991. Cytokines and Growth-Factors Positively and Negatively Regulate Interstitial Collagen Gene-Expression in Human Vascular Smooth-Muscle Cells. *Arteriosclerosis and Thrombosis*, 11, (5) 1223-1230.

Anderson, N.L., Polanski, M., Pieper, R., Gatlin, T., Tirumalai, R.S., Conrads, T.P., Veenstra, T.D., Adkins, J.N., Pounds, J.G., Fagan, R., & Lobley, A. 2004. The human plasma proteome - A nonredundant list developed by combination of four separate sources. *Molecular & Cellular Proteomics*, 3, (4) 311-326.

Arrell, D.K., Neverova, I., & Van Eyk, J.E. 2001. Cardiovascular proteomics - Evolution and potential. *Circulation Research*, 88, (8) 763-773.

Backes, B.J., Harris, J.L., Leonetti, F., Craik, C.S., & Ellman, J.A. 2000. Synthesis of positional-scanning libraries of fluorogenic peptide substrates to define the extended substrate specificity of plasmin and thrombin. *Nature Biotechnology*, 18, (2) 187-193.

Bajaj, M.S., Birktoft, J.J., Steer, S.A., & Bajaj, S.P. 2001. Structure and biology of tissue factor pathway inhibitor. *Thrombosis and Haemostasis*, 86, (4) 959-972.

Bale, M.D. & Mosher, D.F. 1986. Thrombospondin is a substrate for blood coagulation factor XIIIa. *Biochemistry*, 25, (19) 5667-5673.

Bale, M.D., Westrick, L.G., & Mosher, D.F. 1985. Incorporation of thrombospondin into fibrin clots. *J.Biol.Chem.*, 260, (12) 7502-7508.

Barillari, G., Albonici, L., Incerpi, S., Bogetto, L., Pistritto, G., Volpi, A., Ensoli, B., & Manzari, V. 2001. Inflammatory cytokines stimulate vascular smooth muscle cells locomotion and growth by enhancing alpha 5 beta 1 integrin expression and function. *Atherosclerosis*, 154, (2) 377-385.

Barthel, D., Schindler, S., & Zipfel, P. F. Plasminogen is a complement inhibitor. Journal of Biological Chemistry 287, 18831-18842. 2012a.

Barthel, D., Singh, B., Riesbeck, K., & Zipfel, P.F. 2012b. Haemophilus influenzae Uses the Surface Protein E To Acquire Human Plasminogen and To Evade Innate Immunity. *Journal of Immunology*, 188, (1) 379-385.

Barua, R.S., Sy, F., Srikanth, S., Huang, G., Javed, U., Buhari, C., Margosan, D., & Ambrose, J.A. 2010. Effects of Cigarette Smoke Exposure on Clot Dynamics and Fibrin Structure An Ex Vivo Investigation. *Arteriosclerosis Thrombosis and Vascular Biology*, 30, (1) 75-79.

Bauer, R., Hansen, S.L., Jones, G., Suenson, E., Thorsen, S., & Ogendal, L. 1994. Fibrin Structures During Tissue-Type Plasminogen Activator-Mediated Fibrinolysis Studied by Laser-Light Scattering - Relation to Fibrin Enhancement of Plasminogen Activation. *European Biophysics Journal with Biophysics Letters*, 23, (4) 239-252.

Bendixen, E., Borth, W., & Harpel, P.C. 1993. Transglutaminases catalyze cross-linking of plasminogen to fibronectin and human endothelial cells. *J.Biol.Chem.*, 268, (29) 21962-21967.

Bendixen, E., Harpel, P.C., & Sottrup-Jensen, L. 1995. Location of the major epsilon-(gamma-glutamyl)lysyl cross-linking site in transglutaminase-modified human plasminogen. *J.Biol.Chem.*, 270, (30) 17929-17933.

Benelli, R., Morini, M., Carrozzino, F., Ferrari, N., Minghelli, S., Santi, L., Cassatella, M., Noonan, D.M., & Albini, A. 2001. Neutrophils as a key cellular target for angiostatin: implications for regulation of angiogenesis and inflammation. *Faseb Journal*, 15, (14) 267-269.

Bennasr, A., Wistedt, A., Ringdahl, U., & Sjobring, U. 1994. Streptokinase Activates Plasminogen Bound to Human Group-C and Group-G Streptococci Through M-Like Proteins. *European Journal of Biochemistry*, 222, (2) 267-276.

Berhane, B.T., Zong, C.G., Liem, D.A., Huang, A., Le, S., Edmondson, R.D., Jones, R.C., Qiao, X., Whitelegge, J.P., Ping, P.P., & Vondriska, T.M. 2005. Cardiovascular-related proteins identified in human plasma by the HUPO Plasma Proteome Project pilot Phase. *Proteomics*, 5, (13) 3520-3530.

Bhakdi, S. & Tranumjensen, J. 1986. C5B-9 Assembly - Average Binding of One C9 Molecule to C5B-8 Without Poly-C9 Formation Generates A Stable Transmembrane Pore. *Journal of Immunology*, 136, (8) 2999-3005.

Bini, A., Fenoglio, J.J., Mesatejada, R., Kudryk, B., & Kaplan, K.L. 1989. Identification and Distribution of Fibrinogen, Fibrin, and Fibrin(Ogen) Degradation Products in Atherosclerosis - Use of Monoclonal-Antibodies. *Arteriosclerosis*, 9, (1) 109-121.

Biro, A., Thielens, N.M., Cervenak, L., Prohaszka, Z., Fust, G., & Arlaud, G.J. 2007. Modified low density lipoproteins differentially bind and activate the C1 complex of complement. *Molecular Immunology*, 44, (6) 1169-1177.

Bjorhall, K., Miliotis, T., & Davidsson, P. 2005. Comparison of different depletion strategies for improved resolution in proteomic analysis of human serum samples. *Proteomics*, 5, (1) 307-317.

Blankenberg, S., Barbaux, S., & Tiret, L. 2003. Adhesion molecules and atherosclerosis. *Atherosclerosis*, 170, (2) 191-203.

Bockenstedt, P., McDonagh, J., & Handin, R.I. 1986. Binding and covalent cross-linking of purified von Willebrand factor to native monomeric collagen. *J.Clin.Invest*, 78, (2) 551-556.

Bosma, P.J., Rijken, D.C., & Nieuwenhuizen, W. 1988. Binding of Tissue-Type Plasminogen-Activator to Fibrinogen Fragments. *European Journal of Biochemistry*, 172, (2) 399-404.

Brissette, C.A., Haupt, K., Barthel, D., Cooley, A.E., Bowman, A., Skerka, C., Wallich, R., Zipfel, P.F., Kraiczy, P., & Stevenson, B. 2009. Borrelia burgdorferi Infection-Associated Surface Proteins ErpP, ErpA, and ErpC Bind Human Plasminogen. *Infection and Immunity*, 77, (1) 300-306.

Brummel-Ziedins, K.E., Vossen, C.Y., Butenas, S., Mann, K.G., & Rosendaal, F.R. 2005. Thrombin generation profiles in deep venous thrombosis. *Journal of Thrombosis and Haemostasis*, 3, (11) 2497-2505.

Brunnee, T., Laporta, C., Reddigari, S.R., Salerno, V.M., Kaplan, A.P., & Silverberg, M. 1993. Activation of Factor-Xi in Plasma Is Dependent on Factor-Xii. *Blood*, 81, (3) 580-586.

Buono, C., Come, C.E., Witztum, J.L., Maguire, G.F., Connelly, P.W., Carroll, M., & Lichtman, A.H. 2002. Influence of C3 deficiency on atherosclerosis. *Circulation*, 105, (25) 3025-3031.

Burke, A.P., Tracy, R.P., Kolodgie, F., Malcom, G.T., Zieske, A., Kutys, R., Pestaner, J., Smialek, J., & Virmani, R. 2002. Elevated C-reactive protein values and atherosclerosis in sudden coronary death - Association with different pathologies. *Circulation*, 105, (17) 2019-2023.

Burton, P.R., Clayton, D.G., Cardon, L.R., Craddock, N., Deloukas, P., Duncanson, A., Kwiatkowski, D.P., McCarthy, M.I., Ouwehand, W.H., Samani, N.J., Todd, J.A., Donnelly, P., Barrett, J.C., Davison, D., Easton, D., Evans, D., Leung, H.T., Marchini, J.L., Morris, A.P., Spencer, C.C.A., Tobin, M.D., Attwood, A.P., Boorman, J.P., Cant, B., Everson, U., Hussey, J.M., Jolley, J.D., Knight, A.S., Koch, K., Meech, E., Nutland, S., Prowse, C.V., Stevens, H.E., Taylor, N.C., Walters, G.R.,

Walker, N.M., Watkins, N.A., Winzer, T., Jones, R.W., McArdle, W.L., Ring, S.M., Strachan, D.P., Pembrey, M., Breen, G., St Clair, D., Caesar, S., Gordon-Smith, K., Jones, L., Fraser, C., Green, E.K., Grozeva, D., Hamshere, M.L., Holmans, P.A., Jones, I.R., Kirov, G., Moskvina, V., Nikolov, I., O'Donovan, M.C., Owen, M.J., Collier, D.A., Elkin, A., Farmer, A., Williamson, R., McGuffin, P., Young, A.H., Ferrier, I.N., Ball, S.G., Balmforth, A.J., Barrett, J.H., Bishop, D.T., Iles, M.M., Maqbool, A., Yuldasheva, N., Hall, A.S., Braund, P.S., Dixon, R.J., Mangino, M., Stevens, S., Thompson, J.R., Bredin, F., Tremelling, M., Parkes, M., Drummond, H., Lees, C.W., Nimmo, E.R., Satsangi, J., Fisher, S.A., Forbes, A., Lewis, C.M., Onnie, C.M., Prescott, N.J., Sanderson, J., Mathew, C.G., Barbour, J., Mohiuddin, M.K., Todhunter, C.E., Mansfield, J.C., Ahmad, T., Cummings, F.R., Jewell, D.P., Webster, J., Brown, M.J., Lathrop, G.M., Connell, J., Dominiczak, A., Marcano, C.A.B., Burke, B., Dobson, R., Gungadoo, J., Lee, K.L., Munroe, P.B., Newhouse, S.J., Onipinla, A., Wallace, C., Xue, M.Z., Caulfield, M., Farrall, M., Barton, A., Bruce, I.N., Donovan, H., Eyre, S., Gilbert, P.D., Hider, S.L., Hinks, A.M., John, S.L., Potter, C., Silman, A.J., Symmons, D.P.M., Thomson, W., Worthington, J., Dunger, D.B., Widmer, B., Frayling, T.M., Freathy, R.M., Lango, H., Perry, J.R.B., Shields, B.M., Weedon, M.N., Hattersley, A.T., Hitman, G.A., Walker, M., Elliott, K.S., Groves, C.J., Lindgren, C.M., Rayner, N.W., Timpson, N.J., Zeggini, E., Newport, M., Sirugo, G., Lyons, E., Vannberg, F., Brown, M.A., Franklyn, J.A., Heward, J.M., Simmonds, M.J., Hill, A.V.S., Bradbury, L.A., Farrar, C., Pointon, J.J., Wordsmith, P., Gough, S.C.L., Seal, S., Stratton, M.R., Rahman, N., Ban, M., Goris, A., Sawcer, S.J., Compston, A., Conway, D., Jallow, M., Bumpstead, S.J., Chaney, A., Downes, K., Ghori, M.J.R., Gwilliam, R., Inouye, M., Keniry, A., King, E., McGinnis, R., Potter, S., Ravindrarajah, R., Whittaker, P., Withers, D., Easton, D., Pereira-Gale, J., Hallgrimsdottir, I.B., Howie, B.N., Su, Z., Teo, Y.Y., Vukcevic, D., Bentley, D., Caulfield, M., Mathew, C.G., & Worthington, J. 2007. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature*, 447, (7145) 661-678.

Campbell, W., Okada, N., & Okada, H. 2001. Carboxypeptidase R is an inactivator of complement-derived inflammatory peptides and an inhibitor of fibrinolysis. *Immunological Reviews*, 180, 162-167.

Carr, M.E. 1988. Fibrin Formed in Plasma Is Composed of Fibers More Massive Than Those Formed from Purified Fibrinogen. *Thrombosis and Haemostasis*, 59, (3) 535-539.

Carr, M.E. & Alving, B.M. 1995. Effect of Fibrin Structure on Plasmin-Mediated Dissolution of Plasma Clots. *Blood Coagulation & Fibrinolysis*, 6, (6) 567-573.

Carr, M.E. & Carr, S.L. 1995. Fibrin Structure and Concentration Alter Clot Elastic-Modulus But do Not Alter Platelet-Mediated Force Development. *Blood Coagulation & Fibrinolysis*, 6, (1) 79-86.

Carter, A.M., Catto, A.J., & Grant, P.J. 1999. Association of the alpha-fibrinogen Thr312Ala polymorphism with poststroke mortality in subjects with atrial fibrillation. *Circulation*, 99, (18) 2423-2426.

Carter, A.M., Cymbalista, C.M., Spector, T.D., & Grant, P.J. 2007. Heritability of clot formation, morphology, and lysis - The EuroCLOT study. *Arteriosclerosis Thrombosis and Vascular Biology*, 27, (12) 2783-2789.

Carter, A.M., Prasad, U.K., & Grant, P.J. 2009. Complement C3 and C-reactive protein in male survivors of myocardial infarction. *Atherosclerosis*, 203, (2) 538-543.

Castell, J.V., Gomezlechon, M.J., David, M., Fabra, R., Trullenque, R., & Heinrich, P.C. 1990. Acute-Phase Response of Human Hepatocytes - Regulation of Acute-Phase Protein-Synthesis by Interleukin-6. *Hepatology*, 12, (5) 1179-1186.

Cederholm-Williams, SA. Concentration of plasminogen and antiplasmin in plasma and serum. J.Clin.Pathol 34, 979-981. 1981.

Chavakis, T., Athanasopoulos, A., Rhee, J.S., Orlova, V., Schmidt-Woll, T., Bierhaus, A., May, A.E., Celik, I., Nawroth, P.P., & Preissner, K.T. 2005. Angiostatin is a novel anti-inflammatory factor by inhibiting leukocyte recruitment. *Blood*, 105, (3) 1036-1043.

Chen, M.J., Masaki, T., & Sawamura, T. 2002. LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells: implications in endothelial dysfunction and atherosclerosis. *Pharmacology & Therapeutics*, 95, (1) 89-100.

Chesne, S., Villiers, C.L., Arlaud, G.J., Lacroix, M.B., & Colomb, M.G. 1982. Fluid-Phase Interaction of C1 Inhibitor (C1inh) and the Sub-Components C1r and C1s of the first Component of Complement, C1. *Biochemical Journal*, 201, (1) 61-70.

Choay, J., Petitou, M., Lormeau, J.C., Sinay, P., Casu, B., & Gatti, G. 1983. Structure-Activity Relationship in Heparin - A Synthetic Pentasaccharide with High-Affinity for Anti-Thrombin-Iii and Eliciting High Anti-Factor-Xa Activity. *Biochemical and Biophysical Research Communications*, 116, (2) 492-499.

Cleary, D.B. & Maurer, M.C. 2006. Characterizing the specificity of activated factor XIII for glutamine-containing substrate peptides. *Biochimica et Biophysica Acta-Proteins and Proteomics*, 1764, (7) 1207-1217.

Cohen, I., Blankenberg, T.A., Borden, D., Kahn, D.R., & Veis, A. 1980. Factor XIIIa-catalyzed cross-linking of platelet and muscle actin. Regulation by nucleotides. *Biochim.Biophys.Acta*, 628, (3) 365-375.

Cohen, I., Young-Bandala, L., Blankenberg, T.A., Siefring, G.E., Jr., & Bruner-Lorand, J. 1979. Fibrinoligase-catalyzed cross-linking of myosin from platelet and skeletal muscle. *Arch.Biochem.Biophys.*, 192, (1) 100-111.

Collet, J.P., Allali, Y., Lesty, C., Tanguy, M.L., Silvain, J., Ankri, A., Blanchet, B., Dumaine, R., Gianetti, J., Payot, L., Weisel, J.W., & Montalescot, G. 2006. Altered Fibrin Architecture Is Associated With Hypofibrinolysis and Premature Coronary Atherothrombosis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 26, (11) 2567-2573.

Collet, J.P., Park, D., Lesty, C., Soria, J., Soria, C., Montalescot, G., & Weisel, J.W. 2000. Influence of fibrin network conformation and fibrin fiber diameter on

fibrinolysis speed - Dynamic and structural approaches by confocal microscopy. *Arteriosclerosis Thrombosis and Vascular Biology*, 20, (5) 1354-1361.

Collet, J.P., Shuman, H., Ledger, R.E., Lee, S.T., & Weisel, J.W. 2005. The elasticity of an individual fibrin fiber in a clot. *Proceedings of the National Academy of Sciences of the United States of America*, 102, (26) 9133-9137.

Corbett, S.A., Lee, L., Wilson, C.L., & Schwarzbauer, J.E. 1997. Covalent cross-linking of fibronectin to fibrin is required for maximal cell adhesion to a fibronectin-fibrin matrix. *J.Biol.Chem.*, 272, (40) 24999-25005.

Cottrell, B.A., Strong, D.D., Watt, K.W., & Doolittle, R.F. 1979. Amino acid sequence studies on the alpha chain of human fibrinogen. Exact location of cross-linking acceptor sites. *Biochemistry*, 18, (24) 5405-5410.

Cunnion, K.M., Hair, P.S., & Buescher, E.S. 2004. Cleavage of Complement C3b to iC3b on the Surface of Staphylococcus aureus Is Mediated by Serum Complement Factor I. *Infection and Immunity*, 72, (5) 2858-2863.

Daffern, P.J., Pfeifer, P.H., Ember, J.A., & Hugli, T.E. 1995. C3A Is A Chemotaxin for Human Eosinophils But Not for Neutrophils .1. C3A Stimulation of Neutrophils Is Secondary to Eosinophil Activation. *Journal of Experimental Medicine*, 181, (6) 2119-2127.

Dahlback, B. 1991. Protein-S and C4B-Binding Protein - Components Involved in the Regulation of the Protein-C Anticoagulant System. *Thrombosis and Haemostasis*, 66, (1) 49-61.

Dale, G.L., Friese, P., Batar, P., Hamilton, S.F., Reed, G.L., Jackson, K.W., Clemetson, K.J., & Alberio, L. 2002. Stimulated platelets use serotonin to enhance their retention of procoagulant proteins on the cell surface. *Nature*, 415, (6868) 175-179.

Danesh, J., Lewington, S., Thompson, S.G., Lowe, G.D.O., & Collins, R. 2005. Plasma fibrinogen level and the risk of major cardiovascular diseases and nonvascular mortality - An individual participant meta-analysis. *Jama-Journal of the American Medical Association*, 294, (14) 1799-1809.

Davignon, J. & Ganz, P. 2004. Role of endothelial dysfunction in atherosclerosis. *Circulation*, 109, (23) 27-32.

de Cordoba, S.R. & de Jorge, E.G. 2008. Translational mini-review series on complement factor H: genetics and disease associations of human complement factor H. *Clin.Exp.Immunol.*, 151, (1) 1-13.

Del, C., I, Cruz, M.A., Zhang, H., Lopez, J.A., & Afshar-Kharghan, V. 2005. Platelet activation leads to activation and propagation of the complement system. *J.Exp.Med.*, 201, (6) 871-879.

Distelmaier, K., Adlbrecht, C., Jakowitsch, J., Wagner, O., Gerner, C., Lang, I. M., & Kubicek, M. Proteomic profiling of acute coronary thrombosis reveals a local

decrease in pigment epithelium-derived factor in acute myocardial infarction. Clinical Science 123[2], 111-119. 2012.

Distelmaier, K., Adlbrecht, C., Jakowitsch, J., Winkler, S., Dunkler, D., Gerner, C., Wagner, O., Lang, I.M., & Kubicek, M. 2009. Local complement activation triggers neutrophil recruitment to the site of thrombus formation in acute myocardial infarction. *Thromb.Haemost.*, 102, (3) 564-572.

Donahue, M.P., Rose, K., Hochstrasser, D., Vonderscher, J., Grass, P., Chibout, S.D., Nelson, C.L., Sinnaeve, P., Goldschmidt, P., & Granger, C.B. 2006. Discovery of proteins related to coronary artery disease using industrial-scale proteomics analysis of pooled plasma. *American Heart Journal*, 152, (3) 478-485.

Donners, M.M.P.C., Verluyten, M.J., Bouwman, F.G., Mariman, E.C.M., Devreese, B., Vanrobaeys, F., van Beeumen, J., van den Akker, L.H.J.M., Daemen, M.J.A.P., & Heeneman, S. 2005. Proteomic analysis of differential protein expression in human atherosclerotic plaque progression. *Journal of Pathology*, 206, (1) 39-45.

Dunn, E., Philippou, H., Ariens, R., & Grant, P. 2006. Molecular mechanisms involved in the resistance of fibrin to clot lysis by plasmin in subjects with type 2 diabetes mellitus. *Diabetologia*, 49, (5) 1071-1080.

Eastman, D., Wurm, F.M., Vanreis, R., & Higgins, D.L. 1992. A Region of Tissue Plasminogen-Activator That Affects Plasminogen Activation Differentially with Various Fibrin(Ogen)-Related Stimulators. *Biochemistry*, 31, (2) 419-422.

Eggertsen, G., Hellman, U., Lundwall, A., Folkersen, J., & Sjoquist, J. 1985. Characterization of tryptic fragments of human complement factor C3. *Mol.Immunol.*, 22, (8) 833-841.

Ehret, G.B., Munroe, P.B., Rice, K.M., Bochud, M., Johnson, A.D., Chasman, D.I., Smith, A.V., Tobin, M.D., Verwoert, G.C., Hwang, S.J., Pihur, V., Vollenweider, P., O'Reilly, P.F., Amin, N., Bragg-Gresham, J.L., Teumer, A., Glazer, N.L., Launer, L., Zhao, J.H., Aulchenko, Y., Heath, S., Sober, S., Parsa, A., Luan, J.A., Arora, P., Dehghan, A., Zhang, F., Lucas, G., Hicks, A.A., Jackson, A.U., Peden, J.F., Tanaka, T., Wild, S.H., Rudan, I., Igl, W., Milaneschi, Y., Parker, A.N., Fava, C., Chambers, J.C., Fox, E.R., Kumari, M., Go, M.J., van der Harst, P., Kao, W.H.L., Sjogren, M., Vinay, D.G., Alexander, M., Tabara, Y., Shaw-Hawkins, S., Whincup, P.H., Liu, Y.M., Shi, G., Kuusisto, J., Tayo, B., Seielstad, M., Sim, X., Nguyen, K.D.H., Lehtimaki, T., Matullo, G., Wu, Y., Gaunt, T.R., Onland-Moret, N.C., Cooper, M.N., Platou, C.G.P., Org, E., Hardy, R., Dahgam, S., Palmen, J., Vitart, V., Braund, P.S., Kuznetsova, T., Uiterwaal, C.S.P.M., Adeyemo, A., Palmas, W., Campbell, H., Ludwig, B., Tomaszewski, M., Tzoulaki, I., Palmer, N.D., Aspelund, T., Garcia, M., Chang, Y.P.C., O'Connell, J.R., Steinle, N.I., Grobbee, D.E., Arking, D.E., Kardia, S.L., Morrison, A.C., Hernandez, D., Najjar, S., McArdle, W.L., Hadley, D., Brown, M.J., Connell, J.M., Hingorani, A.D., Day, I.N.M., Lawlor, D.A., Beilby, J.P., Lawrence, R.W., Clarke, R., Hopewell, J.C., Ongen, H., Dreisbach, A.W., Li, Y.L., Young, J.H., Bis, J.C., Kahonen, M., Viikari, J., Adair, L.S., Lee, N.R., Chen, M.H., Olden, M., Pattaro, C., Bolton, J.A.H., Kottgen, A., Bergmann, S., Mooser, V., Chaturvedi, N., Frayling, T.M., Islam, M., Jafar, T.H., Erdmann, J., Kulkarni, S.R., Bornstein, S.R., Grassler, J., Groop, L., Voight, B.F., Kettunen, J., Howard, P.,

Taylor, A., Guarrera, S., Ricceri, F., Emilsson, V., Plump, A., Barroso, I.S., Khaw, K.T., Weder, A.B., Hunt, S.C., Sun, Y.V., Bergman, R.N., Collins, F.S., Bonnycastle, L.L., Scott, L.J., Stringham, H.M., Peltonen, L., Perola, M., Vartiainen, E., Brand, S.M., Staessen, J.A., Wang, T.J., Burton, P.R., Artigas, M.S., Dong, Y.B., Snieder, H., Wang, X.L., Zhu, H.D., Lohman, K.K., Rudock, M.E., Heckbert, S.R., Smith, N.L., Wiggins, K.L., Doumatey, A., Shriner, D., Veldre, G., Viigimaa, M., Kinra, S., Prabhakaran, D., Tripathy, V., Langefeld, C.D., Rosengren, A., Thelle, D.S., Corsi, A.M., Singleton, A., Forrester, T., Hilton, G., McKenzie, C.A., Salako, T., Iwai, N., Kita, Y., Ogihara, T., Ohkubo, T., Okamura, T., Ueshima, H., Umemura, S., Eyheramendy, S., Meitinger, T., Wichmann, H.E., Cho, Y.S., Kim, H.L., Lee, J.Y., Scott, J., Sehmi, J.S., Zhang, W.H., Hedblad, B., Nilsson, P., Smith, G.D., Wong, A., Narisu, N., Stancakova, A., Raffel, L.J., Yao, J., Kathiresan, S., O'Donnell, C.J., Schwartz, S.M., Ikram, M.A., Longstreth, W.T., Mosley, T.H., Seshadri, S., Shrine, N.R.G., Wain, L.V., Morken, M.A., Swift, A.J., Laitinen, J., Prokopenko, I., Zitting, P., Cooper, J.A., Humphries, S.E., Danesh, J., Rasheed, A., Goel, A., Hamsten, A., Watkins, H., Bakker, S.J.L., van Gilst, W.H., Janipalli, C.S., Mani, K.R., Yajnik, C.S., Hofman, A., Mattace-Raso, F.U.S., Oostra, B.A., Demirkan, A., Isaacs, A., Rivadeneira, F., Lakatta, E.G., Orru, M., Scuteri, A., Ala-Korpela, M., Kangas, A.J., Lyytikainen, L.P., Soininen, P., Tukiainen, T., Wurtz, P., Ong, R.T.H., Dorr, M., Kroemer, H.K., Volker, U., Volzke, H., Galan, P., Hercberg, S., Lathrop, M., Zelenika, D., Deloukas, P., Mangino, M., Spector, T.D., & Zhai, G.J. 2011. Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. *Nature*, 478, (7367) 103-109.

Eisenhardt, S.U., Habersberger, J., Murphy, A., Chen, Y.C., Woollard, K.J., Bassler, N., Qian, H., von zur Muhlen, C., Hagemeyer, C.E., Ahrens, I., Chin-Dusting, J., Bobik, A., & Peter, K. 2009. Dissociation of Pentameric to Monomeric C-Reactive Protein on Activated Platelets Localizes Inflammation to Atherosclerotic Plaques. *Circulation Research*, 105, (2) 128-137.

Ekdahl, K.N. & Nilsson, B. 1995. Phosphorylation of Complement Component C3 and C3 Fragments by A Human Platelet Protein-Kinase - Inhibition of Factor I-Mediated Cleavage of C3B. *Journal of Immunology*, 154, (12) 6502-6510.

Ekdahl, K.N. & Nilsson, B. 1999. Alterations in C3 activation and binding caused by phosphorylation by a casein kinase released from activated human platelets. *Journal of Immunology*, 162, (12) 7426-7433.

Ekdahl, K.N. & Nilsson, N. 2001. Phosphorylation of C3 by a casein kinase released from activated human platelets increases opsonization of immune complexes and binding to complement receptor type 1. *European Journal of Immunology*, 31, (4) 1047-1054.

Endo, Y., Nakazawa, N., Iwaki, D., Takahashi, M., Matsushita, M., & Fujita, T. 2010. Interactions of Ficolin and Mannose-Binding Lectin with Fibrinogen/Fibrin Augment the Lectin Complement Pathway. *Journal of Innate Immunity*, 2, (1) 33-42.

Engstrom, G., Hedblad, B., Janzon, L., & Lindgarde, F. 2005. Weight gain in relation to plasma levels of complement factor 3: results from a population-based cohort study. *Diabetologia*, 48, (12) 2525-2531.

Entwistle, R.A. & Furcht, L.T. 1988. C1Q Component of Complement Binds to Fibrinogen and Fibrin. *Biochemistry*, 27, (1) 507-512.

Eppihimer, M.J., Wolitzky, B., Anderson, D.C., Labow, M.A., & Granger, D.N. 1996. Heterogeneity of expression of E- and P-selectins in vivo. *Circulation Research*, 79, (3) 560-569.

Esmon, C.T. & Owen, W.G. 1981. Identification of An Endothelial-Cell Cofactor for Thrombin-Catalyzed Activation of Protein-C. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*, 78, (4) 2249-2252.

Essler, M., Retzer, M., Bauer, M., Heemskerk, J.W., Aepfelbacher, M., Siess, W. 1999. Mildly oxidized low density lipoprotein induces contraction of human endothelial cells through activation of Rho/Rho kinase and inhibition of myosin light chain phosphatase. *Journal of Biological Chemistry*, 274, 30361-30364

Fatah, K., Silveira, A., Tornvall, P., Karpe, F., Blomback, M., & Hamsten, A. 1996. Proneness to formation of tight and rigid fibrin gel structures in men with myocardial infarction at a young age. *Thrombosis and Haemostasis*, 76, (4) 535-540.

Fears, R. 1989. Binding of Plasminogen Activators to Fibrin - Characterization and Pharmacological Consequences. *Biochemical Journal*, 261, (2) 313-324.

Fernando, A.N., Furtado, P.B., Clark, S.J., Gilbert, H.E., Day, A.J., Sim, R.B., & Perkins, S.J. 2007. Associative and structural properties of the region of complement factor H encompassing the Tyr402His disease-related polymorphism and its interactions with heparin. *Journal of Molecular Biology*, 368, (2) 564-581.

Flaumenhaft, R. 2006. Formation and fate of platelet microparticles. *Blood Cells Molecules and Diseases*, 36, (2) 182-187.

Fleckenstein, B., Qiao, S.W., Larsen, M.R., Jung, G., Roepstorff, P., & Sollid, L.M. 2004. Molecular characterization of covalent complexes between tissue transglutaminase and gliadin peptides. *Journal of Biological Chemistry*, 279, (17) 17607-17616.

Fleury, V. & Anglescano, E. 1991. Characterization of the Binding of Plasminogen to Fibrin Surfaces - the Role of Carboxy-Terminal Lysines. *Biochemistry*, 30, (30) 7630-7638.

Fontaine, M., Demares, M.J., Koistinen, V., Day, A.J., Davrinche, C., Sim, R.B., & Ripoche, J. 1989. Truncated Forms of Human-Complement Factor-H. *Biochemical Journal*, 258, (3) 927-930.

Forneris, F., Ricklin, D., Wu, J., Tzekou, A., Wallace, R.S., Lambris, J.D., & Gros, P. 2010. Structures of C3b in Complex with Factors B and D Give Insight into Complement Convertase Formation. *Science*, 330, (6012) 1816-1820.

Francis, R.T., McDonagh, J., & Mann, K.G. 1986. Factor V is a substrate for the transamidase factor XIIIa. *J.Biol.Chem.*, 261, (21) 9787-9792.

Fraser, S.R., Booth, N.A., & Mutch, N.J. 2011. The antifibrinolytic function of factor XIII is exclusively expressed through alpha-antiplasmin cross-linking. *Blood*, 117, (23) 6371-6374.

Fredenburgh, J.C. & Nesheim, M.E. 1992. Lys-Plasminogen Is A Significant Intermediate in the Activation of Glu-Plasminogen During Fibrinolysis Invitro. *Journal of Biological Chemistry*, 267, (36) 26150-26156.

Fuchs, H., Wallich, R., Simon, M.M., & Kramer, M.D. 1994. The Outer Surface Protein-A of the Spirochete Borrelia-Burgdorferi Is A Plasmin(Ogen) Receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 91, (26) 12594-12598.

Fujita, T., Gigli, I., & Nussenzweig, V. 1978. Human C4-binding protein. II. Role in proteolysis of C4b by C3b-inactivator. *The Journal of experimental medicine*, 148, (4) 1044-1051.

Fujita, T., Inoue, T., Ogawa, K., Iida, K., & Tamura, N. 1987. The Mechanism of Action of Decay-Accelerating Factor (Daf) Daf Inhibits the Assembly of C-3 Convertases by Dissociating C2A and Bb. *Journal of Experimental Medicine*, 166, (5) 1221-1228.

Fung, T.T., Rimm, E.B., Spiegelman, D., Rifai, N., Tofler, G.H., Willett, W.C., & Hu, F.B. 2001. Association between dietary patterns and plasma biomarkers of obesity and cardiovascular disease risk. *American Journal of Clinical Nutrition*, 73, (1) 61-67.

Gabrielsson, B.G., Johansson, J.M., Lonn, M., Jernas, M., Olbers, T., Peltonen, M., Larsson, I., Lonn, L., Sjostrom, L., Carlsson, B., & Carlsson, L.M.S. 2003. High Expression of Complement Components in Omental Adipose Tissue in Obese Men. *Obesity*, 11, (6) 699-708.

Gage, M.C., Keen, J.N., Buxton, A.T., Bedi, M.K., & Findlay, J.B.C. 2009. Proteomic Analysis of IgE-Mediated Secretion by LAD2 Mast Cells. *Journal of Proteome Research*, 8, (8) 4116-4125.

Gigli, I., Fujita, T., & Nussenzweig, V. 1979. Modulation of the Classical Pathway C-3 Convertase by Plasma-Proteins C-4 Binding-Protein and C3B Inactivator. *Proceedings of the National Academy of Sciences of the United States of America*, 76, (12) 6596-6600.

Golan, M.D., Burger, R., & Loos, M. 1982. Conformational changes in C1q after binding to immune complexes: detection of neoantigens with monoclonal antibodies. *The Journal of Immunology*, 129, (2) 445-447.

Gorg, A., Weiss, W., & Dunn, M.J. 2005. Current two dimensional electrophoresis technology for proteomics (vol 4, pg 3665, 2004). *Proteomics*, 5, (3) 826-827.

Gorman, J.J. & Folk, J.E. 1980. Structural features of glutamine substrates for human plasma factor XIIIa (activated blood coagulation factor XIII). *J.Biol.Chem.*, 255, (2) 419-427.

Granger, J., Siddiqui, J., Copeland, S., & Remick, D. 2005. Albumin depletion of human plasma also removes low abundance proteins including the cytokines. *Proteomics*, 5, (18) 4713-4718.

Greenberg, C.S. & Shuman, M.A. 1982. The Zymogen Forms of Blood-Coagulation Factor-Xiii Bind Specifically to Fibrinogen. *Journal of Biological Chemistry*, 257, (11) 6096-6101.

Grella, D.K. & Castellino, F.J. 1997. Activation of human plasminogen by staphylokinase. Direct evidence that preformed plasmin is necessary for activation to occur. *Blood*, 89, (5) 1585-1589.

Gros, P., Milder, F.J., & Janssen, B.J.C. 2008. Complement driven by conformational changes. *Nature Reviews Immunology*, 8, (1) 48-58.

Gupta, M., Greenberg, C.S., Eckman, D.M., & Sane, D.C. 2007. Arterial vimentin is a transglutaminase substrate: A link between vasomotor activity and remodeling? *Journal of Vascular Research*, 44, (5) 339-344.

Hada, M., Kaminski, M., Bockenstedt, P., & McDonagh, J. 1986. Covalent crosslinking of von Willebrand factor to fibrin. *Blood*, 68, (1) 95-101.

Haffner, S.M., D'Agostino, R., Mykkanen, L., Tracy, R., Howard, B., Rewers, M., Selby, J., Savage, P.J., & Saad, M.F. 1999. Insulin sensitivity in subjects with type 2 diabetes - Relationship to cardiovascular risk factors: the Insulin Resistance Atherosclerosis Study. *Diabetes Care*, 22, (4) 562-568.

Hagel, L. 2001. Unit 10.9 Gel filtration chromatography. *Current Protocols in Molecular Biology*, 44, 10.9.1-10.9.2.

Haiko, J., Laakkonen, L., Juuti, K., Kalkkinen, N., & Korhonen, T.K. 2010. The Omptins of Yersinia pestis and Salmonella enterica Cleave the Reactive Center Loop of Plasminogen Activator Inhibitor 1. *Journal of Bacteriology*, 192, (18) 4553-4561.

Hamad, O.A., Nilsson, P.H., Wouters, D., Lambris, J.D., Ekdahl, K.N., & Nilsson, B. 2010. Complement component C3 binds to activated normal platelets without preceding proteolytic activation and promotes binding to complement receptor 1. *J.Immunol.*, 184, (5) 2686-2692.

Hartmer, R., Kaplan, D.A., Gebhardt, C.R., Ledertheil, T., & Brekenfeld, A. 2008. Multiple ion/ion reactions in the 3D ion trap: Selective reagent anion production for ETD and PTR from a single compound. *International Journal of Mass Spectrometry*, 276, (2-3) 82-90.

Hashimoto, H., Kitagawa, K., Hougaku, H., Shimizu, Y., Sakaguchi, M., Nagai, Y., Iyama, S., Yamanishi, H., Matsumoto, M., & Hori, M. 2001. C-reactive protein is an independent predictor of the rate of increase in early carotid atherosclerosis. *Circulation*, 104, (1) 63-67.

Hatano, Y., Taniuchi, S., Masuda, M., Tsuji, S., Ito, T., Hasui, M., Kobayashi, Y., & Kaneko, K. 2009. Phagocytosis of heat-killed Staphylococcus aureus by eosinophils: comparison with neutrophils. *Apmis*, 117, (2) 115-123.

Henriksson, P., Becker, S., Lynch, G., & McDonagh, J. 1985. Identification of intracellular factor XIII in human monocytes and macrophages. *J.Clin.Invest*, 76, (2) 528-534.

Hernandez-Mijares, A., Banuls, C., Bellod, L., Jover, A., Sola, E., Morillas, C., Victor, V.M., & Rocha, M. 2012. Effect of weight loss on C3 and C4 components of complement in obese patients. *European Journal of Clinical Investigation*, 42, (5) 503-509.

Hess, K., Ajjan, R., Dobo, J., Gal, P., & Schroeder, V. 2012. Effects of MASP-1 of the complement system on activation of coagulation factors and plasma clot formation. *Plos One*, 7, (4), e35690.

Hess, K., Alzahrani, S.H., Mathai, M., Schroeder, V., Carter, A.M., Howell, G., Koko, T., Strachan, M.W., Price, J.F., Smith, K.A., Grant, P.J., & Ajjan, R.A. 2011. A novel mechanism for hypofibrinolysis in diabetes: the role of complement C3. *Diabetologia*, 55, (4), 1103-1113

Hornyak, T.J. & Shafer, J.A. 1992. Interactions of Factor-Xiii with Fibrin As Substrate and Cofactor. *Biochemistry*, 31, (2) 423-429.

Horrevoets, A.J.G., Pannekoek, H., & Nesheim, M.E. 1997. A steady-state template model that describes the kinetics of fibrin-stimulated [Glu(1)]- and [Lys(78)]plasminogen activation by native tissue-type plasminogen activator and variants that lack either the finger or kringle-2 domain. *Journal of Biological Chemistry*, 272, (4) 2183-2191.

Hortin, G.L. & Sviridov, D. 2010. The dynamic range problem in the analysis of the plasma proteome. *Journal of Proteomics*, 73, (3) 629-636.

Hourcade, D.E. 2006. The role of properdin in the assembly of the alternative pathway C3 convertases of complement. *Journal of Biological Chemistry*, 281, (4) 2128-2132.

Howes, J.M., Richardson, VR., Smith, KA., Schroeder, V., Somani, R., Shore, A., Hess, K., Ajjan, R., Pease, R.J., Keen, JN., Standeven, K.F., & Carter, A.M. 2012. Complement C3 is a novel plasma clot component with anti-fibrinolytic properties. *Diabetes and Vascular Disease Research*, 9, (3) 216-225.

Hoylaerts, M., Rijken, D.C., Lijnen, H.R., & Collen, D. 1982. Kinetics of the Activation of Plasminogen by Human-Tissue Plasminogen-Activator - Role of Fibrin. *Journal of Biological Chemistry*, 257, (6) 2912-2919.

Huh, H.Y., Pearce, S.F., Yesner, L.M., Schindler, J.L., & Silverstein, R.L. 1996. Regulated expression of CD36 during monocyte-to-macrophage differentiation: Potential role of CD36 in foam cell formation. *Blood*, 87, (5) 2020-2028.

Huh, M.M., Schick, B.P., Schick, P.K., & Colman, R.W. 1988. Covalent crosslinking of human coagulation factor V by activated factor XIII from guinea pig megakaryocytes and human plasma. *Blood*, 71, (6) 1693-1702.

Husain, S.S., Hasan, A.A.K., & Budzynski, A.Z. 1989. Differences Between Binding of One-Chain and 2-Chain Tissue Plasminogen Activators to Non Cross-Linked and Cross-Linked Fibrin Clots. *Blood*, 74, (3) 999-1006.

Ichinose, A., Tamaki, T., & Aoki, N. 1983. Factor XIII-mediated cross-linking of NH2-terminal peptide of alpha 2-plasmin inhibitor to fibrin. *FEBS Lett.*, 153, (2) 369-371.

Inoguchi, T., Li, P., Umeda, F., Yu, H.Y., Kakimoto, M., Imamura, M., Aoki, T., Etoh, T., Hashimoto, T., Naruse, M., Sano, H., Utsumi, H., Nawata, H. 2000. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes*, 49, (11) 1939-1945.

Janssen, B.J.C., Christodoulidou, A., McCarthy, A., Lambris, J.D., & Gros, P. 2006. Structure of C3b reveals conformational changes that underlie complement activity. *Nature*, 444, (7116) 213-216.

Janssen, B.J.C., Huizinga, E.G., Raaijmakers, H.C.A., Roos, A., Daha, M.R., Nilsson-Ekdahl, K., Nilsson, B., & Gros, P. 2005. Structures of complement component C3 provide insights into the function and evolution of immunity. *Nature*, 437, (7058) 505-511.

Jarvisalo, M.J., Raitakari, M., Toikka, J.O., Putto-Laurila, A., Rontu, R., Laine, S., Lehtimaki, T., Ronnemaa, T., Viikari, J., & Raitakari, O.T. 2004. Endothelial dysfunction and increased arterial intima-media thickness in children with type 1 diabetes. *Circulation*, 109, (14) 1750-1755.

Jennings, L.K., Fox, J.E.B., Edwards, H.H., & Phillips, D.R. 1981. Changes in the Cytoskeletal Structure of Human-Platelets Following Thrombin Activation. *Journal of Biological Chemistry*, 256, (13) 6927-6932.

Jensen, P.H., Schuler, E., Woodrow, G., Richardson, M., Goss, N., Hojrup, P., Petersen, T.E., & Rasmussen, L.K. 1994. A unique interhelical insertion in plasminogen activator inhibitor-2 contains three glutamines, Gln83, Gln84, Gln86, essential for transglutaminase-mediated cross-linking. *J.Biol.Chem.*, 269, (21) 15394-15398.

Johnson-Tidey, R.R., McGregor, J.L., Taylor, P.R., & Poston, R.N. 1994. Increase in the adhesion molecule P-selectin in endothelium overlying atherosclerotic plaques. Coexpression with intercellular adhesion molecule-1. *Am.J.Pathol.*, 144, (5) 952-961.

Jy, W.C., Mao, W.W., Horstman, L.L., Tao, J.G., & Ahn, Y.S. 1995. Platelet microparticles bind, activate and aggregate neutrophils in vitro. *Blood Cells Molecules and Diseases*, 21, (22) 217-231.

Kahn, M.L., Nakanishi-Matsui, M., Shapiro, M.J., Ishihara, H., & Coughlin, S.R. 1999. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *Journal of Clinical Investigation*, 103, (6) 879-887.

Kappelmayer, J., Bacsko, G., Birinyi, L., Zakany, R., Kelemen, E., & Adany, R. 1995. Consecutive appearance of coagulation factor XIII subunit A in macrophages, megakaryocytes, and liver cells during early human development. *Blood*, 86, (6) 2191-2197.

Kaptoge, S., Di Angelantonio, E., Lowe, G., Pepys, M.B., Thompson, S.G., Collins, R., & Danesh, J. 2010. C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. *Lancet*, 375, (9709) 132-140.

Kardys, I., Klaver, C.C., Despriet, D.D., Bergen, A.A., Uitterlinden, A.G., Hofman, A., Oostra, B.A., van Duijn, C.M., de Jong, P.T., & Witteman, J.C. 2006. A common polymorphism in the complement factor H gene is associated with increased risk of myocardial infarction: the Rotterdam Study. *J.Am.Coll.Cardiol.*, 47, (8) 1568-1575.

Kassam, G., Kwon, M., Yoon, Y.S., Graham, K.S., Young, M.K., Gluck, S., & Waisman, D.M. 2001. Purification and characterization of A(61) - An angiostatin-like plasminogen fragment produced by plasmin autodigestion in the absence of sulfhydryl donors. *Journal of Biological Chemistry*, 276, (12) 8924-8933.

Kastl, S.P., Speidl, W.S., Kaun, C., Rega, G., Assadian, A., Weiss, T.W., Valent, P., Hagmueller, G.W., Maurer, G., Huber, K., & Wojta, J. 2006. The complement component C5a induces the expression of plasminogen activator inhibitor-1 in human macrophages via NF-kappa B activation. *Journal of Thrombosis and Haemostasis*, 4, (8) 1790-1797.

Kawashima, S. & Yokoyama, M. 2004. Dysfunction of endothelial nitric oxide synthase and atherosclerosis. *Arteriosclerosis Thrombosis and Vascular Biology*, 24, (6) 998-1005.

Kehrel, B., Wierwille, S., Clemetson, K.J., Anders, O., Steiner, M., Knight, C.G., Farndale, R.W., Okuma, M., & Barnes, M.J. 1998. Glycoprotein VI is a major collagen receptor for platelet activation: It recognizes the platelet-activating quaternary structure of collagen, whereas CD36, glycoprotein IIb/IIIa, and von Willebrand factor do not. *Blood*, 91, (2) 491-499.

Keuren, J.F.W., Magdeleyns, E.J.P., Govers-Riemslag, J.W.P., Lindhout, T., & Curvers, J. 2006. Effects of storage-induced platelet microparticles on the initiation and propagation phase of blood coagulation. *British Journal of Haematology*, 134, (3) 307-313.

Kim, P.Y., Tieu, L.D., Stafford, A.R., Fredenburgh, J.C., & Weitz, J.I. 2011. A high affinity interaction of plasminogen with fibrin is not essential for efficient activation by tissue plasminogen activator. *Journal of Biological Chemistry*, 287, 4652-4661

Kimura, S. & Aoki, N. 1986. Cross-linking site in fibrinogen for alpha 2-plasmin inhibitor. *J.Biol.Chem.*, 261, (33) 15591-15595.

Kimura, S., Tamaki, T., & Aoki, N. 1985a. Acceleration of fibrinolysis by the N-terminal peptide of alpha 2-plasmin inhibitor. *Blood*, 66, (1) 157-160.

Kimura, S., Tamaki, T., & Aoki, N. 1985b. Acceleration of fibrinolysis by the N-terminal peptide of alpha 2-plasmin inhibitor. *Blood*, 66, (1) 157-160.

Kodama, M., Naito, M., Nomura, H., Iguchi, A., Thompson, W.D., Stirk, C.M., & Smith, E.B. 2002. Role of D and E domains in the migration of vascular smooth muscle cells into fibrin gels. *Life Sciences*, 71, (10) 1139-1148.

Koeijvoets, K.C., Mooijaart, S.P., Dallinga-Thie, G.M., Defesche, J.C., Steyerberg, E.W., Westendorp, R.G., Kastelein, J.J., van Hagen, P.M., & Sijbrands, E.J. 2009. Complement factor H Y402H decreases cardiovascular disease risk in patients with familial hypercholesterolaemia. *Eur.Heart J.*, 30, (5) 618-623.

Kolb, W.P. & Mullereberhard, H.J. 1975. Membrane Attack Mechanism of Complement - Isolation and Subunit Composition of C5B-9 Complex. *Journal of Experimental Medicine*, 141, (4) 724-735.

Komaromi, I., Bagoly, Z., & Muszbek, L. 2011. Factor XIII: novel structural and functional aspects. *J.Thromb.Haemost.*, 9, (1) 9-20.

Krarup, A., Gulla, K.C., Gal, P., Hajela, K., & Sim, R.B. 2008. The action of MBL-associated serine protease 1 (MASP1) on factor XIII and fibrinogen. *Biochimica et Biophysica Acta-Proteins and Proteomics*, 1784, (9) 1294-1300.

Krarup, A., Hajela, K., Wallis, R., Presanis, J.S., Gal, P., & Sim, R.B. 2007. Mannan-binding lectin associated serine protease 2 (MASP-2) activates prothrombin directly and initiates low-level clotting. *Molecular Immunology*, 44, (1-3) 198.

Krem, M.M. & Di Cera, E. 2002. Evolution of enzyme cascades from embryonic development to blood coagulation. *Trends in Biochemical Sciences*, 27, (2) 67-74.

Krishnaswamy, S. 1990. Prothrombinase Complex Assembly - Contributions of Protein-Protein and Protein-Membrane Interactions Toward Complex-Formation. *Journal of Biological Chemistry*, 265, (7) 3708-3718.

Kristiansen, G.K. & Andersen, M.D. 2011. Reversible Activation of Cellular Factor XIII by Calcium. *Journal of Biological Chemistry*, 286, (11) 9833-9839.

Kroll, M.H., Harris, T.S., Moake, J.L., Handin, R.I., & Schafer, A.I. 1991. Vonwillebrand-Factor Binding to Platelet Gpib Initiates Signals for Platelet Activation. *Journal of Clinical Investigation*, 88, (5) 1568-1573.

Kukkonen, M., Lahteenmaki, K., Suomalainen, M., Kalkkinen, N., Emody, L., Lang, H., & Korhonen, T.K. 2001. Protein regions important for plasminogen activation and inactivation of alpha(2)-antiplasmin in the surface protease Pla of Yersinia pestis. *Molecular Microbiology*, 40, (5) 1097-1111.

La Bonte, L.R., Pavlov, V.I., Tan, Y.S., Takahashi, K., Takahashi, M., Banda, N.K., Zou, C.H., Fujita, T., & Stahl, G.L. 2012. Mannose-Binding Lectin-Associated Serine Protease-1 Is a Significant Contributor to Coagulation in a Murine Model of Occlusive Thrombosis. *Journal of Immunology*, 188, (2) 885-891.

Laine, M., Jarva, H., Seitsonen, S., Haapasalo, K., Lehtinen, M.J., Lindeman, N., Anderson, D.H., Johnson, P.T., Jarvela, I., Jokiranta, T.S., Hageman, G.S., Immonen, I., & Meri, S. 2007. Y402H polymorphism of complement factor H affects binding affinity to C-reactive protein. *Journal of Immunology*, 178, (6) 3831-3836.

Laine, P., Pentikainen, M.O., Wurzner, R., Penttila, A., Paavonen, T., Meri, S., & Kovanen, P.T. 2002. Evidence for complement activation in ruptured coronary plaques in acute myocardial infarction. *American Journal of Cardiology*, 90, (4) 404-408.

Landi, F., Johansson, C.M., Campopiano, D.J., & Hulme, A.N. 2010. Synthesis and application of a new cleavable linker for "click"-based affinity chromatography. *Organic & Biomolecular Chemistry*, 8, (1) 56-59.

Law, S.K.A. & Dodds, A.W. 1997. The internal thioester and the covalent binding properties of the complement proteins C3 and C4. *Protein Science*, 6, (2) 263-274.

Leander, K., Hallqvist, J., Reuterwall, C., Ahlbom, A., & de Faire, U. 2001. Family history of coronary heart disease, a strong risk factor for myocardial infarction interacting with other cardiovascular risk factors: Results from the Stockholm Heart Epidemiology Program (SHEEP). *Epidemiology*, 12, (2) 215-221.

Lee, K.N., Lee, C.S., Tae, W.C., Jackson, K.W., Christiansen, V.J., & McKee, P.A. 2000. Cross-linking of wild-type and mutant alpha 2-antiplasmins to fibrin by activated factor XIII and by a tissue transglutaminase. *J.Biol.Chem.*, 275, (48) 37382-37389.

Lee, K.N., Lee, C.S., Tae, W.C., Jackson, K.W., Christiansen, V.J., & McKee, P.A. 2001. Crosslinking of alpha 2-antiplasmin to fibrin. *Ann.N.Y.Acad.Sci.*, 936, 335-339.

Lepedda, A.J., Cigliano, A., Cherchi, G.M., Spirito, R., Maggioni, M., Carta, F., Turrini, F., Edelstein, C., Scanu, A.M., & Formato, M. 2009. A proteomic approach to differentiate histologically classified stable and unstable plaques from human carotid arteries. *Atherosclerosis*, 203, (1) 112-118.

Leung, L.L.K., Myles, T., Nishimura, T., Song, J.J., & Robinson, W.H. 2008. Regulation of tissue inflammation by thrombi n-activatable carboxypeptidase B (or TAFI). *Molecular Immunology*, 45, (16) 4080-4083.

Leung, V.W.Y., Yun, S., Botto, M., Mason, J.C., Malik, T.H., Song, W.C., Paixao-Cavalcante, D., Pickering, M.C., Boyle, J.J., & Haskard, D.O. 2009. Decay-Accelerating Factor Suppresses Complement C3 Activation and Retards Atherosclerosis in Low-Density Lipoprotein Receptor-Deficient Mice. *American Journal of Pathology*, 175, (4) 1757-1767.

Lewis, S.D., Janus, T.J., Lorand, L., & Shafer, J.A. 1985. Regulation of Formation of Factor-Xiiia by Its Fibrin Substrates. *Biochemistry*, 24, (24) 6772-6777.

Li, D.Y., Liu, L., Chen, H.J., Sawamura, R., & Mehta, J.L. 2003. LOX-1 mediates oxidized low-density lipoprotein-induced expression of matrix metalloproteinases in human coronary artery endothelial cells. *Circulation*, 107, (4) 612-617.

Li, Z.Y., Howarth, S.P.S., Tang, T., & Gillard, J.H. 2006. How critical is fibrous cap thickness to carotid plaque stability? A flow-plaque interaction model. *Stroke*, 37, (5) 1195-1199.

Linton, M.F. & Fazio, S. 2003. Macrophages, inflammation, and atherosclerosis. *International Journal of Obesity*, 27, S35-S40.

Lisman, T., de Groot, P.G., Meijers, J.C.M., & Rosendaal, F.R. 2005. Reduced plasma fibrinolytic potential is a risk factor for venous thrombosis. *Blood*, 105, (3) 1102-1105.

Liszewski, M.K., Leung, M., Cui, W.Y., Subramanian, V.B., Parkinson, J., Barlow, P.N., Manchester, M., & Atkinson, J.P. 2000. Dissecting sites important for complement regulatory activity in membrane cofactor protein (MCP; CD46). *Journal of Biological Chemistry*, 275, (48) 37692-37701.

Liu, S., Manson, J.E., Lee, I.M., Cole, S.R., Hennekens, C.H., Willett, W.C., & Buring, J.E. 2000. Fruit and vegetable intake and risk of cardiovascular disease: the Women's Health Study. *American Journal of Clinical Nutrition*, 72, (4) 922-928.

Liu, W., Carlisle, C.R., Sparks, E.A., & Guthold, M. 2010. The mechanical properties of single fibrin fibers. *J.Thromb.Haemost.*, 8, (5) 1030-1036.

Loewy, A.G., Kriel, R., Dunathan, K., & WOLFINGE.HL 1961. Fibrinase .1. Purifiction of Substrate and Enzyme. *Journal of Biological Chemistry*, 236, (10) 2625-&.

Longstaff, C. & Whitton, C.M. 2004. A proposed reference method for plasminogen activators that enables calculation of enzyme activities in SI units. *Journal of Thrombosis and Haemostasis*, 2, (8) 1416-1421.

Longstaff, C. & Gaffney, P.J. 1991. Serpin-serine protease binding kinetics: .alpha.2-antiplasmin as a model inhibitor. *Biochemistry*, 30, (4) 979-986.

Loof, T.G., Morgelin, M., Johansson, L., Oehmcke, S., Olin, A.I., Dickneite, G., Norrby-Teglund, A., Theopold, U., & Herwald, H. 2011. A novel role for coagulation factor XIII in the innate immune response against Streptococcus pyogenes. *International Journal of Medical Microbiology*, 301, 46.

Lorand, L. 1983. Post-Translational Pathways for Generating Epsilon(Gamma-Glutamyl)Lysine Cross-Links. *Annals of the New York Academy of Sciences*, 421, (DEC) 10-27.

Lorand, L., Gray, A.J., Brown, K., Credo, R.B., Curtis, C.G., Domanik, R.A., & Stenberg, P. 1974. Dissociation of the subunit structure of fibrin stabilizing factor during activation of the zymogen. *Biochem.Biophys.Res.Commun.*, 56, (4) 914-922.

Lorand, L., Ong, H.H., Lipinski, B., Rule, N.G., Downey, J., & Jacobsen, A. 1966. Lysine As Amine Donor in Fibrin Crosslinking. *Biochemical and Biophysical Research Communications*, 25, (6) 629-&.

Lu, P.D., Galanakis, D.K., Ghebrehiwet, B., & Peerschke, E.I.B. 1999. The receptor for the globular "heads" of C1q, gC1q-R, binds to fibrinogen/fibrin and impairs its polymerization. *Clinical Immunology*, 90, (3) 360-367.

Lynch, G.W., Slayter, H.S., Miller, B.E., & McDonagh, J. 1987. Characterization of thrombospondin as a substrate for factor XIII transglutaminase. *J.Biol.Chem.*, 262, (4) 1772-1778.

Ma, X.W., Chang, Z.W., Qin, M.Z., Sun, Y., Huang, H.L., & He, Y. 2009. Decreased expression of complement regulatory proteins, CD55 and CD59, on peripheral blood leucocytes in patients with type 2 diabetes and macrovascular diseases. *Chinese Medical Journal*, 122, (18) 2123-2128.

Maaroufi, R.M., Jozefowicz, M., Tapon-Bretaudire, J., & Fischer, A.M. 1997. Mechanism of thrombin inhibition by antithrombin and heparin cofactor II in the presence of heparin. *Biomaterials*, 18, (3) 203-211.

Mach, F., Schonbeck, U., Bonnefoy, J.Y., Pober, J.S., & Libby, P. 1997. Activation of monocyte/macrophage functions related to acute atheroma complication by ligation of CD40 - Induction of collagenase, stromelysin, and tissue factor. *Circulation*, 96, (2) 396-399.

Magwenzi, S.G., Ajjan, R.A., Standeven, K.F., Parapia, L.A., & Naseem, K.M. 2011. Factor XIII supports platelet activation and enhances thrombus formation by matrix proteins under flow conditions. *Journal of Thrombosis and Haemostasis*, 9, (4) 820-833.

Malik, T.H., Cortini, A., Carassiti, D., Boyle, J.J., Haskard, D.O., & Botto, M. 2010. The Alternative Pathway Is Critical for Pathogenic Complement Activation in Endotoxin- and Diet-Induced Atherosclerosis in Low-Density Lipoprotein Receptor-Deficient Mice. *Circulation*, 122, (19) 1948-1956.

Mandle, R. & Kaplan, A.P. 1976. Purification of Human Prekallikrein - Its Interaction with High Molecular-Weight (Hmw) Kininogen and Its Mechanism of Activation by Hageman-Factor. *Blood*, 48, (6) 974.

Mandle, R.J., Colman, R.W., & Kaplan, A.P. 1976. Identification of Prekallikrein and High-Molecular-Weight Kininogen As A Complex in Human-Plasma. *Proceedings of the National Academy of Sciences of the United States of America*, 73, (11) 4179-4183.

Marchi-Cappelletti, R. & Suarez-Nieto, N. 2010. Preliminary study of the fibrin structure in hypertensive, dyslipidemic and type 2 diabetic patients. *Investigacion clinica*, 51, (3) 315-324.

Marenberg, M.E., Risch, N., Berkman, L.F., Floderus, B., & Defaire, U. 1994. Genetic Susceptibility to Death from Coronary Heart-Disease in A Study of Twins. *New England Journal of Medicine*, 330, (15) 1041-1046.

Marshall, J., Kupchak, P., Zhu, W.M., Yantha, J., Vrees, T., Furesz, S., Jacks, K., Smith, C., Kireeva, I., Zhang, R., Takahashi, M., Stanton, E., & Jackowski, G. 2003.

Processing of serum proteins underlies the mass spectral fingerprinting of myocardial infarction. *Journal of Proteome Research*, 2, (4) 361-372.

Martel, C., Cointe, S., Maurice, P., Matar, S., Ghitescu, M., Theroux, P., & Bonnefoy, A. 2011. Requirements for Membrane Attack Complex Formation and Anaphylatoxins Binding to Collagen-Activated Platelets. *Plos One*, 6, (4) e18812

Martel, C., Granger, C.B., Ghitescu, M., Stebbins, A., Fortier, A., Armstrong, P.W., Bonnefoy, A., & Theroux, P. 2012. Pexelizumab fails to inhibit assembly of the terminal complement complex in patients with ST-elevation myocardial infarction undergoing primary percutaneous coronary intervention. Insight from a substudy of the Assessment of Pexelizumab in Acute Myocardial Infarction (APEX-AMI) trial. *American Heart Journal*, 164, (1) 43-51.

Martinez, A., Pio, R., Lopez, J., & Cuttitta, F. 2001. Expression of the adrenomedullin binding protein, complement factor H, in the pancreas and its physiological impact on insulin secretion. *J.Endocrinol.*, 170, (3) 503-511.

Mateos-Caceres, P.J., Garcia-Mendez, A., Farre, A.L., Macaya, C., Nunez, A., Gomez, J., Alonso-Org, Carrasco, C., Burgos, M.E., de Andres, R., Granizo, J.J., Farre, J., & Rico, L.A. 2004. Proteomic analysis of plasma from patients during an acute coronary syndrome. *Journal of the American College of Cardiology*, 44, (8) 1578-1583.

Matsuka, Y.V., Medved, L.V., Migliorini, M.M., & Ingham, K.C. 1996. Factor XIIIa-catalyzed cross-linking of recombinant alpha C fragments of human fibrinogen. *Biochemistry*, 35, (18) 5810-5816.

Matsuka, Y.V., Migliorini, M.M., & Ingham, K.C. 1997. Cross-linking of fibronectin to C-terminal fragments of the fibrinogen alpha-chain by factor XIIIa. *J.Protein Chem.*, 16, (8) 739-745.

Mauray, S., de Raucourt, E., Talbot, J.C., Dachary-Prigent, J., Jozefowicz, M., & Fischer, A.M. 1998. Mechanism of factor IXa inhibition by antithrombin in the presence of unfractionated and low molecular weight heparins and fucoidan. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 1387, 184-194.

McDonagh, R.P., McDonagh, J., Petersen, T.E., Thogersen, H.C., Skorstengaard, K., Sottrup-Jensen, L., Magnusson, S., Dell, A., & Morris, H.R. 1981. Amino acid sequence of the factor XIIIa acceptor site in bovine plasma fibronectin. *FEBS Lett.*, 127, (2) 174-178.

Meri, S., Morgan, B.P., Davies, A., Daniels, R.H., Olavesen, M.G., Waldmann, H., & Lachmann, P.J. 1990. Human Protectin (Cd59), An 18,000-20,000 Mw Complement Lysis Restricting Factor, Inhibits C5B-8 Catalyzed Insertion of C9 Into Lipid Bilayers. *Immunology*, 71, (1) 1-9.

Mihlan, M., Blom, A.M., Kupreishvili, K., Lauer, N., Stelzner, K., Bergstrom, F., Niessen, H.W.M., & Zipfel, P.F. 2011. Monomeric C-reactive protein modulates classic complement activation on necrotic cells. *Faseb Journal*, 25, (12) 4198-4210.

Mills, J.D., Ariens, R.A.S., Mansfield, M.W., & Grant, P.J. 2002. Altered fibrin clot structure in the healthy relatives of patients with premature coronary artery disease. *Circulation*, 106, (15) 1938-1942.

Minkoff, M.S., Wong, W.W., & Silberstein, D.S. 1991. Identification of C3 Beta-Chain As the Human Serum Eosinophil Cytotoxicity Inhibitor. *Journal of Experimental Medicine*, 174, (5) 1267-1270.

Misek, D.E., Kuick, R., Wang, H., Galchev, V., Deng, B., Zhao, R., Tra, J., Pisano, M.R., Amunugama, R., Allen, D., Walker, A.K., Strahler, J.R., Andrews, P., Omenn, G.S., & Hanash, S.M. 2005. A wide range of protein isoforms in serum and plasma uncovered by a quantitative intact protein analysis system. *Proteomics*, 5, (13) 3343-3352.

Moaddel, M., Falls, L.A., & Farrell, D.H. 2000. The role of gamma A/gamma ' fibrinogen in plasma factor XIII activation. *Journal of Biological Chemistry*, 275, (41) 32135-32140.

Molecular Probes Inc. CaptAvidin Biotin-binding protein. tools.invitrogen.com/content/sfs/manuals/mp21385.pdf . 2001.

Moller-Kristensen, M., Thiel, S., Sjoholm, A., Matsushita, M., & Jensenius, J.C. 2007. Cooperation between MASP-1 and MASP-2 in the generation of C3 convertase through the MBL pathway. *International Immunology*, 19, (2) 141-149.

Mooijaart, S.P., Koeijvoets, K.M.C., Sijbrands, E.J.G., Daha, M.R., & Westendorp, R.G.J. 2007. Complement Factor H polymorphism Y402H associates with inflammation, visual acuity, and cardiovascular mortality in the elderly population at large. *Experimental Gerontology*, 42, (11) 1116-1122.

Moreno-Navarrete, J.M., Martinez-Barricarte, R., Catalan, V., Sabater, M., Gomez-Ambrosi, J., Ortega, F.J., Ricart, W., Bluher, M., Fruhbeck, G., Rodriguez de, C.S., & Fernandez-Real, J.M. 2010. Complement factor H is expressed in adipose tissue in association with insulin resistance. *Diabetes*, 59, (1) 200-209.

Morgan, B.P., Marchbank, K.J., Longhi, M.P., Harris, C.L., & Gallimore, A.M. 2005. Complement: central to innate immunity and bridging to adaptive responses. *Immunology Letters*, 97, (2) 171-179.

Morgan, H.P., Schmidt, C.Q., Guariento, M., Blaum, B.S., Gillespie, D., Herbert, A.P., Kavanagh, D., Mertens, H.D.T., Svergun, D.I., Johansson, C.M., Uhrin, D., Barlow, P.N., & Hannan, J.P. 2011. Structural basis for engagement by complement factor H of C3b on a self surface. *Nature Structural & Molecular Biology*, 18, (4) 463-470.

Morigi, M., Galbusera, M., Gastoldi, S., Locatelli, M., Buelli, S., Pezzotta, A., Pagani, C., Noris, M., Gobbi, M., Stravalaci, M., Rottoli, D., Tedesco, F., Remuzzi, G., & Zoja, C. 2011. Alternative pathway activation of complement by Shiga toxin promotes exuberant C3a formation that triggers microvascular thrombosis. *J.Immunol.*, 187, (1) 172-180.

Mosesson, M.W., DiOrio, J.P., Siebenlist, K.R., Wall, J.S., & Hainfeld, J.F. 1993. Evidence for a second type of fibril branch point in fibrin polymer networks, the trimolecular junction. *Blood*, 82, (5) 1517-1521.

Mosesson, M.W., Siebenlist, K.R., Meh, D.A., Wall, J.S., & Hainfeld, J.F. 1998. The location of the carboxy-terminal region of gamma chains in fibrinogen and fibrin D domains. *Proc.Natl.Acad.Sci.U.S.A*, 95, (18) 10511-10516.

Mosher, D.F. 1976. Action of fibrin-stabilizing factor on cold-insoluble globulin and alpha2-macroglobulin in clotting plasma. *J.Biol.Chem.*, 251, (6) 1639-1645.

Mosher, D.F. 1984. Cross-linking of fibronectin to collagenous proteins. *Mol.Cell Biochem.*, 58, (1-2) 63-68.

Mosher, D.F. & Schad, P.E. 1979. Cross-linking of fibronectin to collagen by blood coagulation Factor XIIIa. *J.Clin.Invest*, 64, (3) 781-787.

Mosher, D.F., Schad, P.E., & Vann, J.M. 1980. Cross-linking of collagen and fibronectin by factor XIIIa. Localization of participating glutaminyl residues to a tryptic fragment of fibronectin. *J.Biol.Chem.*, 255, (3) 1181-1188.

Muscari, A., Bozzoli, C., Puddu, G.M., Sangiorgi, Z., Dormi, A., Rovinetti, C., Descovich, G.C., & Puddu, P. 1995. Association of Serum C3 Levels with the Risk of Myocardial-Infarction. *American Journal of Medicine*, 98, (4) 357-364.

Muscari, A., Massarelli, G., Bastagli, L., Poggiopollini, G., Tomassetti, V., Drago, G., Martignani, C., Pacilli, P., Boni, P., & Puddu, P. 2000. Relationship of serum C3 to fasting insulin, risk factors and previous ischaemic events in middle-aged men. *European Heart Journal*, 21, (13) 1081-1090.

Muscari, A., Sbano, D., Bastagli, L., Poggiopollini, G., Tomassetti, V., Forti, P., Boni, P., Ravaglia, G., Zoli, M., & Puddu, P. 2005. Effects of weight loss and risk factor treatment in subjects with elevated serum C3, an inflammatory predictor of myocardial infarction. *International Journal of Cardiology*, 100, (2) 217-223.

Mutch, N. J., Moore, N. R., Wang, E., & Booth, N. A. TAFI is cross-linked by its activation peptide to sites on fibrin(ogen) distict from other inhibitors of fibrinolysis. Journal of Thrombosis and Haemostasis 1[Supplement 1], Abstract Number: OC064. 2003.

Mutch, N.J., Thomas, L., Moore, N.R., Lisiak, K.M., & Booth, N.A. 2007. TAFIa, PAI-1 and alpha-antiplasmin: complementary roles in regulating lysis of thrombi and plasma clots. *J.Thromb.Haemost.*, 5, (4) 812-817.

Mutch, N.J., Koikkalainen, J.S., Fraser, S.R., Duthie, K.M., Griffin, M., Mitchell, J., Watson, H.G., Booth, N.A. 2010. Model thrombi formed under flow reveal the role of factor XIII-mediated cross-linking in rsistance to fibrinolysis. *Journal of Thrombosis and Haemostasis*, 8, (9) 2017-2024Myles, T., Nishimura, T., Yun, T.H., Nagashima, M., Morser, J., Patterson, A.J., Pearl, R.G., & Leung, L.L.K. 2003. Thrombin activatable fibrinolysis inhibitor, a potential regulator of vascular inflammation. *Journal of Biological Chemistry*, 278, (51) 51059-51067.

Nagasawa, S. & Stroud, R.M. 1977. Cleavage of C2 by C1s into the antigenically distinct fragments C2a and C2b: Demonstration of binding of C2b to C4b. *Proceedings of the National Academy of Sciences*, 74, (7) 2998-3001.

Nagy, J.A., Henriksson, P., & McDonagh, J. 1986. Biosynthesis of factor XIII B subunit by human hepatoma cell lines. *Blood*, 68, (6) 1272-1279.

Naito, K. & Fujikawa, K. 1991. Activation of Human Blood-Coagulation Factor-Xi Independent of Factor-Xii - Factor-Xi Is Activated by Thrombin and Factor-Xia in the Presence of Negatively Charged Surfaces. *Journal of Biological Chemistry*, 266, (12) 7353-7358.

Nan, R.D., Gor, J., & Perkins, S.J. 2008. Implications of the progressive self-association of wild-type human factor H6 for complement regulation and disease. *Journal of Molecular Biology*, 375, (4) 891-900.

Neth, O., Jack, D.L., Dodds, A.W., Holzel, H., Klein, N.J., & Turner, M.W. 2000. Mannose-Binding Lectin Binds to a Range of Clinically Relevant Microorganisms and Promotes Complement Deposition. *Infection and Immunity*, 68, (2) 688-693.

Neverova, I. & Van Eyk, J.E. 2005. Role of chromatographic techniques in proteomic analysis. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 815, (1-2) 51-63.

Nicklas, B.J., Penninx, B.W.J.H., Cesari, M., Kritchevsky, S.B., Newman, A.B., Kanaya, A.M., Pahor, M., Ding, J.Z., & Harris, T.B. 2004. Association of visceral adipose tissue with incident myocardial infarction in older men and women - The health, aging and body composition study. *American Journal of Epidemiology*, 160, (8) 741-749.

Niessen, S., Hoover, H., & Gale, A.J. 2011. Proteomic analysis of the coagulation reaction in plasma and whole blood using PROTOMAP. *Proteomics*, 11, (12) 2377-2388.

Nikolajsen, C.L., Scavenius, C., & Enghild, J.J. 2012. Human Complement C3 Is a Substrate for Transglutaminases. A Functional Link between Non-Protease-Based Members of the Coagulation and Complement Cascades. *Biochemistry*, 51, (23) 4735-4742.

Nurminskaya, M. & Kaartinen, M.T. 2006. Transglutaminases in mineralized tissues. *Front Biosci.*, 11, 1591-1606.

Ohtsuka, H., Imamura, T., Matsushita, M., Tanase, S., Okada, H., Ogawa, M., & Kambara, T. 1993. Thrombin generates monocyte chemotactic activity from complement factor H. *Immunology*, 80, (1) 140-145.

Ohyama, S., Harada, T., Chikanishi, T., Miura, Y., & Hasumi, K. 2004. Nonlysine-analog plasminogen modulators promote autoproteolytic generation of plasmin(ogen) fragments with angiostatin-like activity. *European Journal of Biochemistry*, 271, (4) 809-820.

Okemefuna, A.I., Gilbert, H.E., Griggs, K.M., Ormsby, R.J., Gordon, D.L., & Perkins, S.J. 2008. The regulatory SCR-1/5 and cell surface-binding SCR-16/20 fragments of factor H reveal partially folded-back solution structures and different self-associative properties. *Journal of Molecular Biology*, 375, (1) 80-101.

Oksjoki, R., Jarva, H., Kovanen, P.T., Laine, P., Meri, S., & Pentikainen, M.O. 2003. Association between complement factor H and proteoglycans in early human coronary atherosclerotic lesions - Implications for local regulation of complement activation. *Arteriosclerosis Thrombosis and Vascular Biology*, 23, (4) 630-636.

Oksjoki, R., Laine, P., Helske, S., Kreula, P.V., Mayranpaa, M.I., Gasque, P., Kovanen, P.T., & Pentikainen, M.O. 2007. Receptors for the anaphylatoxins C3a and C5a are expressed in human atherosclerotic coronary plaques. *Atherosclerosis*, 195, (1) 90-99.

Onat, A., Hergenc, G., Can, G., Kaya, Z., & Yuksel, H. 2010. Serum complement C3: a determinant of cardiometabolic risk, additive to the metabolic syndrome, in middle-aged population. *Metabolism-Clinical and Experimental*, 59, (5) 628-634.

Onat, A., Uzunlar, B., Hergenc, G., Yazici, M., Sari, I., Uyarel, H., Can, G., & Sansoy, V. 2005. Cross-sectional study of complement C3 as a coronary risk factor among men and women. *Clinical Science*, 108, (2) 129-135.

Orcutt, S.J. & Krishnaswamy, S. 2004. Binding of substrate in two conformations to human prothrombinase drives consecutive cleavage at two sites in prothrombin. *Journal of Biological Chemistry*, 279, (52) 54927-54936.

Orru, S., Caputo, I., D'Amato, A., Ruoppolo, M., & Esposito, C. 2003. Proteomics identification of acyl-acceptor and acyl-donor substrates for transglutaminase in a human intestinal epithelial cell line. Implications for celiac disease. *J.Biol.Chem.*, 278, (34) 31766-31773.

Osterud, B. & Rapaport, S.I. 1977. Activation of Factor-Ix by Reaction-Product of Tissue Factor and Factor-Vii-Additional Pathway for Initiating Blood-Coagulation. *Proceedings of the National Academy of Sciences of the United States of America*, 74, (12) 5260-5264.

Pai, J.K., Manson, J.E., Rexrode, K.M., Albert, C.M., Hunter, D.J., & Rimm, E.B. 2007. Complement factor H (Y402H) polymorphism and risk of coronary heart disease in US men and women. *Eur.Heart J.*, 28, (11) 1297-1303.

Palabrica, T., Lobb, R., Furie, B.C., Aronovitz, M., Benjamin, C., Hsu, Y.M., Sajer, S.A., & Furie, B. 1992. Leukocyte Accumulation Promoting Fibrin Deposition Is Mediated Invivo by P-Selectin on Adherent Platelets. *Nature*, 359, (6398) 848-851.

Palikhe, A., Sinisalo, J., Seppanen, M., Haario, H., Meri, S., Valtonen, V., Nieminen, M.S., & Lokki, M.L. 2007. Serum complement C3/C4 ratio, a novel marker for recurrent cardiovascular events. *American Journal of Cardiology*, 99, (7) 890-895.

Pangburn, M.K. & Mullereberhard, H.J. 1983. Initiation of the Alternative Complement Pathway Due to Spontaneous Hydrolysis of the Thioester of C-3. *Annals of the New York Academy of Sciences*, 421, (DEC) 291-298.

Pangburn, M.K. & Rawal, N. 2002. Structure and function of complement C5 convertase enzymes. *Biochemical Society Transactions*, 30, 1006-1010.

Pangburn, M.K., Rawal, N., Cortes, C., Alam, M.N., Ferreira, V.P., & Atkinson, M.A.L. 2009. Polyanion-Induced Self-Association of Complement Factor H. *Journal of Immunology*, 182, (2) 1061-1068.

Pangburn, M.K., Schreiber, R.D., & Mullereberhard, H.J. 1981. Formation of the Initial C-3 Convertase of the Alternative Complement Pathway - Acquisition of C3B-Like Activities by Spontaneous Hydrolysis of the Putative Thioester in Native C-3. *Journal of Experimental Medicine*, 154, (3) 856-867.

Pangburn, M.K. 2002. Cutting Edge: Localization of the Host Recognition Functions of Complement Factor H at the Carboxyl-Terminal: Implications for Hemolytic Uremic Syndrome. *The Journal of Immunology*, 169, (9) 4702-4706.

Pedersen, E.D., Waje-Andreassen, U., Vedeler, C.A., Aamodt, G., & Mollnes, T.E. 2004. Systemic complement activation following human acute ischaemic stroke. *Clinical and Experimental Immunology*, 137, (1) 117-122.

Peerschke, E.I.B., Yin, W., Grigg, S.E., & Ghebrehiwet, B. 2006. Blood platelets activate the classical pathway of human complement. *Journal of Thrombosis and Haemostasis*, 4, (9) 2035-2042.

Peng, H.B., Rajavashisth, T.B., Libby, P., & Liao, J.K. 1995. Nitric-Oxide Inhibits Macrophage-Colony-Stimulating Factor Gene-Transcription in Vascular Endothelial-Cells. *Journal of Biological Chemistry*, 270, (28) 17050-17055.

Perlmutter, D.H., Dinarello, C.A., Punsal, P.I., & Colten, H.R. 1986. Cachectin Tumor-Necrosis-Factor Regulates Hepatic Acute-Phase Gene-Expression. *Journal of Clinical Investigation*, 78, (5) 1349-1354.

Persson, L., Boren, J., Robertson, A.K.L., Wallenius, V., Hansson, G.K., & Pekna, M. 2004. Lack of complement factor C3, but not factor B, increases hyperlipidemia and atherosclerosis in apolipoprotein E-/- low-density lipoprotein receptor-/- mice. *Arteriosclerosis Thrombosis and Vascular Biology*, 24, (6) 1062-1067.

Piercy-Kotb, S.A., Mousa, A., Al-Jallad, H.F., Myneni, V.D., Chicatun, F., Nazhat, S.N., & Kaartinen, M.T. 2012. Factor XIIIA transglutaminase expression and secretion by osteoblasts is regulated by extracellular matrix collagen and the MAP kinase signaling pathway. *Journal of Cellular Physiology*, 227, (7) 2936-2946.

Pieters, M., Covic, N., Loots, D.T., van der Westhuizen, F.H., van Zyl, D.G., Rheeder, P., Jerling, J.C., & Weisel, J.W. 2006. The effect of glycaemic control on fibrin network structure of type 2 diabetic subjects. *Thrombosis and Haemostasis*, 96, (5) 623-629.

Pizzo, S.V., Schwartz, M.L., Hill, R.L., & Mckee, P.A. 1973. Effect of Plasmin on Subunit Structure of Human Fibrin. *Journal of Biological Chemistry*, 248, (13) 4574-4583.

Pober, J.S., Gimbrone, M.A., Lapierre, L.A., Mendrick, D.L., Fiers, W., Rothlein, R., & Springer, T.A. 1986. Overlapping Patterns of Activation of Human-Endothelial Cells by Interleukin-1, Tumor-Necrosis-Factor, and Immune Interferon. *Journal of Immunology*, 137, (6) 1893-1896.

Podrez, E.A., Poliakov, E., Shen, Z.Z., Zhang, R.L., Deng, Y.J., Sun, M.J., Finton, P.J., Shan, L., Febbraio, M., Hajjar, D.P., Silverstein, R.L., Hoff, H.F., Salomon, R.G., & Hazen, S.L. 2002. A novel family of atherogenic oxidized phospholipids promotes macrophage foam cell formation via the scavenger receptor CD36 and is enriched in atherosclerotic lesions. *Journal of Biological Chemistry*, 277, (41) 38517-38523.

Polkinghorne, V.R., Standeven, K.F., Schroeder, V., & Carter, A.M. 2009. Role of proteomic technologies in understanding risk of arterial thrombosis. *Expert Review of Proteomics*, 6, (5) 539-550.

Polley, M.J. & Nachman, R.L. 1983. Human-Platelet Activation by C3A and C3A Des-Arg. *Journal of Experimental Medicine*, 158, (2) 603-615.

Presanis, J.S., Hajela, K., Ambrus, G., Gal, P., & Sim, R.B. 2003. Differential substrate and inhibitor profiles for human MASP-1 and MASP-2. *Molecular Immunology*, 40, (2-4) 172.

Presanis, J.S., Hajela, K., Ambrus, G., Gal, P., & Sim, R.B. 2004. Differential substrate and inhibitor profiles for human MASP-1 and MASP-2. *Molecular Immunology*, 40, (13) 921-929.

Price, J.F., Mowbray, P.I., Lee, A.J., Rumley, A., Lowe, G.D.O., & Fowkes, F.G.R. 1999. Relationship between smoking and cardiovascular risk factors in the development of peripheral arterial disease and coronary artery disease - Edinburgh Artery Study. *European Heart Journal*, 20, (5) 344-353.

Procyk, R. & Blomback, B. 1988. Factor XIII-induced crosslinking in solutions of fibrinogen and fibronectin. *Biochim.Biophys.Acta*, 967, (2) 304-313.

Purves, L., Purves, M., & Brandt, W. 1987. Cleavage of Fibrin-Derived D-Dimer Into Monomers by Endopeptidase from Puff Adder Venom (Bitis-Arietans) Acting at Cross-Linked Sites of the Gamma-Chain - Sequence of Carboxy-Terminal Cyanogen-Bromide Gamma-Chain Fragments. *Biochemistry*, 26, (15) 4640-4646.

Qin, X., Goldfine, A., Krumrei, N., Grubissich, L., Acosta, J., Chorev, M., Hays, A.P., & Halperin, J.A. 2004. Glycation Inactivation of the Complement Regulatory Protein CD59. *Diabetes*, 53, (10) 2653-2661.

Reddy, K.S. 2004. Cardiovascular disease in non-Western countries. *New England Journal of Medicine*, 350, (24) 2438-2440.

Reycampos, J., Baezasanz, D., & Decordoba, S.R. 1990. Physical Linkage of the Human Genes-Coding for Complement Factor-H and Coagulation Factor-Xiii B-Subunit. *Genomics*, 7, (4) 644-646.

Richardson, V. R., Schroeder, V., Grant, P. J., Standeven, K. F., & Carter, A. M. 2013. Complement C3 is a substrate for activated factor XIII that is cross-linked to fibrin during clot formation. *British Journal of Haematology*, 160, (1) 116-119.

Richardson, V.R., Cordell, P., Standeven, K.F., & Carter, A.M. 2013. Substrates of Factor XIII-A: roles in thrombosis and wound healing. *Clinical Science*, 124, (3) 123-137.

Ridker, P.M. 1998. C-reactive protein and risks of future myocardial infarction and thrombotic stroke. *European Heart Journal*, 19, (1) 1-3.

Ridker, P.M., Buring, J.E., Cook, N.R., & Rifai, N. 2003. C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events. *Circulation*, 107, (3) 391-397.

Ridker, P.M., Buring, J.E., Shih, J., Matias, M., & Hennekens, C.H. 1998. Prospective study of C-reactive protein and the risk of future cardiovascular events among apparently healthy women. *Circulation*, 98, (8) 731-733.

Ritchie, H., Lawrie, L.C., Crombie, P.W., Mosesson, M.W., & Booth, N.A. 2000. Cross-linking of plasminogen activator inhibitor 2 and alpha 2-antiplasmin to fibrin(ogen). *J.Biol.Chem.*, 275, (32) 24915-24920.

Ritchie, H., Lawrie, L.C., Mosesson, M.W., & Booth, N.A. 2001. Characterization of crosslinking sites in fibrinogen for plasminogen activator inhibitor 2 (PAI-2). *Ann.N.Y.Acad.Sci.*, 936, 215-218.

Ritchie, H., Robbie, L.A., Kinghorn, S., Exley, R., & Booth, N.A. 1999. Monocyte plasminogen activator inhibitor 2 (PAI-2) inhibits u-PA-mediated fibrin clot lysis and is cross-linked to fibrin. *Thromb.Haemost.*, 81, (1) 96-103.

Rodriguez de, C.S., Esparza-Gordillo, J., Goicoechea de Jorge, E., Lopez-Trascasa, M., & Sanchez-Corral, P. 2004. The human complement factor H: functional roles, genetic variations and disease associations. *Molecular Immunology*, 41, (4) 355-367.

Ruoppolo, M., Orru, S., D'Amato, A., Francese, S., Rovero, P., Marino, G., & Esposito, C. 2003. Analysis of transglutaminase protein substrates by functional proteomics. *Protein Science*, 12, (6) 1290-1297.

Rutledge, J.C., Curry, F.R., Lenz, J.F., Davis, P.A. 1990. Low density lipoprotein transport across a microvascular endothelial barrier after permeability is increased. *Circulation Research*, 66. 486-495

Saenko, E.L., Shima, M., Gilbert, M., & Scandella, D. 1996. Slowed release of thrombin-cleaved factor VIII from von Willebrand factor by a monoclonal and a human antibody is a novel mechanism for factor VIII inhibition. *Journal of Biological Chemistry*, 271, (44) 27424-27431.

Sahu, A., Isaacs, S.N., Soulika, A.M., & Lambris, J.D. 1998. Interaction of vaccinia virus complement control protein with human complement proteins: Factor I-

mediated degradation of C3b to iC3b(1) inactivates the alternative complement pathway. *Journal of Immunology*, 160, (11) 5596-5604.

Sahu, A., Kozel, T.R., & Pangburn, M.K. 1994. Specificity of the Thioester-Containing Reactive-Site of Human C3 and Its Significance to Complement Activation. *Biochemical Journal*, 302, 429-436.

Sahu, A. & Lambris, J.D. 2001. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. *Immunological Reviews*, 180, 35-48.

Sakata, Y. & Aoki, N. 1980. Cross-linking of alpha 2-plasmin inhibitor to fibrin by fibrin-stabilizing factor. *J.Clin.Invest*, 65, (2) 290-297.

Sakata, Y. & Aoki, N. 1982. Significance of cross-linking of alpha 2-plasmin inhibitor to fibrin in inhibition of fibrinolysis and in hemostasis. *J.Clin.Invest*, 69, (3) 536-542.

Sakuma, M., Morooka, T., Wang, Y.M., Shi, C., Croce, K., Gao, H.Y., Strainic, M., Medof, M.E., & Simon, D.I. 2010. The Intrinsic Complement Regulator Decay-Accelerating Factor Modulates the Biological Response to Vascular Injury. *Arteriosclerosis Thrombosis and Vascular Biology*, 30, (6) 1196-1202.

Sane, D.C., Moser, T.L., Pippen, A.M., Parker, C.J., Achyuthan, K.E., & Greenberg, C.S. 1988. Vitronectin is a substrate for transglutaminases. *Biochem.Biophys.Res.Commun.*, 157, (1) 115-120.

Sarma, J.V. & Ward, P.A. 2011. The complement system. *Cell Tissue Res.*, 343, (1) 227-235.

Sarrias, M.R., Franchini, S., Canziani, G., Argyropoulos, E., Moore, W.T., Sahu, A., & Lambris, J.D. 2001. Kinetic analysis of the interactions of complement receptor 2 (CR2, CD21) with its ligands C3d, iC3b, and the EBV glycoprotein gp350/220. *Journal of Immunology*, 167, (3) 1490-1499.

Scarborough, P., Bhatnagar, P., Kaur, A., Smolina, K., Wickramasinghe, K., & Rayner, M. 2010a, *Ethnic differences in cardiovascular disease*, British Heart Foundation.

Scarborough, P., Bhatnagar, P., Wickramasinghe, K., Smolina, K., Mitchell, C., & Rayner, M. 2010b, *Coronary heart disease statistics*, British Heart Foundation.

Schielen, W.J.G., Adams, H.P.H.M., Voskuilen, M., Tesser, G.J., & Nieuwenhuizen, W. 1991. Structural Requirements of Position-A-Alpha-157 in Fibrinogen for the Fibrin-Induced Rate Enhancement of the Activation of Plasminogen by Tissue-Type Plasminogen-Activator. *Biochemical Journal*, 276, 655-659.

Scholz, T., Temmler, U., Krause, S., Heptinstall, S., & Losche, W. 2002. Transfer of tissue factor from platelets to monocytes: Role of platelet-derived microvesicles and CD62P. *Thrombosis and Haemostasis*, 88, (6) 1033-1038.

Schroeder, V., Carter, A.M., Dunne, J., Mansfield, M.W., & Grant, P.J. 2010. Proinflammatory and hypofibrinolytic phenotype in healthy first-degree relatives of patients with Type 2 diabetes. *Journal of Thrombosis and Haemostasis*, 8, (9) 2080-2082.

Schwartz, M.L., Pizzo, S.V., Hill, R.L., & McKee, P.A. 1973. Human Factor XIII from plasma and platelets. Molecular weights, subunit structures, proteolytic activation, and cross-linking of fibrinogen and fibrin. *J.Biol.Chem.*, 248, (4) 1395-1407.

Seifert, P.S., Hugo, F., Hansson, G.K., & Bhakdi, S. 1989. Prelesional Complement Activation in Experimental Atherosclerosis - Terminal C5B-9 Complement Deposition Coincides with Cholesterol Accumulation in the Aortic Intima of Hypercholesterolemic Rabbits. *Laboratory Investigation*, 60, (6) 747-754.

Serrano, K. & Devine, D.V. 2002. Intracellular factor XIII crosslinks platelet cytoskeletal elements upon platelet activation. *Thromb.Haemost.*, 88, (2) 315-320.

Shah, P.K., Falk, E., Badimon, J.J., Fernandezortiz, A., Mailhac, A., Villareallevy, G., Fallon, J.T., Regnstrom, J., & Fuster, V. 1995. Human Monocyte-Derived Macrophages Induce Collagen Breakdown in Fibrous Caps of Atherosclerotic Plaques - Potential Role of Matrix-Degrading Metalloproteinases and Implications for Plaque Rupture. *Circulation*, 92, (6) 1565-1569.

Sharma, V., Eng, J.K., Feldman, S., von Haller, P.D., MacCoss, M.J., & Noble, W.S. 2010. Precursor Charge State Prediction for Electron Transfer Dissociation Tandem Mass Spectra. *Journal of Proteome Research*, 9, (10) 5438-5444.

Shats-Tseytlina, E., Nair, C., & Dhall, D. 1994. Complement activation: a new participant in the modulation of fibrin gel characteristics and the progression of atherosclerosis? *Blood Coagul.Fibrinolysis*, 5, (4), 529-535.

Shemirani, A.H., Haramura, G., Bagoly, Z., & Muszbek, L. 2006. The combined effect of fibrin formation and factor XIII A subunit Val34Leu polymorphism on the activation of factor XIII in whole plasma. *Biochimica et Biophysica Acta-Proteins and Proteomics*, 1764, (8) 1420-1423.

Shen, L. & Lorand, L. 1983. Contribution of fibrin stabilization to clot strength. Supplementation of factor XIII-deficient plasma with the purified zymogen. *J.Clin.Invest*, 71, (5) 1336-1341.

Shen, L.L., McDonagh, R.P., McDonagh, J., & Hermans, J., Jr. 1974. Fibrin gel structure: influence of calcium and covalent cross-linking on the elasticity. *Biochem.Biophys.Res.Commun.*, 56, (3) 793-798.

Shields, K.J., Stolz, D., Watkins, S.C., & Ahearn, J.M. 2011. Complement Proteins C3 and C4 Bind to Collagen and Elastin in the Vascular Wall: A Potential Role in Vascular Stiffness and Atherosclerosis. *Cts-Clinical and Translational Science*, 4, (3) 146-152.

Siebenlist, K.R., Meh, D.A., & Mosesson, M.W. 1996. Plasma factor XIII binds specifically to fibrinogen molecules containing gamma' chains. *Biochemistry*, 35, (32) 10448-10453.

Siebenlist, K.R., Meh, D.A., & Mosesson, M.W. 2001. Protransglutaminase (factor XIII) mediated crosslinking of fibrinogen and fibrin. *Thromb.Haemost.*, 86, (5) 1221-1228.

Siebenlist, K.R. & Mosesson, M.W. 1994. Progressive cross-linking of fibrin gamma chains increases resistance to fibrinolysis. *J.Biol.Chem.*, 269, (45) 28414-28419.

Siezenga, M.A., Shaw, P.K.C., van der Geest, R.N., Mollnes, T.E., Daha, M.R., Rabelink, T.J., & Berger, S.P. 2009. Enhanced complement activation is part of the unfavourable cardiovascular risk profile in South Asians. *Clinical and Experimental Immunology*, 157, (1) 98-103.

Silverberg, S.A., Nemerson, Y., & Zur, M. 1977. Kinetics of Activation of Bovine Coagulation Factor-X by Components of Extrinsic Pathway - Kinetic-Behavior of 2-Chain Factor-Vii in Presence and Absence of Tissue Factor. *Journal of Biological Chemistry*, 252, (23) 8481-8488.

Sim, R.B. & Discipio, R.G. 1982. Purification and Structural Studies on the Complement-System Control Protein Beta-1H (Factor-H). *Biochemical Journal*, 205, (2) 285-293.

Sims, P.J., Ginsberg, M.H., Plow, E.F., & Shattil, S.J. 1991. Effect of Platelet Activation on the Conformation of the Plasma-Membrane Glycoprotein-Iib-Iiia Complex. *Journal of Biological Chemistry*, 266, (12) 7345-7352.

Skorstengaard, K., Halkier, T., Hojrup, P., & Mosher, D. 1990. Sequence location of a putative transglutaminase cross-linking site in human vitronectin. *FEBS Lett.*, 262, (2) 269-274.

Smith, K.A., Adamson, P.J., Pease, R.J., Brown, J.M., Balmforth, A.J., Cordell, P.A., Ariens, R.A.S., Philippou, H., & Grant, P.J. 2011. Interactions between factor XIII and the alpha C region of fibrinogen. *Blood*, 117, (12) 3460-3468.

Sobel, J.H. & Gawinowicz, M.A. 1996. Identification of the alpha chain lysine donor sites involved in factor XIIIa fibrin cross-linking. *J.Biol.Chem.*, 271, (32) 19288-19297.

Soff, G.A. 2000. Angiostatin and angiostatin-related proteins. *Cancer and Metastasis Reviews*, 19, (1-2) 97-107.

Somani, R., Grant, P.J., Kain, K., Catto, A.J., & Carter, A.M. 2006. Complement C3 and C-reactive protein are elevated in South Asians independent of a family history of stroke. *Stroke*, 37, (8) 2001-2006.

Somani, R., Richardson, V.R., Standeven, K.F., Grant, P.J., & Carter, A.M. 2012. Elevated Properdin and Enhanced Complement Activation in First-Degree Relatives of South Asian Subjects With Type 2 Diabetes. *Diabetes Care*, 35, (4) 894-899.

Sottrup-Jensen, L., Stepanik, T.M., Wierzbicki, D.M., Jones, C.M., Lonblad, P.B., Kristensen, T., Mortensen, S.B., Petersen, T.E., & Magnusson, S. 1983. The primary structure of alpha 2-macroglobulin and localization of a Factor XIIIa cross-linking site. *Ann.N.Y.Acad.Sci.*, 421, 41-60.

Speidl, W.S., Exner, M., Amighi, J., Kastl, S.P., Zorn, G., Maurer, G., Wagner, O., Huber, K., Minar, E., Wojta, J., & Schillinger, M. 2005. Complement component C5a predicts future cardiovascular events in patients with advanced atherosclerosis. *European Heart Journal*, 26, (21) 2294-2299.

Speidl, W.S., Kastl, S.P., Hutter, R., Katsaros, K.M., Kaun, C., Bauriedel, G., Maurer, G., Huber, K., Badimon, J.J., & Wojta, J. 2011. The complement component C5a is present in human coronary lesions in vivo and induces the expression of MMP-1 and MMP-9 in human macrophages in vitro. *Faseb Journal*, 25, (1) 35-44.

Spross, J. & Sinz, A. 2012. Monolithic columns with immobilized monomeric avidin: preparation and application for affinity chromatography. *Analytical and Bioanalytical Chemistry*, 402, (7) 2395-2405.

Stack, M.S., Gately, S., Bafetti, L.M., Enghild, J.J., & Soff, G.A. 1999. Angiostatin inhibits endothelial and melanoma cellular invasion by blocking matrix-enhanced plasminogen activation. *Biochemical Journal*, 340, 77-84.

Stahl, A.L., Vaziri-Sani, F., Heinen, S., Kristoffersson, A.C., Gydell, K.H., Raafat, R., Gutierrez, A., Beringer, O., Zipfel, P.F., & Karpman, D. 2008. Factor H dysfunction in patients with atypical hemolytic uremic syndrome contributes to complement deposition on platelets and their activation. *Blood*, 111, (11) 5307-5315.

Standeven, K.F., Carter, A.M., Grant, P.J., Weisel, J.W., Chernysh, I., Masova, L., Lord, S.T., & Ariens, R.A. 2007. Functional analysis of fibrin {gamma}-chain cross-linking by activated factor XIII: determination of a cross-linking pattern that maximizes clot stiffness. *Blood*, 110, (3) 902-907.

Standeven, K.F., Grant, P.J., Carter, A.M., Scheiner, T., Weisel, J.W., & Ariens, R.A. 2003. Functional analysis of the fibrinogen Aalpha Thr312Ala polymorphism: effects on fibrin structure and function. *Circulation*, 107, (18) 2326-2330.

Stark, K., Neureuther, K., Sedlacek, K., Hengstenberg, W., Fischer, M., Baessler, A., Wiedmann, S., Jeron, A., Holmer, S., Erdmann, J., Schunkert, H., & Hengstenberg, C. 2007. The common Y402H variant in complement factor H gene is not associated with susceptibility to myocardial infarction and its related risk factors. *Clinical Science*, 113, (3-4) 213-218.

Strainic, M.G., Liu, J.B., Huang, D.P., An, F., Lalli, P.N., Muqim, N., Shapiro, V.S., Dubyak, G.R., Heeger, P.S., & Medof, M.E. 2008. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4(+) T cells. *Immunity*, 28, (3) 425-435.

Strandberg, T.E. & Tilvis, R.S. 2000. C-reactive protein, cardiovascular risk factors, and mortality in a prospective study in the elderly. *Arteriosclerosis Thrombosis and Vascular Biology*, 20, (4) 1057-1060.

Strieter, R.M., Wiggins, R., Phan, S.H., Wharram, B.L., Showell, H.J., Remick, D.G., Chensue, S.W., & Kunkel, S.L. 1989. Monocyte Chemotactic Protein Gene-Expression by Cytokine-Treated Human-Fibroblasts and Endothelial-Cells. *Biochemical and Biophysical Research Communications*, 162, (2) 694-700.

Suenson, E., Lutzen, O., & Thorsen, S. 1984. Initial Plasmin-Degradation of Fibrin As the Basis of A Positive Feedback Mechanism in Fibrinolysis. *European Journal of Biochemistry*, 140, (3) 513-522.

Sun, Y.H. & Gailani, D. 1996. Identification of a factor IX binding site on the third apple domain of activated factor XI. *Journal of Biological Chemistry*, 271, (46) 29023-29028.

Sundsmo, J.S. & Wood, L.M. 1981. Activated Factor B (Bb) of the Alternative Pathway of Complement Activation Cleaves and Activates Plasminogen. *Journal of Immunology*, 127, (3) 877-880.

Suzuki, K., Dahlback, B., & Stenflo, J. 1982. Thrombin-Catalyzed Activation of Human Coagulation Factor-V. *Journal of Biological Chemistry*, 257, (11) 6556-6564.

Szasz, R. & Dale, G.L. 2002. Tbrombospondin and fibrinogen bind serotonin-derivatized proteins on COAT-platelets. *Blood*, 100, (8) 2827-2831.

Szeplaki, G., Szegedi, R., Hirschberg, K., Gombos, T., Varga, L., Karadi, I., Entz, L., Szeplaki, Z., Garred, P., Prohaszka, Z., & Fust, G. 2009. Strong complement activation after acute ischemic stroke is associated with unfavorable outcomes. *Atherosclerosis*, 204, (1) 315-320.

Tack, B.F. & Prahl, J.W. 1976. Third component of human complement: purification from plasma and physicochemical characterization. *Biochemistry*, 15, (20) 4513-4521.

Takagi, J., Aoyama, T., Ueki, S., Ohba, H., Saito, Y., & Lorand, L. 1995. Identification of factor-XIIIa-reactive glutaminyl residues in the propolypeptide of bovine von Willebrand factor. *Eur.J.Biochem.*, 232, (3) 773-777.

Takagi, T. & Doolittle, R.F. 1974. Amino acid sequence studies on factor XIII and the peptide released during its activation by thrombin. *Biochemistry*, 13, (4) 750-756.

Takahashi, K., Chang, W.C., Takahashi, M., Paylov, V., Ishida, Y., La Bonte, L., Shi, L., Fujita, T., Stahl, G.L., & Van Cott, E.M. 2011. Mannose-binding lectin and its associated proteases (MASPs) mediate coagulation and its deficiency is a risk factor in developing complications from infection, including disseminated intravascular coagulation. *Immunobiology*, 216, (1-2) 96-102.

Takebe, M., Soe, G., Kohno, I., Sugo, T., & Matsuda, M. 1995. Calcium Ion-Dependent Monoclonal-Antibody Against Human Fibrinogen - Preparation, Characterization, and Application to Fibrinogen Purification. *Thrombosis and Haemostasis*, 73, (4) 662-667.

Talens, S., Leebeek, F.W.G., Demmers, J.A.A., & Rijken, D.C. 2012. Identification of Fibrin Clot-Bound Plasma Proteins. *Plos One*, 7, (8) e41966

Tamam, Y., Iltumur, K., & Apak, I. 2005. Assessment of acute phase proteins in acute ischemic stroke. *Tohoku Journal of Experimental Medicine*, 206, (2) 91-98.

Tammen, H., Schulte, L., Hess, R., Menzel, C., Kellmann, M., Mohring, T., & Schulz-Knappe, P. 2005. Peptidomic analysis of human blood specimens: Comparison between plasma specimens and serum by differential peptide display. *Proteomics*, 5, (13) 3414-3422.

Thermo fisher scientific Inc. Thermo Scientific Avidin-Biotin Technical Handbook. www.piercenet.com/files/1601675_AvBi_HB_INTL.pdf . 2009.

Thermo fisher scientific Inc. Pierce Monomeric Avidin Kit. http://piercenet.com/instructions/2160340.pdf . 2011. 8-10-2012.

Thiel, S., VorupJensen, T., Stover, C.M., Schwaeble, W., Laursen, S.B., Poulsen, K., Willis, A.C., Eggleton, P., Hansen, S., Holmskov, U., Reid, K.B., & Jensenius, J.C. 1997. A second serine protease associated with mannan-binding lectin that activates complement. *Nature*, 386, (6624) 506-510.

Thogersen, A.M., Jansson, J.H., Boman, K., Nilsson, T.K., Weinehall, L., Huhtasaari, F., & Hallmans, G. 1998. High plasminogen activator inhibitor and tissue plasminogen activator levels in plasma precede a first acute myocardial infarction in both men and women - Evidence for the fibrinolytic system as an independent primary risk factor. *Circulation*, 98, (21) 2241-2247.

Thompson, R.E., Mandle, R., & Kaplan, A.P. 1978. Characterization of Human High Molecular-Weight Kininogen - Procoagulant Activity Associated with Light Chain of Kinin-Free High Molecular-Weight Kininogen. *Journal of Experimental Medicine*, 147, (2) 488-499.

Thung, S.N., Wang, D.F., Fasy, T.M., Hood, A., & Gerber, M.A. 1989. Hepatitis-B Surface-Antigen Binds to Human-Serum Albumin Cross-Linked by Transglutaminase. *Hepatology*, 9, (5) 726-730.

Torzewski, J., Torzewski, M., Bowyer, D.E., Frohlich, M., Koenig, W., Waltenberger, J., Fitzsimmons, C., & Hombach, V. 1998a. C-reactive protein frequently colocalizes with the terminal complement complex in the intima of early atherosclerotic lesions of human coronary arteries. *Arteriosclerosis Thrombosis and Vascular Biology*, 18, (9) 1386-1392.

Torzewski, M., Klouche, M., Hock, J., Messner, M., Dorweiler, B., Torzewski, J., Gabbert, H.E., & Bhakdi, S. 1998b. Immunohistochemical demonstration of enzymatically modified human LDL and its colocalization with the terminal complement complex in the early atherosclerotic lesion. *Arteriosclerosis Thrombosis and Vascular Biology*, 18, (3) 369-378.

Tran-Thang, C., Kruithof, E.K.O., Atkinson, J., & Bachmann, F. 1986. High-affinity binding sites for human Glu-plasminogen unveiled by limited plasmic degradation of human fibrin. *European Journal of Biochemistry*, 160, (3) 599-604.

Tsurupa, G. & Medved, L. 2001. Identification and characterization of novel tPA- and plasminogen-binding sites within fibrin(ogen) alpha C-domains. *Biochemistry*, 40, (3) 801-808.

Usui, T., Takagi, J., & Saito, Y. 1993. Propolypeptide of von Willebrand factor serves as a substrate for factor XIIIa and is cross-linked to laminin. *J.Biol.Chem.*, 268, (17) 12311-12316.

Vaisar, T., Pennathur, S., Green, P.S., Gharib, S.A., Hoofnagle, A.N., Cheung, M.C., Byun, J., Vuletic, S., Kassim, S., Singh, P., Chea, H., Knopp, R.H., Brunzell, J., Geary, R., Chait, A., Zhao, X.Q., Elkon, K., Marcovina, S., Ridker, P., Oram, J.F., & Heinecke, J.W. 2007. Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL. *Journal of Clinical Investigation*, 117, (3) 746-756.

Valnickova, Z. & Enghild, J.J. 1998. Human procarboxypeptidase U, or thrombin-activable fibrinolysis inhibitor, is a substrate for transglutaminases. Evidence for transglutaminase-catalyzed cross-linking to fibrin. *J.Biol.Chem.*, 273, (42) 27220-27224.

Van de Wouwer, M. & Conway, E.M. 2004. Novel functions of thrombomodulin in inflammation. *Critical Care Medicine*, 32, (5) S254-S261.

Vaziri-Sani, F., Hellwage, J., Zipfel, P.F., Sjoholm, A.G., Iancu, R., & Karpman, D. 2005. Factor H binds to washed human platelets. *J.Thromb.Haemost.*, 3, (1) 154-162.

Volcik, K.A., Ballantyne, C.M., Braun, M.C., Coresh, J., Mosley, T.H., & Boerwinkle, E. 2008. Association of the Complement Factor H Y402H Polymorphism With Cardiovascular Disease Is Dependent Upon Hypertension Status: The ARIC Study. *Am J Hypertens*, 21, (5) 533-538.

Wallenfeldt, K., Hulthe, J., Bokemark, L., Wikstrand, J., & Fagerberg, B. 2001. Carotid and femoral atherosclerosis, cardiovascular risk factors and C-reactive protein in relation to smokeless tobacco use or smoking in 58-year-old men. *Journal of Internal Medicine*, 250, (6) 492-501.

Wang, W. 2011. Identification of respective lysine donor and glutamine acceptor sites involved in factor XIIIa-catalyzed fibrin alpha chain cross-linking. *J.Biol.Chem.*, 286, (52) 44952-44964.

Wang, W., Boffa, M.B., Bajzar, L., Walker, J.B., & Nesheim, M.E. 1998. A study of the mechanism of inhibition of fibrinolysis by activated thrombin-activable fibrinolysis inhibitor. *J.Biol.Chem.*, 273, (42) 27176-27181.

Wang, Z., Wilhelmsson, C., Hyrsl, P., Loof, T.G., Dobes, P., Klupp, M., Loseva, O., Morgelin, M., Ikle, J., Cripps, R.M., Herwald, H., & Theopold, U. 2010. Pathogen entrapment by transglutaminase--a conserved early innate immune mechanism. *PLoS.Pathog.*, 6, (2) e1000763.

Wannamethee, S.G., Shaper, A.G., Whincup, P.H., Lennon, L., & Sattar, N. 2011. Impact of Diabetes on Cardiovascular Disease Risk and All-Cause Mortality in Older Men Influence of Age at Onset, Diabetes Duration, and Established and Novel Risk Factors. *Archives of Internal Medicine*, 171, (5) 404-410.

Weisel, J.W. 2005. Fibrinogen and fibrin. *Adv.Protein Chem.*, 70, 247-299.

Weisel, J.W., Veklich, Y., & Gorkun, O. 1993. The sequence of cleavage of fibrinopeptides from fibrinogen is important for protofibril formation and enhancement of lateral aggregation in fibrin clots. *J.Mol.Biol.*, 232, (1) 285-297.

Werfel, T., Kirchhoff, K., Wittmann, M., Begemann, G., Kapp, A., Heidenreich, F., Gotze, O., & Zwirner, J.+. 2000. Activated Human T Lymphocytes Express a Functional C3a Receptor. *The Journal of Immunology*, 165, (11) 6599-6605.

Wiedmer, T., Esmon, C.T., & Sims, P.J. 1986. On the mechanism by which complement proteins C5b-9 increase platelet prothrombinase activity. *J.Biol.Chem.*, 261, (31) 14587-14592.

Wilken, H.C., Gotze, O., Werfel, T., & Zwirner, J. 1999. C3a(desArg) does not bind to and signal through the human C3a receptor. *Immunology Letters*, 67, (2) 141-145.

Wilson, P.W.F., D'Agostino, R.B., Sullivan, L., Parise, H., & Kannel, W.B. 2002. Overweight and obesity as determinants of cardiovascular risk - The Framingham experience. *Archives of Internal Medicine*, 162, (16) 1867-1872.

Wing, R.R., Lang, W., Wadden, T.A., Safford, M., Knowler, W.C., Bertoni, A.G., Hill, J.O., Brancati, F.L., Peters, A., & Wagenknecht, L. 2011. Benefits of Modest Weight Loss in Improving Cardiovascular Risk Factors in Overweight and Obese Individuals With Type 2 Diabetes. *Diabetes Care*, 34, (7) 1481-1486.

Wojta, J., Gallicchio, M., Zoellner, H., Hufnagl, P., Last, K., Filonzi, E.L., Binder, B.R., Hamilton, J.A., & Mcgrath, K. 1993. Thrombin Stimulates Expression of Tissue-Type Plasminogen-Activator and Plasminogen-Activator Inhibitor Type-1 in Cultured Human Vascular Smooth-Muscle Cells. *Thrombosis and Haemostasis*, 70, (3) 469-474.

Wojta, J., Huber, K., & Valent, P. 2003. New aspects in thrombotic research: Complement induced switch in mast cells from a profibrinolytic to a prothrombotic phenotype. *Pathophysiology of Haemostasis and Thrombosis*, 33, (5-6) 438-441.

Wojta, J., Kaun, C., Zorn, G., Ghannadan, M., Hauswirth, A.W., Sperr, W.R., Fritsch, G., Printz, D., Binder, B.R., Schatzl, G., Zwirner, J., Maurer, G., Huber, K., & Valent, P. 2002. C5a stimulates production of plasminogen activator inhibitor-1 in human mast cells and basophils. *Blood*, 100, (2) 517-523.

Wolberg, A.S. 2007. Thrombin generation and fibrin clot structure. *Blood Reviews*, 21, (3) 131-142.

Woo, K.S., Chook, P., Yu, C.W., Sung, R.Y.T., Qiao, M., Leung, S.S.F., Lam, C.W.K., Metreweli, C., & Celermajer, D.S. 2004. Overweight in children is

associated with arterial endothelial dysfunction and intima-media thickening. *International Journal of Obesity*, 28, (7) 852-857.

Wu, G.X., Hu, W.G., Shahsafaei, A., Song, W.P., Dobarro, M., Sukhova, G.K., Bronson, R.R., Shi, G.P., Rother, R.P., Halperin, J.A., & Qin, X.B. 2009a. Complement Regulator CD59 Protects Against Atherosclerosis by Restricting the Formation of Complement Membrane Attack Complex. *Circulation Research*, 104, (4) 550-558.

Wu, J., Wu, Y.Q., Ricklin, D., Janssen, B.J.C., Lambris, J.D., & Gros, P. 2009b. Structure of complement fragment C3b-factor H and implications for host protection by complement regulators. *Nature Immunology*, 10, (7) 728-U79.

Yakovlev, S., Makogonenko, E., Kurochkina, N., Nieuwenhuizen, W., Ingham, K., & Medved, L. 2000. Conversion of fibrinogen to fibrin: Mechanism of exposure of tPA- and plasminogen-binding sites. *Biochemistry*, 39, (51) 15730-15741.

Yasojima, K., Schwab, C., McGeer, E.G., & McGeer, P.L. 2001. Generation of C-Reactive Protein and Complement Components in Atherosclerotic Plaques. *The American Journal of Pathology*, 158, (3) 1039-1051.

Yasuda, M., Takeuchi, K., Hiruma, M., Iida, H., Tahara, A., Itagane, H., Toda, I., Akioka, K., Teragaki, M., Oku, H., Kanayama, Y., Takeda, T., Kolb, W.P., & Tamerius, J.D. 1990. The Complement-System in Ischemic-Heart-Disease. *Circulation*, 81, (1) 156-163.

Yates, J.R., Ruse, C.I., & Nakorchevsky, A. 2009. Proteomics by Mass Spectrometry: Approaches, Advances, and Applications. *Annual Review of Biomedical Engineering*, 11, 49-79.

Yavlovich, A., Higazi, A.A.R., & Rottem, S. 2001. Plasminogen binding and activation by Mycoplasma fermentans. *Infection and Immunity*, 69, (4) 1977-1982.

Yin, W., Ghebrehiwet, B., Weksler, B., & Peerschke, E.I. 2007. Classical pathway complement activation on human endothelial cells. *Molecular Immunology*, 44, (9) 2228-2234.

Yin, W. & Peerschke, E.I.B. 2007. Localization of classical complement components and C1 inhibitor on platelet microparticles. *Arteriosclerosis Thrombosis and Vascular Biology*, 27, (6) E54.

Zee, R.Y., Diehl, K.A., & Ridker, P.M. 2006. Complement factor H Y402H gene polymorphism, C-reactive protein, and risk of incident myocardial infarction, ischaemic stroke, and venous thromboembolism: a nested case-control study. *Atherosclerosis*, 187, (2) 332-335.

Zhou, M., Lucas, D.A., Chan, K.C., Issaq, H.J., Petricoin, E.F., Liotta, L.A., Veenstra, T.D., & Conrads, T.R. 2004. An investigation into the human serum "interactome". *Electrophoresis*, 25, (9) 1289-1298.

Zwirner, J., Gotze, O., Begemann, G., Kapp, A., Kirchhoff, K., & Werfel, T. 1999. Evaluation of C3a receptor expression on human leucocytes by the use of novel monoclonal antibodies. *Immunology*, 97, (1) 166-172.

9.1. Appendix A: Identification of complement proteins in perfused, solubilised plasma clots

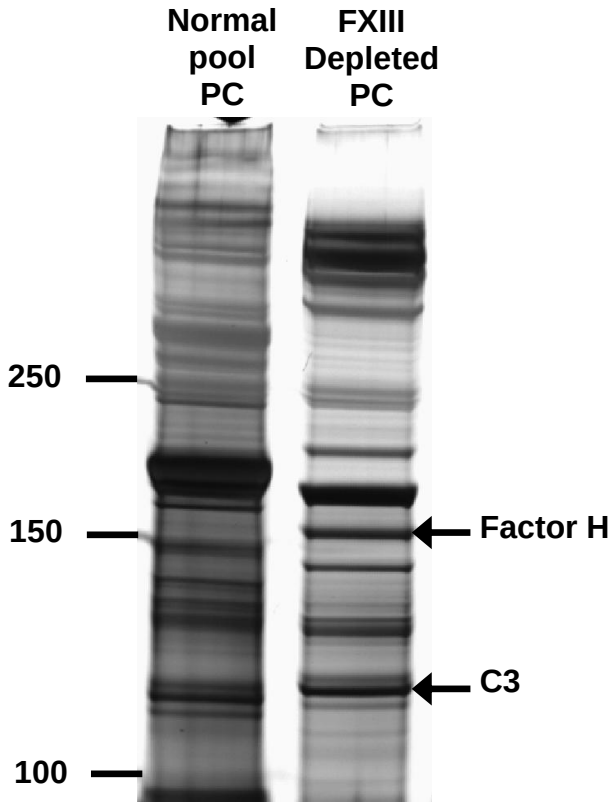

Figure 68: Identification of complement proteins in perfused, solubilised plasma clots.

SDS-PAGE of normal pool plasma clots (PC) and FXIII depleted plasma clots (PC) on a 3-8% Tris- acetate gel (Work carried out by Dr JM Howes) Factor H and C3 identified by MALDI-MS of trypsin digested bands.

9.2. Appendix B: Buffers and Solutions

C3 PURIFICATION	
PEG I (pH 7.4)	15% PEG 4000 100 mM Sodium phosphate 15 mM EDTA 150 mM NaCl 0.5 mM PMSF
PEG II (pH 7.4)	26% PEG 4000 100 mM Sodium phosphate 15 mM EDTA 150 mM NaCl 0.5 mM PMSF
Lysine Sepharose I (pH 7.4)	100 mM Sodium phosphate 15 mM EDTA 150 mM NaCl 0.5 mM PMSF
Lysine Sepharose II (pH 7.4)	100 mM Sodium phosphate 15 mM EDTA 150 mM NaCl 200 mM ε-aminocaproic acid (εACA) 0.5 mM PMSF
Ion Exchange I (pH 7.0)	25 mM Sodium phosphate 5 mM EDTA
Ion Exchange II (pH 7.0)	25 mM Sodium phosphate 5 mM EDTA 25 mM NaCl
Ion Exchange III (pH 7.0)	25 mM Sodium phosphate 5 mM EDTA 300 mM NaCl
Gel Filtration Buffer (pH 7.4)	100 mM Sodium phosphate 5 mM EDTA 150 mM NaCl 50 mM εACA 0.5 mM PMSF
Potassium Phosphate Dialysis Buffer (pH 7.4)	25 mM Potassium phosphate

	100 mM KCl 50 mM εACA
C5 Elution Buffer (pH 7.4)	25 mM Potassium phosphate 2 M KCl
C3 Elution Buffer I (pH 7.4)	250 mM Potassium phosphate 100 mM KCl
C3 Elution Buffer II (pH7.4)	400 mM Potassium phosphate 100 mM KCl
TBS (pH 7.4)	100 mM NaCl 50 mM Tris

FIBRINOGEN AND FXIII PURIFICATION	
Fibrinogen Equilibration Buffer (pH 7.4)	20 mM Tris 300 mM NaCl 1 mM $CaCl_2$
Fibrinogen Wash Buffer I (pH 7.4)	20 mM Tris 1 M NaCl 1 mM $CaCl_2$
Fibrinogen Wash Buffer II (pH 6.0)	50 mM Na-Acetate 300 mM NaCl 1 mM $CaCl_2$
Fibrinogen Elution Buffer (pH 7.4)	20 mM Tris 300 mM NaCl 5 mM EDTA
TBS (pH 7.4)	100 mM NaCl 50 mM Tris
ASSAY BASED BUFFERS	
Transfer Buffer	190 mM Glycine 20 mM Tris 40% Methanol
PBS (pH 7.2)	100 mM Sodium phosphate 140 mM NaCl
PBS-Tween (pH 7.2)	100 mM Sodium phosphate 500 mM NaCl

	0.002% Tween 20
TBS (pH 7.4)	100 mM NaCl 50 mM Tris
TBS1 (pH 7.4)	140 mM NaCl 40 mM Tris
TBS1-BSA (pH 7.4)	140 mM NaCl 40 mM Tris 1% BSA
TBS1-T (pH 7.4)	140 mM NaCl 40 mM Tris 0.01% Tween
Diethanolamine Buffer (pH 9.8)	1M Diethanolamine 0.5 mM $MgCl_2$
TBS2 (pH 7.4)	110 mM NaCl 50 mM Tris
TBS2-T (pH 7.4)	110 mM NaCl 50 mM Tris 0.01% Tween
TBS2-blocking buffer (pH 7.4)	140 mM NaCl 50 mM Tris 0.01% Tween 3% BSA
Plasmin assay high salt buffer (pH 7.4)	750 mM NaCl 50 mM Tris
Fixing solution	40% Methanol 10% Acetic acid
Silver nitrate solution	2 mg/ml Silver nitrate 0.02% Formaldehyde
Silver nitrate developing solution	30 mg/ml Sodium carbonate 8.75 µg/ml Pentahydrate sodium thiosulphate 0.05% Formaldehyde

9.3. Appendix C: Letter of ethical approval

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Dr A Carter
Principal Research Fellow
Division of Cardiovascular & Diabetes Research
The LIGHT Laboratories
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University of Leeds
LS2 9JT

23rd March 2009

Dear Angela

Re ref no: **HSLT/08/002**

Title: **Relationship of Complement C3 to Clot Structure/Function & Platelet//Fibrin Interactions**

I am pleased to inform you that the above research application has been reviewed by the Leeds Institute of Health Sciences (LIHS) and Leeds Institute of Genetics, Health and Therapeutics (LIGHT) joint ethics committee and I can confirm a favourable ethical opinion on the basis described in the application form and any supporting documentation submitted at date of this letter.

Please notify the committee if you intend to make any amendments to the original research as submitted at date of this approval. This includes recruitment methodology and all changes must be ethically approved prior to implementation. Please contact the Faculty Research Ethics and Governance Administrator for further information (r.e.desouza@leeds.ac.uk)

Ethical approval does not infer you have the right of access to any member of staff or student or documents and the premises of the University of Leeds. Nor does it imply any right of access to the premises of any other organisation, including clinical areas. The committee takes no responsibility for you gaining access to staff, students and/or premises prior to, during or following your research activities.

Please note: You are expected to keep a record of all your approved documentation, as well as documents such as sample consent forms, and other documents relating to the study. This should be kept in your study file, which should be readily available for audit purposes. You will be given a two week notice period if your project is to be audited.

It is our policy to remind everyone that it is your responsibility to comply with Health and Safety, Data Protection and any other legal and/or professional guidelines there may be.

I wish you every success with the project.

Yours sincerely

Laura Stroud

Professor Alastair Hay/Mrs Laura Stroud/Dr David Jayne
Chairs, LIHS/LIGHT/LIMM REC

9.4. Appendix D: Binding of C3 to fibrin

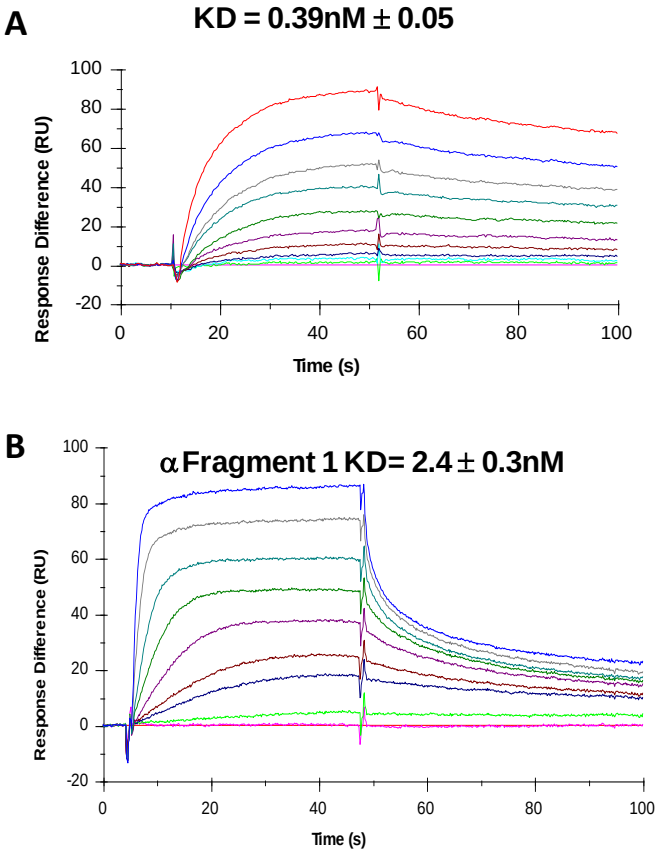

Figure 69: Binding interactions between C3 and fibrinogen.

Experiments performed by Dr Kerrie Smith**. Panel A**. Binding of C3 to immobilised fibrin **Panel B**. Binding of a recombinant αC fragment (233-425) to immobilised C3 analysed by SPR using a Biacore 3000.

9.5. Appendix E: Turbidity and lysis measurements in South Asian first degree relatives of patients with T2DM and South Asian controls with no family history

Table 18: Plasma clot turbidity and lysis measurements in South Asian first degree relatives of patients with T2DM and South Asian controls with no family history

Experiments and data analysis performed by Dr Riyaz Somani. Data presented as mean and 95% confidence intervals. Differences between the two groups were assessed using the Students t-test and were found to have an increase in maximum absorbances (MaxAbs) and a decrease in lysis time in South Asian relatives compared with South Asian controls.

	South Asian Controls (n=119)	South Asian Relatives (n=119)	P Value
Turbidity			
Lag time	338 (319, 357)	337 (318, 356)	0.949
MaxAbs	0.369 (0.351, 0.386)	0.399 (0.378, 0.419)	0.033
Concurrent turbidity and lysis			
Lag time	375 (357, 393)	360 (343, 377)	0.241
MaxAbs	0.254 (0.239, 0.271)	0.291 (0.272, 0.311)	0.004
Lysis time	1658 (1625, 1691)	1566 (1530, 1603)	0.001
AUC	242 (222, 263)	258 (238, 278)	0.287

AUC (area under the lysis curve)